Abstract— Apoptosis of smooth muscle cells (SMC) due to changes in extracellular matrix has emerged as an important factor underlying vascular and visceral disease progression. The calcium signal induced by extracellular matrix fragmentation also plays a pivotal role in cell death and disease evolution. Here, we found that KDGEA, a particular motif of type I collagen, induced rat duodenal SMC death in cultures through specific interaction with $\alpha_5\beta_1$ integrin signaling as suggested by (1) the absence of KDGAA effect, an inactive analog of KDGEA, or LDV, a fibronectin fragment that recognizes $\alpha_5\beta_1$ integrin, and (2) the inhibition of SMC death induced by KDGEA when $\alpha_5\beta_1$ expression was knocked down by antisense oligonucleotide. DNA fragmentation was also detected in KDGEA-treated cells, thus suggesting activation of an apoptotic process. These KDGEA-activated processes were totally blunted by pharmacological modulation of Ca$^{2+}$ entry through T-type Ca$^{2+}$ channels and antisense oligonucleotides directed against these channels. KDGEA also increased caffeine-induced calcium release and this effect was abolished when cultures were treated with either antisense oligonucleotide or T-type channel blockers. These results suggested that type I collagen peptide KDGEA can induce the death of SMC through T-type Ca$^{2+}$ channel activation and alteration of calcium release from sarcoplasmic reticulum.

Keywords — apoptosis, calcium entry, extracellular matrix, integrin

I. INTRODUCTION

Many diseases are associated with extracellular matrix (ECM) remodeling characterized in some cases by a pronounced type I collagen deposition, as observed during tissue fibrosis and remodeling (1, 2). For example, in scleroderma, collagen degradation coincides with SMC rarefaction surrounded by areas of focal fibrosis (3) resulting in hypomotility, lumen dilatation, tensile rigidity and loss of organ functions (4) in pulmonary, peripheral vascular, renal, cardiac and gastrointestinal systems. In vessels, atherosclerosis lesions are rich in type I and III collagens constituting up to 60% of the total plaque proteins (5) that contribute to plaque stability. As atherosclerosis progresses, degradation of fibrous cap collagens and SMC apoptosis coincide with sites of plaque rupture and have both been implied in pathogenesis (6, 7). In other vascular diseases such as aneurysms and dissections of ascending aorta, the inflammatory process leads to protease-mediated degradation of ECM and collagen disruption. These processes act in concert with SMC death to progressively weaken the aortic wall, resulting in dilatation and aneurysm formation or aortic dissections (8, 9).

Although contribution of collagen remodeling and SMC death to various diseases has been well documented, precise mechanisms involved in this phenomenon remain unclear.

Integrins represent a large family of 24 ECM receptors with different ligand specificity, in which $\alpha_1\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$ and $\alpha_6\beta_1$ form a subset of collagen-binding integrins (10). ECM binding to integrin can change intracellular Ca$^{2+}$ concentration ([Ca$^{2+}]_i$) in many cell types including platelets, neutrophils, monocytes, lymphocytes, fibroblasts, endothelial, epithelial and SMC. As a result, a myriad of cellular functions such as differentiation, contraction, relaxation or apoptosis are affected (11-13). In vascular smooth muscle for example, damage to ECM after tissue injury or remodeling could be involved in vascular tone modulation. Indeed, the use of peptides and specific antibodies showed that ligands of $\alpha_5\beta_1$ or $\alpha_6\beta_1$ (fibronectin receptors) and $\alpha_1\beta_1$ (vitronectin receptor) integrins reciprocally regulated Ca$^{2+}$ flow through L-type Ca$^{2+}$ current in myocytes of rat skeletal muscle arterioles (12-14).

Disturbances of Ca$^{2+}$ regulation by sarcoplasmic reticulum (SR) can play an important role in cell death (15). SR Ca$^{2+}$ overload or depletion can lead to calpain activation, which induces apoptosis through BAD, Bid and caspase-12 (16). These studies suggest a strong link between abnormal myocyte Ca$^{2+}$ handling, SR and cell death.

