Integrin Ligands Mobilize $\text{Ca}^{2+}$ from Ryanodine Receptor-gated Stores and Lysosome-related Acidic Organelles in Pulmonary Arterial Smooth Muscle Cells*

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Extracellular matrix (ECM)2 protein receptors, or integrins, participate in vascular remodeling and the systemic myogenic response. Synthetic ligands and ECM fragments regulate the vascular smooth muscle cell contractile state by altering intracellular $\text{Ca}^{2+}$ levels ([Ca$^{2+}$]). Information on the $\text{Ca}^{2+}$ effect of integrins in vascular smooth muscle cells is limited, but nonexistent in pulmonary arterial smooth muscle cells (PASMCs). We therefore characterized integrin expression in endothelium-denuded pulmonary arteries, and explored [Ca$^{2+}$], mobilization pathways induced by soluble ligands in rat PASMCs. Reverse transcriptase-PCR showed mRNA expression of integrins $\alpha_1$, $\alpha_2$, $\alpha_3$, $\alpha_5$, $\alpha_6$, $\alpha_v$, $\beta_1$, $\beta_3$, and $\beta_4$, and immunoblots of $\alpha_6$, $\alpha_v$, $\beta_1$, and $\beta_3$ confirmed protein expression. Exposure of PASMCs to integrin-binding peptides (0.5 mM) containing the arginine-glycine-aspartate (RGD) motif elicited [Ca$^{2+}$]$_i$ responses with an order of potency of GRGDNP > GRGDSP > GRGDTP = cyclo-RGD. Pharmacological analysis revealed that the GRGDSP-induced $\text{Ca}^{2+}$ response was unrelated to Ca$^{2+}$ influx and the inositol triphosphate receptor-gated Ca$^{2+}$ store, but partially blocked by ryanodine or inhibition of lysosome-related acidic organelles with bafilomycin A1. Simultaneous inhibition of both pathways was necessary to abolish the response. GRGDSP treatment increased cyclic ADP-ribose, the endogenous activator of ryanodine receptors, by 70%. GRGDSP also rapidly reduced Lysotracker Red accumulation, confirming direct modulation of acidic organelles. These data are the first demonstration of integrin-mediated $\text{Ca}^{2+}$ regulation in PASMCs. The presence of an array of integrins, and activation of ryanodine-sensitive Ca$^{2+}$ stores and lysosome-like organelles by GRGDSP suggest important roles for integrin-dependent $\text{Ca}^{2+}$ signaling in regulating PASMC function.

Extracellular matrix (ECM)2 protein receptors, or integrins, comprise a superfamily of structurally related heterodimeric

leucine-aspartic acid-valine; cADPR, cyclic ADP-ribose; HBSS, HEPES-buffered salt solution; PBS, phosphate-buffered saline; PBBST, PBS containing Tween 20; IP$_i$, inositol trisphosphate; RyR$_i$, ryanodine receptor; NAADP, nicotinic acid adenine dinucleotide phosphate; V-ATPase, vacuolar H$^+$ ATPase; SMC, smooth muscle cell; RT, reverse transcriptase.
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EXPERIMENTAL PROCEDURES

Isolation and Culture of PASMCs—PASMCs were enzymatically isolated and transiently cultured as previously described (17). Briefly, lungs were removed from male Wistar rats (150–200 g) anesthetized with sodium pentobarbital (130 mg/kg intraperitoneal), upon which intrapulmonary arteries were dissected in HEPES-buffered salt solution (HBSS) containing (in mM) 130 NaCl, 5 KCl, 1.2 MgCl$_2$, 1.5 CaCl$_2$, 10 HEPES, 10 glucose (pH 7.4). Pulmonary arteries were carefully cleaned of connective tissue, and the endothelial layer removed by gently rubbing the luminal surface with a cotton swab. Arteries were incubated in ice-cold HBSS (30 min), and then in reduced Ca$^{2+}$ (20 μM) HBSS (20 min, room temperature), upon which they were digested in reduced Ca$^{2+}$ HBSS containing collagenase (Type I, 1750 units/ml), papain (9.5 units/ml), bovine serum albumin (2 mg/ml), and dithiothreitol (1 mM), at 37 °C for 18 min. After washing with Ca$^{2+}$-free HBSS, single smooth muscle cells were gently dispersed by the tissues by trituration in Ca$^{2+}$-free HBSS, and cultured (16–24 h, 37 °C, 5% CO$_2$) on 25-mm glass coverslips in Ham’s F-12 medium (with l-glutamine) supplemented with 0.5% fetal calf serum, 100 units/ml streptomycin, and 0.1 mg/ml penicillin.

RNA Isolation and RT-PCR—Intralobar pulmonary arteries and aorta were removed, cleaned of connective tissue, denuded of endothelium as above, frozen in liquid nitrogen, and kept at −80 °C until use. Tissues were homogenized in TRIzol reagent (Invitrogen) using a PowerGen homogenizer (Fisher Scientific), and total RNA was isolated according to the protocol supplied with the TRIzol reagent. Ethanol-precipitated RNA samples were dissolved in diethyl pyrocarbonate-treated water and quantified using a BioPhotometer spectrophotometer (Eppendorf, Hamburg, Germany).

Total RNA samples were subsequently used for cDNA synthesis with SuperScript II (Invitrogen). Primers for PCR (Table 1) were carefully designed to regions specific for each integrin subtype, based on sequence alignments generated by a Clustal W algorithm. At least one of each primer pair was designed to exon-exon junctions, to minimize the possibility of amplifying genomic DNA. Where it was not feasible to design primers to exon-exon junctions, primers were designed to span intronic regions, to differentiate between genomic DNA and cDNA. PCR was carried out for 30 cycles using PlatinumTaq DNA polymerase (Invitrogen), which involved denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min. The resulting RT-PCR products were analyzed by 1.5% agarose gel electrophoresis.

Preparation of Protein Samples and Western Analysis—Total protein was isolated from endothelium-denuded pulmonary arteries and aorta. Briefly, upon dissection, tissues were frozen in liquid nitrogen and pulverized. Samples were subsequently homogenized with a Dounce homogenizer (30 strokes) in ice-cold Tris–HCl buffer (50 mM, pH 7.4) containing phenylmethylsulfonyl fluoride (1 mM) and protease mixture inhibitor (Roche Applied Sciences). Homogenized tissues were centrifuged (3000 × g, 4 °C, 10 min), upon which the protein concentrations of the post-nuclear supernatant were measured with the BCA Protein Assay Kit (Pierce). Total protein from cultured PASMCs were isolated by scraping cells with a rubber policeman in the ice-cold Tris–HCl buffer and processed as described for the smooth muscle tissue.