Here, we investigated the role of KDGEA (Lys-Asp-Gly-Glu-Ala), a short soluble collagen type I-derived motif specifically recognized by the collagen-binding integrin...
αβ₁ (17) on SMC survival and Ca²⁺ signaling. We found that KDGEA induced SMC death via Ca²⁺ entry through T-type Ca²⁺ channels causing SR Ca²⁺ signal alteration.

II. METHODS

Cell culture - Investigations conform to the European Community and French guiding principles for care and use of animals. Authorization to perform animal experiments (A-33-00031 to NM) was obtained from the Préfecture de la Gironde (France). Rat duodenum SMC were obtained as previously described (18). Then, SMC were seeded on glass slides (density: 10⁵ cells/mm²) in 24-well plates and maintained in short term primary culture (37°C, 95% air, 5% CO₂) for 6 days in M199 medium containing 10% fetal calf serum, 20U/ml penicillin and 20µg/ml streptomycin. Before treatment, SMC cultures were serum-deprived to 0.5% serum overnight.

Cell Treatment - KDGEA (Lys-Asp-Gly-Glu-Ala), KDGAA (Lys-Asp-Gly-Ala-Ala) and LDV-containing peptide (Glu-Ile-Leu-Asp-Val-Pro-Thr) were purchased from Eurogentec (Serain, Belgium). For survival assessment, peptides were added to cultures (day 6) at different concentrations (10 to 200 µM) for 24 hours before analysis by WST-1 test or DNA gel electrophoresis. In some experiments, tetra acetoxymethyl ester (BAPTA-AM, 10⁻⁶ M, 1,2-bis(2-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid (Glu-Ile-Leu-Asp-Val-Pro-Thr) were purchased from Eurogentec (Serain, Belgium). For survival assessment, peptides were added to cultures (day 6) at different concentrations (10 to 200 µM) for 24 hours before analysis by WST-1 test or DNA gel electrophoresis. In some experiments, tetra acetoxymethyl ester (BAPTA-AM, 10⁻⁶ M, 1,2-bis(2-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid (T-AM, 10 µM, Fluoprobes-Interchim, France) was added for 30 minutes and removed before KDGEA addition. In other experiments, pharmacological drugs such as Nickel (Ni²⁺, 50 µM), Mibebradil (1 µM), Pimozide (1 µM), Isradipine (1 µM), Verapamil (10 µM), purchased from Sigma-Aldrich, were applied 30 minutes before KDGEA-treatment. In calcium and electrophysiological experiments, peptides were added 3 hours before analyzes.

Survival assessment - SMC cultures grown to confluence in 24-well plates were treated in quadruplicate for 24 hours as indicated above. At the end of experiment, cells were washed with PBS. Remaining viable adherent cells were assessed using WST-1 test following manufacturer’s instructions (Roche diagnostic, France). Briefly, WST-1 diluted to 1/10 in culture medium was added and plates were incubated (37°C, 30 minutes). The absorbance was measured at 440 nm. Each experiment was performed in quadruplicate in 3 independent cultures.

DNA ladders - In order to determine DNA ladder fragmentation, a hallmark of apoptosis, cell cultures used for WST-1 test were further submitted to total genomic DNA extraction using DNA preparation kit from Epicentre (Madison, WI, USA) and according to manufacturer’s instructions. DNA concentration was analyzed by biophotometer (Eppendorf, Le Pecq, France) and 10 µg was separated by 2% agarose gel electrophoresis containing 0.5 µg/mL ethidium bromide. Gels were photographed under UV illumination by EDAS120 and analyzed with KDS1D 2.0 software (Kodak Digital Science, Paris, France). Experiments were performed in duplicate in 3 independent cultures.

RT-PCR reactions - Total RNA was extracted using RNA preparation kit (Epicentre, Madison, WI), following supplier instructions. Three conditions were tested: RNA from longitudinal layers of duodenal smooth muscle (DR), from SMC freshly isolated (J₀) or from our model of cell cultured for 6 days in control (J₄), or after antisense oligonucleotide treatment. RNA concentration was determined by biophotometer (Eppendorf, Le Pecq, France). Reverse transcription and polymerase chain reactions were performed as described previously using Qiagen kits (Qiagen, Hilden, Germany) (18). PCR annealing temperatures were: 59.2°C, 57.5°C or 60°C for α₂, α₄ integrins or T-type channel primer pairs, respectively. Sense (s) and antisense (as) oligonucleotides were designed using lasergene software (DNASTAR, Madison, WI, USA) according to sequences deposited in GenBank (accession numbers BC099151, NM001107737 for α₂ and α₄ integrins, respectively). Nucleotide sequences for each primer pair were: α₂: s: 5’-TGAGGATACAAAAACAGAAG-3’, as: 5’-CATAGCCACACAGATTCC-3’, α₄: s: 5’-CAATGCCTCGTGTCACACT-3’, as: 5’-GGGGCCCATGCAATCAT-3’, β₂-microglobuline: s: 5’-ATTCAAGAAAATCTCCCAAATTCAGT-3’, as: 5’-GATTACATGTCCTCGTTCAGG-3’. In some experiments, amplicon relative amounts were determined and normalized to that of β₂-microglobuline. Each experiment was repeated twice in 3 independent experiments.

Antisense oligonucleotide treatment - Phosphorothioate antisense oligonucleotides labelled by 5’-Cy5 indocarbocyanin to visualize cellular uptake were designed based on rat α₂ integrin sequence in regions lacking known splice variants (asα₂: 5’-CCCCGTCAGATACAGAAGGAGTGTG-3’) and synthesized by Eurogentec. T-type calcium channel phosphorothioate antisense oligonucleotide has been previously published (asCaV₃: 5’-TCCACCCACGAGGCCAAATCATGTT-3’) (19). To set free results from Jet-Pei™ transfection, we used as control the sequence 5’-TCAACCCCCGACCACACCAACTGTT-3’ named scrambled (SCR) which was not complementary to any rat registered nucleotide sequences. Antisense oligonucleotides (50 nM) were reconstituted in NaCl 150 mM before transfection in myocytes cultured for 2-4 days with Jet-Pei™ solution according to manufacturer’s instructions. Experiments were performed in 3-5 independent cultures.

Membrane current recordings - Voltage-clamp and current-clamp recordings were made with a standard patch-clamp technique using List EPC-7 patch-clamp amplifier (Darmstadt-Eberstadt, Germany). Whole-cell recordings were performed at room temperature with patch pipettes (resistances: 2-5 MΩ). Membrane potential and current records were analyzed using Pclamp system software (Molecular Devices, Foster City, CA). Junction potential was electronically compensated before acquisition with EPC-7 patch-clamp amplifier (7.1 ± 1 mV, n=5 for K⁺ solution and 4.5 ± 0.8 mV for Cs⁺ solution). T-type currents were measured by test pulse from -80 to -30 mV, a potential where T-type channels were mostly activated. L-type currents were measured at 0 mV from a holding potential of -60 mV where T-type channels are inactivated (20). Cells were superfused with extracellular...
solution (Ca\textsuperscript{2+} solution) containing (mM): 130 NaCl, 5.6 KCl, 1 MgCl\textsubscript{2}, 2 CaCl\textsubscript{2}, 11 glucose, 10 HEPES, pH 7.4 with NaOH. The basic pipette solution (K\textsuperscript{+} solution) contained (mM): 5 NaCl, 50 KCl, 65 K\textsubscript{2}SO\textsubscript{4}, 2 MgCl\textsubscript{2}, 2 ATP, 10 HEPES, pH 7.3 with KOH. To record the T-type current more accurately, without contamination by K\textsuperscript{+} currents, an intracellular Cs\textsuperscript{+} solution was used (mM): 130 CsCl, 10 EGTA, 5 ATPNa\textsubscript{2}, 2 MgCl\textsubscript{2}, 10 HEPES, pH 7.3 with CsOH. Drugs were applied on cells via pressure ejection from a glass pipette.