Protein samples were analyzed by SDS-PAGE and Western blot. Samples were treated with Laemmlli sample buffer with or without β-mercaptoethanol (100 °C, 5 min), separated by an 8% polyacrylamide gel at a concentration of 20 μg/lane, and then electrotransferred onto Immobilon P membranes (0.45 mm, Millipore) using a tank transfer system (80 V, 3 h, 4 °C). Upon blocking (1 h, room temperature) with 5% skim milk in PBS containing 0.05% Tween 20 (PBST), membranes were incubated with primary antibodies diluted in PBST containing 1% bovine serum albumin (bovine serum albumin/PBST) at 4 °C overnight. The following primary antibodies were used: α$_5$ (1:1000, catalog number AB1949, Chemicon International, Temecula, CA); α$_v$ (1:250, catalog number 611012, BD Biosciences, San Diego, CA); β$_1$ (1:2000, catalog number AB1952, Chemicon International); and β$_3$ (1:500, catalog number 4702, Cell Signaling, Beverly, MA). After washing in PBST, membranes were incubated with horseradish peroxidase-coupled donkey anti-rabbit or sheep anti-mouse secondary antibodies (Amersham Biosciences) diluted in 1% bovine serum albumin/PBST (1 h, room temperature), again washed extensively, and ultimately detected using enhanced chemiluminescence (Amersham Biosciences). The anti-CD38 immunoblot was performed essentially as described above, except that for the negative control, the antibody (1:200, catalog number sc-7049, Santa Cruz Biotechnology, Santa Cruz, CA) was preadsorbed with the antigenic peptide (5 times excess by weight, sc-7049P, Santa Cruz) prior to use (overnight, 4 °C).

Calcium Imaging—PASMCs were loaded with Fluo-3 AM (10 μM) dissolved in Me$_2$SO containing 20% pluronic acid for 45 min at room temperature (Molecular Probes, Eugene, OR). Upon washing thoroughly with Tyrode solution containing (in mM) 137 NaCl, 5 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 d-glucose, and 10 NaHEPES (pH 7.4), the cytosolic dye was allowed to de-esterify for 20 min at room temperature.

Laser scanning confocal microscopy was used to monitor the fluorescence changes upon treatment of PASMCs with the respective agents. Images were acquired with a Zeiss LSM-510 inverted confocal microscope (Carl Zeiss Inc., Germany) using...
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**TABLE 1**

| Subtype | Primer sequence | Expected size (bases) |
|---------|-----------------|-----------------------|
| α\textsubscript{1} | AGCAGACACACAGTCCGCCATT | 464 |
| α\textsubscript{2} | CAAACTGCTTTTAACAACTT | 800 |
| α\textsubscript{3} | ACGAGAAGCTCGACGGTTG | 487 |
| α\textsubscript{4} | ATCCTGTTTATTACAGACATGTT | 524 |
| α\textsubscript{5} | GCTAGTGACAGCAAGCATTTTGCTG | 685 |
| α\textsubscript{6} | GCTGCCTCAGATTTCTGATGAGTG | 722 |
| α\textsubscript{7} | TGGACCCACCCACACAGAGAG | 548 |
| α\textsubscript{8} | TATTGGGGATGACAACCCTCCTG | 500 |
| β\textsubscript{1} | ATGAGGTTAGGGAGAGAC | 515 |
| β\textsubscript{2} | GACTGCTGGCCTCCATCTGCA | 598 |
| β\textsubscript{3} | GCCATGTCACAAGGAGAGT | 498 |
| β\textsubscript{4} | GCGAGCTCAGAAAGGAGTT | 498 |

**FIGURE 1.** RT-PCR shows the presence of multiple integrin subtypes in pulmonary artery (A) and aortic (B) smooth muscle. Table 1 gives the specifics of the primers used, as well as the expected sizes, which correspond with each of the products obtained in this figure. All primers were tested in other tissue types to verify their capability in efficiently and robustly amplifying the product of the expected size. This figure is a representative image of an RT-PCR, which was repeated a total of 3 times.

A Zeiss Plan-Neofluor ×40 oil immersion objective (NA = 1.3) and an excitation wavelength of 488 nm. Fluorescence, measured at wavelengths >505 nm, was acquired in a framescan mode at either 5- (Figs. 3–6) or 10-s (Fig. 7) intervals. Photobleaching and laser damage to cells were minimized by attenuating the laser to ~1% of its maximum power (25 milliwatts). In all cases during fluorescence recordings, reagents were applied manually but with care, so as not to disturb the cells or cause an artificial change in fluorescence.

In some experiments, Fluoro-3 fluorescence was detected using a Nikon Diaphot microscope equipped with epifluorescence attachments and a microfluorometer (Biomedical Instrument Group, University of Pennsylvania). Protocols were executed and data collected on-line with a Digidata analog-to-digital interface and the pClamp software package (Axon Instruments, Inc., Foster City, CA).

Changes in fluorescence intensity were used to calculate the cellular concentrations of Ca\textsuperscript{2+}, using the following equation: $[\text{Ca}^{2+}]_i = \frac{(K_D/R)[[\text{Ca}^{2+}]]_{\text{rest}} + 1 - R}{F_0 / F_R}$, where $R$ is $F_0 / F_R$, $K_D$, and the resting $[\text{Ca}^{2+}]_{\text{rest}}$ is 100 nM. Changes in $[\text{Ca}^{2+}]_{\text{rest}}$ (i.e. $\Delta[\text{Ca}^{2+}]_i$) were the differences between the calculated values and the initial $[\text{Ca}^{2+}]_i$ at rest (i.e. 100 nM) (18). Concentrations of Ca\textsuperscript{2+} were similarly calculated for data obtained with the epifluorescence microscope, upon obtaining a background fluorescence value in an area devoid of cells by using the Ca\textsuperscript{2+} ionophore 4-Br-A23187 (EMD Biosciences) followed by Mn\textsuperscript{2+} quenching.