\textbf{[Ca\textsuperscript{2+}]\textsubscript{i} measurement} - Measurements of free [Ca\textsuperscript{2+}]\textsubscript{i} were assessed as described previously (21, 22). Briefly, myocytes were loaded (30 minutes, 37°C) with membrane permeant Ca\textsuperscript{2+} dye indo-1-AM (10 \textmu M, Molecular probes-Interchim, France). Fluorescence intensities (F405 and F485) were measured for one excitation (\lambda\textsubscript{ex} 340 nm) at two emission wavelengths (\lambda\textsubscript{em} 405 and 485 nm). Changes in [Ca\textsuperscript{2+}]\textsubscript{i} were estimated from the changes in the F405/F480 fluorescence ratio (R) as described previously (23). R\textsubscript{basal} is the R value obtained in basal conditions (during 10 s before stimulation). Response amplitudes are expressed as \Delta R / R\textsubscript{basal} (\delta R = maximal R value obtained after stimulation - R\textsubscript{basal}). Caffeine was applied by pressure ejection from a glass pipette. All experiments were carried out at 26±1°C.

\textbf{Statistical analysis} - Student's t-tests or for multiple comparisons, one way ANOVA combined with Dunnett and Student-Newman-Keuls post-hoc test were used and considered significant at p<0.05. Results are presented as mean±s.e.m. and n is the number of independent experiments.

III. RESULTS

KDGEA peptide induced smooth muscle cell death.

KDGEA is a particular soluble motif of type I collagen that specifically recognizes \alpha\textsubscript{2}\beta\textsubscript{1} integrin [17]. To assess peptide effects on cell survival, duodenal SMC primary cultures were treated by KDGEA increasing concentrations for 24 hours.

![Fig. 1. KDGEA-induced apoptosis of duodenal SMC in culture. A. Statistical representation of myocyte survival assessed by WST-1 method after 24h treatment with KDGEA, KDGAA (100 \textmu M) or LDV-containing peptide (100 \textmu M), (n=3 in quadruplicate; ***p<0.001). B. KDGEA-induced internucleosomal DNA cleavage. C. RT-PCR expression of \alpha\textsubscript{2} and \alpha\textsubscript{4} integrins in duodenal smooth muscle tissue (DR), and duodenal smooth muscle cells freshly isolated (J\textsubscript{0}) or cultured for 6 days (J\textsubscript{6}).](image-url)

For low concentrations (10, 50 \textmu M), KDGEA did not modify cell survival (96.8±1.1% and 94.7±2.4%, respectively, n=3) assessed using WST-1 test (Fig. 1A). However, for higher concentrations (100, 200 \textmu M), KDGEA dramatically reduced myocyte survival (62.7±2.5%, n=10 and 54.3±1.2%, n=3, respectively, p<0.001 vs control). Oligonucleosomal-length DNA fragmentation, a hallmark of apoptosis (24), was detected by agarose gel electrophoresis (Fig. 1B) suggesting that KDGEA (100 \textmu M) induces cell death through an apoptotic mechanism.

Other peptides were tested on myocyte survival to check whether KDGEA cell death was mediated specifically by \alpha\textsubscript{2}\beta\textsubscript{1} integrin. These experiments showed that neither KDGAA (100 \textmu M), the inactive analog of KDGEA, nor LDV-containing peptide (100 \textmu M), a fibronectin sequence that specifically binds \alpha\textsubscript{4}\beta\textsubscript{1} integrin, induced cell death (104.6±4.9%, n=4 and 92.0±5.8%, n=3, respectively, NS vs control) (Fig. 1A).
result was corroborated by the absence of oligonucleosomal DNA fragmentation (Fig. 1B). \(\alpha_2\) and \(\alpha_4\) integrin expressions in longitudinal layers of duodenal smooth muscle as well as in SMC freshly isolated or cultured for 6 days were confirmed by RT-PCR (Fig. 1C).

To confirm that KDGEA-induced cell death was mediated through \(\alpha_2\beta_1\) integrin activation, we used two antisense oligonucleotides, one to inhibit \(\alpha_2\beta_1\) expression, and a scrambled one used as control to set results free from Jet-Pei™ transfection. To demonstrate antisense oligonucleotide efficiency, we analyzed endogenous \(\alpha_2\) mRNA expression by RT-PCR in tested conditions. Values are expressed as relative \(\alpha_2\) mRNA amounts compared to \(\beta_2\)-microglobuline levels (Fig. 2A). The \(\alpha_2\) expression was comparable in control and SCR conditions, whereas it was dramatically decreased in as\(\alpha_2\)-treated cultures.

KDGEA-induced cell death was sensitive to calcium entry through \(T\)-type calcium channels.

Changes in [Ca\(^{2+}\)] are involved in numerous intracellular events, including triggering of cell death [24,25]. By buffering intracellular Ca\(^{2+}\) to assess its putative role in our apoptotic model, we found that BAPTA-AM (10 \(\mu\)M), a permeant Ca\(^{2+}\) chelator, totally blunted KDGEA-induced cell death (102.9±15.3\%, \(n=3\) independent cultures, NS vs control) (Fig. 3A) and suppressed oligonucleosomal DNA fragmentation (data not shown).

In these conditions, in the absence of KDGEA, cell survival showed no difference between SCR and as-\(\alpha_2\)-treated conditions (Fig. 2B). In contrast, after KDGEA application myocyte survival was highly reduced in SCR conditions (55.4±7.1\% \(n=3\), \(p<0.001\)) but not in as-\(\alpha_2\)-knockdown cultures (99.9±4.4\%, \(n=3\); Fig. 2B).

Taken together, these results indicated that KDGEA induced duodenal SMC death through specific interaction with \(\alpha_2\beta_1\) integrin signaling.

The putative extracellular origin of Ca\(^{2+}\) was first assessed pharmacologically. In control conditions, Ca\(^{2+}\) channel blockers did not modify cell survival (Fig. 3A). In the presence of KDGEA, inhibitors of L-type Ca\(^{2+}\) channels, namely Verapamil (10 \(\mu\)M) and Isradipine (1 \(\mu\)M) did not modify KDGEA-induced apoptosis while T-type Ca\(^{2+}\) channel blockers Pimozide (1 \(\mu\)M), Mibebradil (1 \(\mu\)M) or Nickel (50 \(\mu\)M) totally abrogated it (Fig. 3A). Moreover, RT-PCR analysis revealed that in duodenal smooth muscle as well as in SMC freshly isolated or cultured for 6 days, expression of \(\alpha_{1G}\) and \(\alpha_{1H}\) subunits but not of \(\alpha_{1I}\) were detected (Fig. 3B).

Voltage-gated Ca\(^{2+}\) currents were recorded by a standard patch-clamp technique to functionally confirm their presence in duodenal smooth muscle cells. Figure 4A represents a typical whole-cell Ca\(^{2+}\) current with a two-component inward current evoked by a long depolarization pulse from -80 to -10 mV. Each component could be isolated on the basis of their voltage dependence of activation and inactivation. The first component was a transient peak inward current (open square) that presented typical features of T-type Ca\(^{2+}\) currents (22).
Transient component was followed by a sustained inward component (filled square) that was activated for higher voltage step and inactivated more slowly (Fig. 4A-B), as described for L-type calcium currents (22).

Having demonstrated that T- and L-type currents coexist in duodenal SMC, we tested if their pharmacological profile was consistent with survival data obtained previously. T-type currents were elicited by test pulse from -80 to -30 mV, a potential where they were mostly activated. L-type calcium currents were measured at 0 mV from a holding potential of -30 mV, a holding potential where T-type channels were inactivated (22). In these conditions, Nickel application (50 µM) inhibited T-type currents but partially reduced L-type currents (Fig. 4B). The ability of Calcium channel blockers to discriminate between T-type and L-type currents is summarized in Figure 4C. These results showed that Mibefradil (1 µM) inhibited preferentially (−70%) T-type currents, whereas Isradipine (1 µM) and Verapamil (10 µM) produced a significant inhibition of L-type currents with a lower alteration of T-type currents.