**Imaging Lysosomes**—Acidic organelles were labeled with the acidotropic dye Lysotracker Red DND-99 (Molecular Probes) diluted in Tyrode’s solution (50 nM) at room temperature for 30–45 min. Labeled PASMCs were imaged with a Zeiss LSM-510 inverted microscope as described above, but with excitation and emission wavelengths of 543 and >560 nm, respectively. Images were acquired in framescan mode at 2-min intervals, and cellular changes in fluorescence intensity were represented as $F/F_0$, with $F_0$ being the fluorescence level immediately preceding treatment with the respective agent.

**Detection of Cyclic ADP-ribose (cADPR)**—cADPR levels were measured in cultured PASMCs at passage 3–4. Cells were isolated and cultured in Ham’s F-12 media overnight as described above, and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum thereafter. Prior to treatment with GRGDSP (1.25 mM), cells were washed three times with PBS, and equilibrated in Tyrode’s solution for at least 30 min. Upon GRGDSP treatment, nucleotides were extracted from cells with 10% (w/v) trichloroacetic acid at 4 °C, followed by removal with water-saturated diethyl ether. The aqueous layer containing cADPR was adjusted to pH 8 with 1M Tris and contaminating nucleotides other than cADPR were removed with a mixture containing hydrolytic enzymes with the following final concentrations: 0.44 unit/ml nucleotide pyrophosphatase, 12.5 units/ml alkaline phosphatase, 0.0625 unit/ml NADase, 2.5 mM MgCl\textsubscript{2}. Incubation proceeded at 37 °C for 2 h. Detection of cADPR was performed with some modifications to the cycling method described recently (19). Briefly, 0.1 ml of cADPR standard or nucleotides extracted from cell samples were incubated with 100 µl of cycling reagent containing (final concentrations) 0.3 µg/ml ADP-ribosyl cyclase, 4 mM...
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FIGURE 2. Representative image of immunoblot analysis of pulmonary artery and aortic smooth muscle. Total protein (20 μg/lane) treated with Laemmli sample buffer either with (+) or without (−) β-mercaptoethanol, were separated by 8% SDS-PAGE and subsequently analyzed for integrin immunoreactivity. Antibodies to integrin α2, αv, β1, and β3 were used as indicated under “Experimental Procedures.”

FIGURE 3. Order of potency of RGD peptides in eliciting PASMC [Ca$^{2+}$], transients. A, four different RGD peptides (GRGDSP, GRGDNP, GRGDTP, and cyclo-RGD) and 2 control peptides (GRGESP and GRADSP) were applied (0.5 mM) at time = 50 s and changes in fluorescence were recorded and expressed as changes in [Ca$^{2+}$]. B, calculated changes in peak levels of [Ca$^{2+}$], are summarized. Only GRGDNP and GRGDSP significantly elevated [Ca$^{2+}$], levels (asterisks, $p < 0.05$). Data acquired from the following numbers of cells from 6 to 8 separate dishes were averaged to obtain the values in the figure: 82, 89, 61, 21, 76, and 62 for GRGDNP, GRGDSP, GRGDTP, cyclo-RGD, GRGESP, and GRADSP, respectively. Cells were prepared from individual animals, and were repeated in cells obtained from two to four replicate animals.

nicotinamide, 100 mM sodium phosphate (pH 8), 0.8% (v/v) ethanol, 40 μg/ml alcohol dehydrogenase, 8 mM resazurin, 0.04 units/ml diaphorase, and 4 μM flavin mononucleotide. Following a 2-h incubation at room temperature, the increase in resorufin fluorescence was measured using a Spectramax XPS fluorescent plate reader with excitation and emission wavelengths of 544 and 590 nm, respectively (Molecular Devices, Sunnyvale, CA). Results are the averages of three to four independent experiments and are normalized to the value at time 0.

Chemicals—GRGDSP, GRGDNP, GRGDTP, GRADSP, thapsigargin, nifedipine, SKF96365, ryanodine, Xestospongin C, bafilomycin A1, and 4-Br-A23187 were purchased from EMD Biosciences (San Diego, CA); GRGESP and cyclo-RGD (cyclo-GRGDSPA) were from Bachem (Torrance, CA); caffeine was from Sigma.

RESULTS

Integrin Expression in PASMCs—To determine the repertoire of integrins expressed in endothelium-denuded rat pulmonary arteries, RT-PCR was performed using primers designed to integrins α1, α2, α3, α5, α6, α7, α9, β1, β3, and β4 (Table 1). Fig. 1 shows a representative image of an RT-PCR, in which positive mRNA expression was observed for all integrin subtypes examined (Fig. 1a). We further compared the integrin expression profile to that in endothelium-denuded aorta (Fig. 1b). As illustrated in Fig. 1, the pattern of integrin expression was similar in both tissue types, except that integrin α4 consistently could not be detected in the aortic smooth muscle. Sizes of the PCR products obtained in all cases corresponded to that expected (see Table 1). All PCR primers were tested prior to use in other tissues (e.g. heart, small intestine, and brain) and shown to efficiently amplify products of the expected size (data not shown). Negative controls, in which the reverse transcriptase was omitted, yielded no PCR products (data not shown).

We focused our attention on a few integrin subtypes that have been characterized most extensively in other smooth muscles and examined the protein expression in endothelium-denuded pulmonary arteries and aorta. Western analysis of integrins α5, αv, β1, and β3 confirmed the presence of these proteins in both tissue subtypes (Fig. 2). Immunoblots were performed using both reduced and non-reduced protein samples, as certain antibodies were generated against reduced or non-reduced antigens. Some integrin α subunits including α5 and αv contain a disulfide-linked cleavage site at the C terminus of the protein, yielding 2 polypeptides in the presence of reducing agents (20). β Subunits have internally disulfide-bonded cysteine-rich domains in the C termini that influence mobility on SDS-PAGE gels depending on the presence of reducing agents (21). A number of antibodies from various companies were tested for each of the integrin subtypes, and those giving the most specific products of the expected size were chosen. The anti-α5 antibody recognized a broad band of ∼150 kDa under non-reducing conditions in both PA and aorta, but was not reactive with the reduced protein as indicated by the manufacturer. Two bands of about 120 and 150 kDa were immunoreactive to the anti-αv antibody, presumably representing the reduced and non-reduced forms, respectively, because the 150-kDa band was more prominent under non-reducing conditions. A single band of about 120 kDa was detected by anti-β1 in both tissues regardless of the presence of
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![Graph](image1)

**FIGURE 4.** Concentration dependence of $\text{[Ca}^{2+}]_{i}$ response to GRGDSP. A, time course of the change in $\text{[Ca}^{2+}]_{i}$, evoked by the application of 3 different (0.5, 1.25, and 2.5 mM) GRGDSP concentrations. GRGDSP was applied at time $t = 0$ s. B, comparison of $\Delta [\text{Ca}^{2+}]_{i}$ levels induced upon exposure to 0.5, 1.25, and 2.5 mM GRGDSP at 7.5 min post-application. Values are the average of data acquired from individual cells in multiple dishes for experiments (i.e. 0.5 mM, $n = 89$ cells from 8 dishes; 1.25 mM, $n = 71$ cells from 8 dishes; 2.5 mM, $n = 102$ cells from 8 dishes). Cells were obtained from individual rats, and experiments were repeated in cells prepared from at least 3 replicate rats.

![Graph](image2)

**FIGURE 5.** $\text{Ca}^{2+}$ influx is not involved in the GRGDSP-elicited $\text{[Ca}^{2+}]_{i}$ response. The GRGDSP-induced (1.25 mM) $\text{Ca}^{2+}$ response was monitored upon pretreating PASMCs with: A, nifedipine (1 mM, 20 min) to block L-type $\text{Ca}^{2+}$ channels; B, SKF 96365 (30 mM, 30 min) to block non-selective $\text{Ca}^{2+}$ channels; and C, 0 mM $\text{Ca}^{2+}$ Tyrode buffer containing 2 mM EGTA to create conditions of no external $\text{Ca}^{2+}$, to eliminate $\text{Ca}^{2+}$ influx. Arrow represents the time of GRGDSP application. D, peak $\Delta [\text{Ca}^{2+}]_{i}$ elicited by GRGDSP, showing that $\text{Ca}^{2+}$ influx does not contribute to the GRGDSP-induced response. Values in the figure reflect the average of data acquired from individual cells in multiple dishes. A, control, $n = 74$ cells from 7 dishes; nifedipine, $n = 65$ cells from 7 dishes; B, control, $n = 46$ cells, 7 dishes; SKF, $n = 34$ cells, 5 dishes; C, control, $n = 67$ cells, 8 dishes; 0 Ca, $n = 60$ cells, 7 dishes. Cells were obtained from individual animals, and experiments were repeated using cells prepared from at least 2 different rats.

![Graph](image3)

**FIGURE 5A.** Mobilized by GRGDSP—We proceeded to examine pharmacologically the source of $\text{Ca}^{2+}$ mobilized by GRGDSP in PASMCs (Figs. 5–7). To determine whether the GRGDSP-induced rise in $\text{[Ca}^{2+}]_{i}$ resulted from extracellular $\text{Ca}^{2+}$ influx through L-type $\text{Ca}^{2+}$ channels, PASMCs were treated with nifedipine (1 mM) for 20 min prior to GRGDSP application (Fig. 5A). Compared with time-matched controls ($\Delta [\text{Ca}^{2+}]_{i} = 200.9 \pm 12.9 \text{ nM, } n = 74$ cells), the GRGDSP elicited changes in $\text{[Ca}^{2+}]_{i}$, did not differ significantly with nifedipine treatment ($\Delta [\text{Ca}^{2+}]_{i} = 171.6 \pm 15.7 \text{ nM, } n = 65$ cells, $p > 0.05$). Treatment of PASMCs with SKF96365 (30 mM, reducing agents, and a single 100-kDa band was recognized by the anti-$\beta_3$ antibody only under reducing conditions.

**Effect of Integrin Ligands on $\text{[Ca}^{2+}]_{i}$**—One of the initial events in the signal transduction cascade following integrin activation is a change in $\text{[Ca}^{2+}]_{i}$ levels (6, 22). For many integrin ligands, the amino acid sequence immediately following the active RGD motif determines receptor selectivity (23). To see if integrin activation was capable of eliciting changes in $\text{[Ca}^{2+}]_{i}$, PASMCs, 4 RGD peptides (GRGDSP, GRGDNP, GRGDTP, and cyclo-RGD) and 2 control peptides (GRGESP, GRADSP) were exogenously applied at a concentration of 0.5 mM and $\text{[Ca}^{2+}]_{i}$ levels were monitored (Fig. 3). At 0.5 mM, GRGDNP and GRGDSP caused significant changes in $\text{[Ca}^{2+}]_{i}$, and subsequent cell contraction, increasing $\text{[Ca}^{2+}]_{i}$ levels by 177.1 ± 7.1 and 16.5 ± 4.2 nm, respectively. GRGDTP, cyclo-RGD, and control peptides were unable to significantly increase $\text{[Ca}^{2+}]_{i}$, above baseline levels, although at higher concentrations (2.5 mM), GRGDTP and cyclo-RGD induced $\text{[Ca}^{2+}]_{i}$ elevations (data not shown).

After establishing that the active motif of integrin ligands indeed elicited $\text{[Ca}^{2+}]_{i}$ changes in rat PASMCs, we further characterized the $\text{[Ca}^{2+}]_{i}$ effects of GRGDSP, because this is the more commonly occurring sequence in endogenous integrin ligands. The $\text{[Ca}^{2+}]_{i}$ response evoked by GRGDSP was concentration-dependent (Fig. 4). A concentration of 1.25 mM GRGDSP was used in all subsequent experiments, as this concentration elicited a consistently robust response.

**Characterization of the Source of $\text{[Ca}^{2+}]_{i}$ Mobilized by GRGDSP**—We proceeded to examine pharmacologically the source of $\text{Ca}^{2+}$ mobilized by GRGDSP in PASMCs (Figs. 5–7). To determine whether the GRGDSP-induced rise in $\text{[Ca}^{2+}]_{i}$ resulted from extracellular $\text{Ca}^{2+}$ influx through L-type $\text{Ca}^{2+}$ channels, PASMCs were treated with nifedipine (1 mM) for 20 min prior to GRGDSP application (Fig. 5A). Compared with time-matched
cells) in the time-matched controls, to 73.2 ± 8.2 nm (n = 115 cells, p < 0.05) in ryanodine-treated cells, corresponding to 44% of control values (Fig. 6A). On the other hand, treatment with Xestospongin C (10 μM) for 30 min (Δ[Ca^{2+}]) = 135.9 ± 11.7 nm, n = 105 cells), had no significant effect on the GRGDSP-induced [Ca^{2+}]_i response compared with that of control (Δ[Ca^{2+}]) = 136.2 ± 8.1 nm, n = 103 cells, p > 0.05) (Fig. 6B).