To confirm T-type channels’ involvement in KDGEA-induced cell death, we used two antisense oligonucleotides, one to inhibit the expression of T-type channels (asCav3), and a scrambled one used as control to set results free from Jet-Pei™ transfection. RT-PCR expression of T-type channels detected previously was used to assess antisense oligonucleotide efficiency (Fig. 5A). Values are expressed as relative α1G and α1H mRNA amounts compared to β2-microglobuline levels. α1GI and α1HI mRNA expressions were comparable in control and SCR conditions, whereas they were dramatically decreased in asCav3-treated cultures.

In these conditions, myocyte survival assessment showed no significant differences between SCR and asCav3 cultures (Fig. 5B). However, in KDGEA-treated cells myocyte survival was highly reduced in SCR condition, whereas in asCav3 condition KDGEA did not modify myocyte survival.

All together, these results suggested that the KDGEA impairment of SMC survival was highly dependent upon calcium entry through T-type calcium channels.
KDGEA treatment enhanced calcium entry and modified sarcoplasmic reticulum calcium signaling.

We further examined the regulation of calcium entry through T-type calcium channel by measuring the effect of KDGEA peptide on T-type current activated by a step depolarisation from -80 to -30 mV. The transient increase in [Ca²⁺], in 3h-KDGEA-treated cells was higher than in control cells, as illustrated in Figure 6A and 6B.

Fig. 6. KDGEA-treated cells exhibited a higher [Ca²⁺], increase induced by T-type channel activities. A. Typical Ca²⁺ response elicited by a step depolarization from -80 to -30 mV in control condition or after a 3h-KDGEA treatment. B. Statistical representation of basal [Ca²⁺], after a 3h-KDGEA treatment (left) and KDGEA calcium response elicited by a step depolarization (right). Calcium responses were measured as difference between resting basal level and peak of Ca²⁺ transient. Cells were loaded with indo-1-AM (*p<0.05; n=7 experiments).

Since calcium entry could induce apoptosis through alteration of [Ca²⁺], mitochondrial and/or SR Ca²⁺ contents (25), [Ca²⁺], changes were recorded using indo-1-AM, in resting conditions or following short applications (10 seconds) of either (1) CCCP, a mitochondrial uncoupler that triggers Ca²⁺ release from mitochondria (25), or (2) caffeine, an activator of ryanodine receptor that triggers Ca²⁺ release from SR. In 3h-KDGEA-treated cultures, basal [Ca²⁺], did not differ from control conditions (1.37±0.03, n=16 cells in KDGEA-treated conditions vs 1.34±0.04, n=20 cells in control) (Fig. 6B), suggesting that cytosolic calcium homeostasis was conserved during the first hours of KDGEA application.

Application of CCCP caused a slow and transient rise in [Ca²⁺], with no difference between control and KDGEA-treated cells (data not shown). In contrast, caffeine application induced a transient increase in [Ca²⁺], with a magnitude greater in KDGEA-treated cells than in controls (Fig. 7A). This signal depended upon α₂ integrin activation since α₂α₂-treated cultures presented a magnitude of caffeine-induced calcium increase similar to control levels (Fig. 7B). Moreover, in the presence of calcium channel blockers, SR function assessment by caffeine application showed that Isradipine did not prevent calcium signal increase. In contrast, Mibebradil reduced this phenomenon to a control level (Fig. 7B). Furthermore, asCav3 prevented SR calcium increase evoked by caffeine (Fig. 7B).

Fig. 7. SR-calcium alteration evoked by caffeine (10 mM) in KDGEA-treated SMC. A. Intracellular calcium signal recorded following a 10-sec caffeine application in control or KDGEA-treated cells loaded with indo-1-AM. B. Statistical representation of caffeine-induced [Ca²⁺]; amplitude in various tested conditions (***p<0.001 as compared with CTL, *p<0.001 as compared with KDGEA; n=7 experiments).

Finally, the acute application of KDGEA could increase the T-type Ca²⁺ current by 5% (not shown).