It has recently been shown that in addition to ryanodine and IP3, receptor-gated intracellular Ca^{2+} stores, that PASMCs contain an active intracellular Ca^{2+} store of lysosomal origin that is sensitive to nicotinic acid adenine dinucleotide phosphate (NAADP), but insensitive to thapsigargin treatment (24, 25). To test the possibility that GRGDSP was promoting Ca^{2+} release through a lysosome-related acidic organelle, we treated the PASMCs with bafilomycin A1 (Fig. 6C). Bafilomycin A1 is a macroside antibiotic that selectively inhibits the vacuolar H^+-ATPase (V-ATPase) at nanomolar to low micromolar concentrations, and the P-type ATPases at high micromolar concentrations (26, 27). It potently inhibits both ATPase and H^+ pumping activities of the V-ATPase, causing dissipation of the proton gradient that energizes Ca^{2+} uptake into these compartments, thereby inhibiting the NAADP-sensitive Ca^{2+} activity (26–29). As reported by Kinnear et al. (24), bafilomycin A1 treatment alone elevated [Ca^{2+}] in PASMCs, presumably arising from the lysosome-related acidic compartments as a result of inhibiting the lysosomal proton motive force. In addition, the GRGDSP-induced response in the bafilomycin A1-treated cells was significantly reduced to 50.4% of control values (Δ[Ca^{2+}] (control) = 79.0 ± 15.9 nm, n = 11 dishes versus Δ[Ca^{2+}] (bafilomycin) = 39.9 ± 11.6 nm, n = 11 dishes, p < 0.1). The GRGDSP-induced [Ca^{2+}]_i response was taken to be the peak [Ca^{2+}]_i elicited by GRGDSP relative to the [Ca^{2+}]) just prior to GRGDSP application. The inset to Fig. 6C shows the same dishes treated with the Ca^{2+} ionophore 4Br-A23187 upon completion of the experiment. Treatment with 4Br-A23187 caused [Ca^{2+}]_i changes in the micromolar range in both the presence and absence of bafilomycin A1, demonstrating that Fluo-3 fluorescence intensity had not been saturated by GRGDSP treatment.

The fact that none of the above pharmacological treatments completely eliminated the response to GRGDSP suggested that multiple Ca^{2+} signaling pathways were involved. This was verified by depletion of both intracellular and extracellular Ca^{2+}...
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**FIGURE 7.** Simultaneous inhibition of ryanodine- and bafilomycin A1-sensitive intracellular Ca\(^{2+}\) stores abolishes the GRGDSP-induced Ca\(^{2+}\) response. A, GRGDSP-induced Ca\(^{2+}\) response in nominally Ca\(^{2+}\)-free conditions in which ryanodine and IP\(_3\)-gated intracellular Ca\(^{2+}\) stores were depleted with thapsigargin (black trace); and lysosome-related acidic organelles was additionally blocked with bafilomycin A1 (gray trace). B, treatment of PASMCs with ryanodine (50 \(\mu\)M) and bafilomycin A1 (3 \(\mu\)M) completely abrogated the GRGDSP-elicted response (\(\Delta[Ca^{2+}]\)i = 4.4 \pm 1.7 nM, \(n = 9\) dishes) in comparison to control cells (\(\Delta[Ca^{2+}]\)i = 53.9 \pm 11.2 nM, \(n = 8\) dishes, \(p < 0.05\)). As in Fig. 6C, treatment of cells with ryanodine and bafilomycin A1 caused elevations in \([Ca^{2+}]\)i, whose peak (\([Ca^{2+}]\)i = 48.8 \pm 5.5 nM) was close to that elicited by GRGDSP in control cells (\([Ca^{2+}]\)i = 54.8 \pm 9.9 nM), suggesting that the levels of intracellular Ca\(^{2+}\) released by GRGDSP was equivalent to that released by bafilomycin A1 and ryanodine. The results from Fig. 7 collectively show that simultaneous disruption of both ryanodine/cADPR- and bafilomycin A1-sensitive intracellular Ca\(^{2+}\) stores was necessary and sufficient to abolish the GRGDSP-induced Ca\(^{2+}\) response in rat PASMCs.

*Effect of GRGDSP on cADPR Levels*—The pyridine nucleotide, cADPR, is thought to be the endogenous activator of ryanodine receptors (30). To test whether GRGDSP affected cADPR levels and thereby verify the pharmacological results of Figs. 6A and 7, we measured cADPR directly in cultured PASMCs upon exposure to GRGDSP. In unstimulated PASMCs, the concentrations of cADPR ranged from 0.5 to 2 pmol/mg of protein, with an average of 1.2 \pm 0.3 pmol/mg of total protein. Treatment of cultured PASMCs indeed increased cADPR levels as early as 30 s after treatment with GRGDSP (Fig. 8B). Maximum levels were reached by 1 min, and were sustained through 5 min, but were decreased by 45 min post-application. GRGDSP treatment elevated cADPR levels by –70% compared with untreated controls.

**FIGURE 7.** Simultaneous inhibition of ryanodine- and bafilomycin A1-sensitive intracellular Ca\(^{2+}\) stores abolishes the GRGDSP-induced Ca\(^{2+}\) response. A, GRGDSP-induced Ca\(^{2+}\) response in nominally Ca\(^{2+}\)-free conditions in which ryanodine and IP\(_3\)-gated intracellular Ca\(^{2+}\) stores were depleted with thapsigargin (black trace); and lysosome-related acidic organelles was additionally blocked with bafilomycin A1 (gray trace). B, treatment of PASMCs with ryanodine (50 \(\mu\)M) and bafilomycin A1 (3 \(\mu\)M) completely abrogated the GRGDSP-elicted response (\(\Delta[Ca^{2+}]\)i = 4.4 \pm 1.7 nM, \(n = 9\) dishes) in comparison to control cells (\(\Delta[Ca^{2+}]\)i = 53.9 \pm 11.2 nM, \(n = 8\) dishes, \(p < 0.05\)). As in Fig. 6C, treatment of cells with ryanodine and bafilomycin A1 caused elevations in \([Ca^{2+}]\)i, whose peak (\([Ca^{2+}]\)i = 48.8 \pm 5.5 nM) was close to that elicited by GRGDSP in control cells (\([Ca^{2+}]\)i = 54.8 \pm 9.9 nM), suggesting that the levels of intracellular Ca\(^{2+}\) released by GRGDSP was equivalent to that released by bafilomycin A1 and ryanodine. The results from Fig. 7 collectively show that simultaneous disruption of both ryanodine/cADPR- and bafilomycin A1-sensitive intracellular Ca\(^{2+}\) stores was necessary and sufficient to abolish the GRGDSP-induced Ca\(^{2+}\) response in rat PASMCs.

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**Note:** The text is a summary of scientific findings from a research paper, discussing the role of integrins in mobilizing intracellular Ca\(^{2+}\) in PASMCs, particularly in response to GRGDSP, and the role of cADPR as an endogenous activator in these processes. The figures illustrate experimental data and methods used to support these findings. The experimental setup involves using thapsigargin and bafilomycin to deplete Ca\(^{2+}\) from intracellular stores and then testing the response to GRGDSP in Ca\(^{2+}\)-free conditions. The results show that simultaneous inhibition of both ryanodine and acidic organelle-mediated Ca\(^{2+}\) stores is necessary for the GRGDSP-induced Ca\(^{2+}\) response, and that cADPR levels are significantly increased upon exposure to GRGDSP, indicating a role for this nucleotide in Ca\(^{2+}\) mobilization.
CD38 is a transmembrane glycoprotein residing on the plasma membrane that possesses ADP ribosyl cyclase activity (30). Cultured PASMCs were shown to express CD38 via Western analysis (Fig. 8A). Lane 1 shows post-nuclear supernatant of PASMCs probed with the anti-CD38 antibody, whereas lane 2 shows the same protein probed with the antigen-pretreated anti-CD38 antibody. A broad band of ~45 kDa (arrow) that is observed in lane 1 is absent in lane 2, indicating the specific band.

**Effect of GRGDSP on Acidic Organelles**—The pharmacological results of Figs. 6C and 7, suggesting the involvement of lysosome-like acidic organelles, was substantiated by imaging PASMCs labeled with the acidotropic dye Lysotracker Red DND-99. The fluorescence intensity of Lysotracker Red is based on the accumulation of the dye to regions of acidic pH. Cells labeled with Lysotracker Red demonstrated punctate intracellular staining patterns, reflecting areas of acidic pH (Figs. 9A and 10A, 0 min). Application of bafilomycin A1 (0.5 μM) to Lysotracker Red-labeled PASMCs (Fig. 9A, lower panel) significantly reduced the fluorescence intensity over 30 min as compared with vehicle (Me2SO, Fig. 9A, upper panel). A similar initial drop in Lysotracker Red fluorescence was seen in both vehicle and bafilomycin A1-treated cells. However, fluorescence levels were sustained thereafter in the vehicle-treated cells, whereas levels continued to decrease significantly in bafilomycin A1-treated cells (Fig. 9B). Bafilomycin A1 reduced the fluorescence by ~80% (n = 56 cells) over time compared with vehicle (n = 45), which caused an approximate 30% decrease.

Most intriguingly, treatment of PASMCs with GRGDSP (Fig. 10) affected Lysotracker Red fluorescence intensities in a concentration-dependent manner. The decrease in Lysotracker Red fluorescence elicited by GRGDSP was transient in nature as it showed a partial recovery with time. Effects of photobleaching were evident from control experiments in which vehicle (Tyrode's solution, Fig. 10B) was applied, but significantly differed from the peptide-induced effects (Fig. 10C). The degree to which GRGDSP affected the Lysotracker Red fluorescence, as well as its transient nature differed from the effect elicited by bafilomycin A1 (Fig. 9), which dissipates the lysosomal pH gradient through inhibition of the V-ATPase. These data directly demonstrate the modulation of the properties of lysosome-related acidic organelles by GRGDSP.

**DISCUSSION**

Here we characterize intracellular Ca\textsuperscript{2+} signaling by integrins in rat PASMCs. We show the presence of multiple integrins in PASMCs, and the initiation of intracellular Ca\textsuperscript{2+} signaling by soluble ligands. The Ca\textsuperscript{2+} response is independent of Ca\textsuperscript{2+} entry and IP\textsubscript{3} receptor-gated intracellular Ca\textsuperscript{2+} stores, but dependent on both RyR-gated Ca\textsuperscript{2+} stores and the lysosome-related acidic organelles. We further demonstrate the ability of soluble integrin ligands to directly affect RyR-gated Ca\textsuperscript{2+} stores by increasing levels of cADPR, as well as alter the properties of lysosome-related acidic compartments through Lysotracker Red imaging. These data illustrate a novel mode of Ca\textsuperscript{2+} regulation by integrins, and underscore both the RyR-gated Ca\textsuperscript{2+} store and lysosome-related acidic organelle as important players in influencing intracellular Ca\textsuperscript{2+} homeostasis in PASMCs.

We examined the largest number of integrin subtypes expressed in native rat PASM, and detected α\textsubscript{1,2,3,4,5}, α\textsubscript{6}, α\textsubscript{7}, α\textsubscript{β\textsubscript{1}, β\textsubscript{2}, β\textsubscript{3}, and β\textsubscript{4}. To date, integrins α\textsubscript{1,2,3,4,5,6}, α\textsubscript{7}, α\textsubscript{β\textsubscript{1}, β\textsubscript{2}, β\textsubscript{3}, and β\textsubscript{4} have been identified in vascular smooth muscle cells (5). A few studies have probed for integrin expression in the pulmonary vasculature (31–39). Our data largely corroborate previous reports showing the presence of α\textsubscript{1,2,3,4,5}, α\textsubscript{6}, α\textsubscript{7}, and β\textsubscript{3} in human PSM, as well as in the rat SMC line PAC-1, which possesses α\textsubscript{1,2,3,4,5,6}, and α\textsubscript{6} in human PSM, as well as in the rat SMC line PAC-1, which possesses α\textsubscript{1,2,3,4,5,6}, α\textsubscript{7}, α\textsubscript{β\textsubscript{3}, and β\textsubscript{4}} (33, 34, 36, 38). The presence of α\textsubscript{6}, α\textsubscript{7}, and β\textsubscript{3} is also consistent with that reported in rat lung tissues (32, 35, 37, 39). The lack of α\textsubscript{4} and α\textsubscript{6} in human PSM disagree with this study as well as those in mouse and PAC-1, presumably due to species differences (31, 33, 34). Another minor difference is the absence of α\textsubscript{4} in PAC-1, perhaps due to the effects of cell culture (34). Of all subtypes examined, only aortic α\textsubscript{4} was repeatedly negative. This is reminiscent of the aortic SMC line, suggesting subtle differences in integrin expression between pulmonary and systemic arteries (34).