All together, these results suggested that, in duodenal SMC, SR calcium signalling was altered in KDGEA-induced cell death through α₂ integrin interaction without alterations of cytosolic or mitochondrial calcium contents. This phenomenon involved Ca²⁺ entry through T-type calcium channels.

IV. DISCUSSION

In this study, we provide several lines of evidence that the collagen fragment KDGEA peptide impaired cell survival of cultured duodenal SMC through T-type Ca²⁺ channel activation and alteration of SR Ca²⁺ release.

We observed a dramatic reduction of cell survival after treatment of duodenal SMC with KDGEA (100 µM), a fragment of type I collagen. This is in line with previous studies which showed that degraded type I collagen can promote initiated calpain-mediated disassembly of focal adhesions and loss of vascular SMC attachment to ECM (1). This phenomenon is at least partly dependent on α₂α₂ and α₂β₃-integrins and could promote SMC proliferation, migration and invasion into arterial intima (26, 27). Recently, two studies showed that the same type I collagen fragments could either activate apoptosis of vascular SMC by calpain-mediated inactivation of anti-apoptotic proteins such as xIAP (X-chromosome-linked inhibitor of apoptosis), or promote survival by a mechanism involving αβ₃-dependent NF-κB activation with consequent activation of IAPs (28, 29). Here we showed that, as vascular SMC, duodenal SMC also activated a cellular death process in the presence of a collagen fragment. This SMC death presented the DNA fragmentation feature of apoptosis. Moreover, by our peptidic approach, we have identified that the KDGEA sequence was sufficient to induce this process. KDGEA...
contains the minimal active recognition sequence for \( \alpha_2 \beta_1 \) integrin that corresponds to the 435-438 residues of alpha 1(I)-CB3 fragment of type I collagen (17). This collagen often predominates in neomatrix of fibrotic smooth muscle tissues such as peripheral vascular and gastrointestinal systems of systemic sclerosis patients or during progression of atherosclerosis (3-9).

KDGEA-induced cell death seemed independent from cellular adhesion since \( \alpha_2 \beta_1 \) integrin knockdown treatment did not modify cell survival, as compared to the scrambled condition. This is consistent with the fact that \( \alpha_2 \beta_1 \)-deficient mice are viable, fertile and develop normally (30). This suggests that in our experimental model KDGEA played a pivotal role in cell death induction through its interaction with \( \alpha_2 \beta_1 \) integrin signaling. However fibrosis, and especially collagen degradation and turnover, could produce several peptides which can present different biological activities. The cellular decision between life and death would then be governed by integration of signals from all engaged integrins and could have an impact on disease progression.

Whatever was the cell death activated by KDGEA, the important feature of this study is that this process could be totally blunted by calcium signal modulation. In SMC, as in many other cell types, calcium signaling is a universal signal transduction mediating extracellular effects of environmental signals into a specific cellular response such as contraction, proliferation or apoptosis (31). In the osteoblast-like cell line SaOS-2 (32) as in human dermal fibroblasts (33), the DGEA peptide, which is more acidic than KDGEA, induced Ca\(^{2+}\) transient by mobilizing calcium from intracellular stores when applied at millimolar ranges. In our study, KDGEA at lower concentration (100 \( \mu \)M) impaired cell survival by a calcium-dependent mechanism as shown by the suppression of cell death in cultures pre-treated with the intracellular calcium chelator BAPTA-AM. In SMC, calcium homeostasis is a complex process highly controlled by various sarcolemmal calcium channels and intracellular calcium stores which may be altered during apoptosis. In our model, pharmacological modulation of membrane calcium channels or reduction of T-type calcium channel expression by antisense oligonucleotides blunted KDGEA-induced cell death. This suggested that KDGEA interaction with \( \alpha_2 \beta_1 \) integrin reduced cell survival via calcium entry through T-type calcium channels. Few studies have reported that pharmacological inhibition of T-type calcium channels could protect against apoptosis. For example, T-type calcium channel blockers Mibebradil, Kurtoxin, Nickel, Zinc or Pimozide protected neurons from delayed ischemia-induced damage (34). In a spermatogenic cell model, Mishra et al. showed that, in SMC, T-type channel activation was involved in cell death induction. Moreover, interaction between \( \alpha_2 \beta_1 \) integrin and its ligand KDGEA was needed for their activation.

Several lines of evidence suggest that L-type channels are regulated by integrin-dependent signaling. In vascular SMC for example, at least three different integrins modulate their activity and alter vascular tone in arterioles. Whole-cell recordings show that insoluble and soluble \( \alpha_2 \beta_1 \) integrin ligands (RGD peptides, vitronectin and fibronectin fragments) inhibit L-type currents and elicit vasodilatation (13). In contrast, soluble \( \alpha_2 \beta_1 \) (LDV peptide) as well as insoluble \( \alpha_2 \beta_1 \) ligands enhance L-type calcium currents and vasoconstriction (13, 14), indicating that ECM interactions with integrins modulate vascular tone.

However, T-type channels are quantitatively a weak source of calcium entry compared with L-type channels or with SR calcium release during smooth muscle contraction. Thus, our observations raise the question as to how T-type channels can have a specific effect on SMC death. In cardiac myocytes for example, persistent increases in calcium influx through L-type channels induced SR calcium overload, correlated with apoptosis induction (36). In another study, SR calcium overload or depletion activated calpains, which induced apoptosis through BAD, Bid and caspase-12 (16). Interestingly, T-type channels can sustain a continuous calcium influx by a window current mechanism, corresponding to a voltage range where a channel population fraction is always opened and does not inactivate (20). This property could be responsible for the increase in caffeine-induced calcium release observed in KDGEA-treated SMC. Indeed, in cells treated with T-type inhibitors or antisense oligonucleotides, KDGEA-induced cell death was blunted and caffeine-induced SR calcium signal returned to a control level. Taken together, these results suggest a possible increase in SR calcium loading. This is in agreement with studies showing that SR is a component of inducible apoptosis (37). Indeed, SERCA overexpression in non-mycocytes was shown to be proapoptotic (38), whereas cells devoid of SR Ca\(^{2+}\) release channels (IP3 receptor) are resistant to apoptosis (39). In addition, proapoptotic Bel-2 proteins (BAX and BAK) induced apoptosis in part by increasing SR content, whereas antiapoptotic Bel-2 proteins protected by decreasing SR calcium content (15, 37, 40). More recently, a study performed on spermatogenic cells showed that Ca\(^{2+}\) entry through T-type channels could regulate the ratio of proapoptotic Bel-xS and antiapoptotic Bel-xL expression (35). However, our study could not exclude an enhanced ryanodine receptor activity. Indeed, in SMC, RGD-containing ligands can also mobilize ryanodine-sensitive Ca\(^{2+}\) stores through increased cyclic ADP ribose, the endogenous activator of ryanodine receptors (41).

T-type Ca\(^{2+}\) channels have been suggested to be implicated in cell death via integrin pathway because Mibebradil could activate apoptosis via the modulation of membrane potential and the modification of integrin expression in HLE-B3 cell line (42). Our results showed that integrin activation by KDGEA increases the window current of T-type Ca\(^{2+}\) channels the SR...
Ca\textsuperscript{2+} loading and/or Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release known to activate apoptosis. The pathway between integrin and T-type Ca\textsuperscript{2+} channel activation remains unknown. However, integrin-activated increases of L-type Ca\textsuperscript{2+} channel activity involved phosphorylation of the channel (43) by src and PKA (44, 45). T-type Ca\textsuperscript{2+} channels has also been shown to be activated via PKA in SMC (46) and src in neuroblastoma cell line (47). Therefore, PKA and src are good candidates to link T-type Ca\textsuperscript{2+} channels activation to integrin stimulation by KDGEA in SMC.

V. CONCLUSIONS

Collectively, these data suggest a crucial role for T-type channel activation and SR calcium signal in smooth muscle cell death induced by KDGEA. This study could give new perspectives of treatment in pathogenesis processes in which SMC rarefaction coincide with type I collagen deposition and alteration of Ca\textsuperscript{2+} signaling.

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References