Of the vascular smooth muscle integrins, α\textsubscript{4}β\textsubscript{1}, α\textsubscript{5}β\textsubscript{1}, α\textsubscript{6}β\textsubscript{1}, and α\textsubscript{6}β\textsubscript{3} have thus far been implicated in the regulation of...
target both $\alpha\beta_3$ and $\alpha\beta_1$, with GRGDNP showing higher specificity for the latter (23). The four peptides elicit differential $[\text{Ca}^{2+}]_i$ changes in rat PASMCs, in which the order of potency $i.e.$ GRGDNP $> \text{GRGDSP} > \text{cyclo-RGD} = \text{GRGDTP}$ suggests a vital role for $\alpha\beta_3$, rather than $\alpha\beta_1$, in PASMC function. Indeed, soluble fibronectin elicits concentration-dependent $[\text{Ca}^{2+}]_i$ changes, albeit of lower magnitude (data not shown). Despite the fact that GRGDNP elicited the greatest changes in $[\text{Ca}^{2+}]_i$, we focused this study on the effects of GRGDSP, the most thoroughly characterized and widely occurring integrin-binding peptide sequence in endogenous ECM proteins. Micromolar concentrations of RGD peptides have been shown to inhibit, whereas millimolar concentrations have been demonstrated as activating integrins (41).

Integrin ligation causes $[\text{Ca}^{2+}]_i$ changes in many cell types, but the source of $\text{Ca}^{2+}$ mobilized is both integrin and cell-type specific. For example, endothelial integrin ligation causes $\text{Ca}^{2+}$ influx and $\text{Ca}^{2+}$ release from $\text{IP}_3$-sensitive intracellular stores (42, 43). In Jurkat T cells, integrin ligation activates $\text{IP}_3$ and cADPR-sensitive stores as well as capacitative $\text{Ca}^{2+}$ entry (44). Smooth muscle $\text{Ca}^{2+}$ signaling by integrins has as yet only been studied in rat cremaster arterioles and renal SMCs. In rat cremaster arterioles, integrins differentially regulate the L-type $\text{Ca}^{2+}$ channel, where $\alpha\beta_3$ and $\alpha\beta_1$ activate and $\alpha\beta_3$ inhibits the L-type $\text{Ca}^{2+}$ channels, causing vasoconstriction and vasodilation, respectively (8, 12, 40). Stimulation of rat renal vascular smooth muscle cells by soluble GRGDSP causes $\text{Ca}^{2+}$ release entirely from RyR-sensitive stores (10, 11).

Our investigations into the source of $\text{Ca}^{2+}$ mobilized by GRGDSP offer unique information on signaling by integrins. Specifically, $\text{Ca}^{2+}$ entry and $\text{IP}_3$-sensitive $\text{Ca}^{2+}$ stores are uninhibited, whereas inhibition of the RyR-gated $\text{Ca}^{2+}$ store and the lysosome-related acidic organelle each reduce the GRGDSP-mediated $\text{Ca}^{2+}$ response by $\sim 50\%$. Furthermore, simultaneous inhibition of the RyR-gated $\text{Ca}^{2+}$ store and the lysosome-related acidic organelle is essential to completely abolish the GRGDSP-induced response.
Ryanodine receptor-gated intracellular Ca\(^{2+}\) stores have been implicated pharmacologically as contributing to integrin-mediated [Ca\(^{2+}\)]\(_i\) increases, as in the case of rat renal vascular smooth muscle cells, cardiomyocytes, and T cells (10, 11, 44, 45). However, our study is the first to directly demonstrate that integrin binding peptides increase the levels of cADPR, which is the endogenous activator of RyRs. It has been well established that cADPR contributes to the regulation of arterial smooth muscle tone by altering [Ca\(^{2+}\)]\(_i\) homeostasis. In the pulmonary vasculature, cADPR plays important roles in the development of hypoxic pulmonary vasoconstriction (46). The resting cADPR levels obtained in this study from cultured PASMCs (~1.2 pmol/mg of protein) is in agreement with that reported in the PASM tissue (~1.5 pmol/mg of protein) (47). These concentrations are also similar to those described in airway smooth muscle (~0.8 pmol/mg of protein) as well as uterine smooth muscle (~1 pmol/mg of protein) (48–50). GRGDSP causes increases in cADPR levels as early as 30 s post-application. This time course is consistent with that observed for [Ca\(^{2+}\)]\(_i\) measurements, where [Ca\(^{2+}\)]\(_i\) levels were on the rise by 30 s, and increased further at 1 and 5 min. The [Ca\(^{2+}\)]\(_i\) at 5 min post-GRGDSP treatment is generally double of that observed at 1-min post-application. This differs from that seen for [cADPR], whose normalized values vary only by 5% between 1 and 5 min post-treatment, and can be due to a contribution of the cADPR-independent, bafilomycin-sensitive intracellular Ca\(^{2+}\) store.

Thapsigargin-insensitive intracellular Ca\(^{2+}\) stores have increasingly received attention over the last decade, and are responsive to NAADP, which is structurally related to the coenzyme NADP (51). This NAADP-sensitive intracellular Ca\(^{2+}\) store has been described in various systems including PASMCs, and has been ascribed to a lysosome-related acidic compartment in sea urchin eggs, pancreatic acinar cells, MIN6 cells, and PASMCs (25, 29, 52). However, research in this field is still in its infancy and therefore controversial. Some studies show NAADP targeting a unique receptor in the lysosome, whereas others argue that it directly activates either the ryanodine or IP\(_3\) receptors, as in skeletal muscle and T-lymphocytes (53, 54). Furthermore, some assert that NAADP receptors are functionally coupled to IP\(_3\) and/or ryanodine receptors, such that Ca\(^{2+}\) mobilization by NAADP causes Ca\(^{2+}\)-induced Ca\(^{2+}\)-release, generating global [Ca\(^{2+}\)]\(_i\) transients mediated by the IP\(_3\) and/or ryanodine receptors (55–57).

The sensitivity of the GRGDSP-elicited [Ca\(^{2+}\)]\(_i\) response to ryanodine and bafilomycin A1 is consistent with that observed by Evans and colleagues (25). They have proposed that NAADP evokes spatially localized “calcium bursts” that trigger RyR-mediated Ca\(^{2+}\)-induced Ca\(^{2+}\)-release through “lysosome-SR” junctions, because RyR inhibition eliminates the global Ca\(^{2+}\) transients described in this study.

**FIGURE 10.** GRGDSP affects lysosome-related acidic organelles in PASMCs in a concentration-dependent manner. *A*, representative images of the effect of control (Tyrode’s solution), 1.25 mM GRGDSP, and 5 mM GRGDSP on the Lysotracker Red staining of PASMCs before and 6 min after treatment. *B*, average Lysotracker Red fluorescence intensities over time. Time 0 indicates the point immediately prior to treatment (arrow).
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transients without affecting Ca\(^{2+}\) bursts (24, 25). However, our results cannot be explained entirely by this model, because thapsigargin treatment or inhibition of RyRs alone did not eliminate the global [Ca\(^{2+}\)] increase. Rather, in our preparation the RyR-gated Ca\(^{2+}\) store and lysosome-related acidic compartments each contributed to half of the GRGDSP-induced Ca\(^{2+}\) response, and had to be inhibited together to abrogate the response. Whereas it is conceivable that these two sources of intracellular Ca\(^{2+}\) may be functionally coupled, GRGDSP does not seem to evoke Ca\(^{2+}\)-induced Ca\(^{2+}\) release from either store. Instead our results suggest that it activates Ca\(^{2+}\) release from these two stores via independent pathways.

Based on the fact that: 1) bafilomycin A1 reduces the intracellular Ca\(^{2+}\) response to GRGDSP, and 2) both bafilomycin A1 and GRGDSP directly affect the properties of acidic organelles as visualized by Lysotracker Red imaging, we can conclude that GRGDSP stimulates lysosome-related organelles. The seemingly transient nature of the GRGDSP-induced decrease in Lysotracker Red fluorescence (Fig. 10B) suggests that it causes a loss of organelle acidification, rather than disrupting the integrity of the lysosome as in the case of compounds such as glycyl-l-phenylalanine 2-naphthylamide (58, 59). A correlation between lysosomal pH and lysosomal [Ca\(^{2+}\)] ([Ca\(^{2+}\)]\(_{lys}\)) has been shown in macrophages, where alkalinization of lysosomes resulted in proportional decreases in [Ca\(^{2+}\)]\(_{lys}\) (28). The mechanism of [Ca\(^{2+}\)]\(_{lys}\) regulation in macrophages was likened to that in yeast vacuoles, in which the V-ATPase maintains a proton gradient that in turn drives [Ca\(^{2+}\)]\(_{lys}\) uptake through a Ca\(^{2+}\)/H\(^+\) exchanger in the lysosomal membrane (28). The fact that GRGDSP reduces the Lysotracker Red fluorescence therefore suggests a proportional decrease in [Ca\(^{2+}\)]\(_{lys}\), which is accompanied by a transient alkalinization of the lysosome. Because bafilomycin A1 also promotes lysosomal alkalinization, it is possible that pretreatment of PASMCs with bafilomycin A1 (Figs. 6C and 7B) causes concomitant [Ca\(^{2+}\)]\(_{lys}\) depletion, such that this Ca\(^{2+}\) source is no longer available for stimulation by GRGDSP. Given that NAADP evokes Ca\(^{2+}\) release from lysosome-related acidic organelles in a bafilomycin A1-sensitive manner, we hypothesize that GRGDSP mobilizes lysosomal Ca\(^{2+}\) in part via NAADP (24, 25, 29, 52). However, we have yet to establish a direct link between GRGDSP treatment and induction of the NAADP pathway. Future studies on the change in cellular NAADP concentrations upon GRGDSP treatment should address this pressing issue.

This study raises interesting questions regarding the mechanism of integrin-mediated Ca\(^{2+}\) signaling. In particular, how does integrin activation couple to Ca\(^{2+}\) mobilization from both RyR-gated stores and acidic organelles? One plausible explanation is that GRGDSP activates ADP-ribosyl cyclase. ADP-ribosyl cyclase synthesizes both cADPR and NAADP from β-NAD and β-NADP, respectively, and its activation by GRGDSP may thus explain the near equivalent contribution of these two Ca\(^{2+}\) stores to the peptide-induced response (60). High levels of ADP-ribosyl cyclase activity have in fact been reported in the PASM tissue (47). We have also evidenced robust levels of ADP-ribosyl cyclase activity in cultured PASMCs, using NGD\(^{+}\) as an alternative substrate to measure cyclic GDP ribose formation (data not shown), and have, furthermore, shown the expression of CD38 in these cells (this study). The lung is among a group of tissues containing a novel enzyme with ADP-ribosyl cyclase activity in addition to CD38 (61–63). This notion is supported by data from CD38\(^{−/−}\) mice containing an appreciable amount of cADPR in the lung albeit at lower levels than in the wild type, as well as the fact that cADPR synthesis has been measured in microsomal fraction of PASM homogenates (47, 63, 64).

Stimulation by GRGDSP may result in Ca\(^{2+}\) mobilization from the RyR-gated stores and acidic organelles, alternatively, by causing integrin clustering and subsequent protein recruitment to form focal adhesion complexes, which tether integrins to the cytoskeleton. Many components of the focal adhesion complex are either substrates of tyrosine kinases or tyrosine kinase themselves, and are activated upon integrin ligation in various cell types, including endothelial and vascular smooth muscle cells (65, 66). Moreover, in rat cremaster arterial cells, [Ca\(^{2+}\)] modulation through the activation of tyrosine kinase pathways has been shown for α\(_{i}\)β\(_{i}\) and α\(_{i}\)β\(_{i}\) stimulation (12, 67).

In summary, we have demonstrated the modulation of [Ca\(^{2+}\)] in PASMCs by integrin agonists. The information obtained adds a level of complexity in PASMC regulation, uncovering a unique mode of Ca\(^{2+}\) signaling by integrins in which RyR-gated Ca\(^{2+}\) stores and acidic organelles are recruited. Modulation of PASMCs by integrin ligands has important implications in pulmonary diseases including chronic obstructive pulmonary disease, pulmonary fibrosis, and pulmonary hypertension, where ECM remodeling as well as induction of matrix metalloproteinases that degrade ECM molecules is observed (68, 69). ECM remodeling and degradation expose cryptic integrin binding sites including RGD motifs (69). Therefore our work on [Ca\(^{2+}\)] regulation by soluble RGD peptides highlights the importance of the ECM in influencing pulmonary function and may provide valuable insights in the progression of pulmonary disease.

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