Effects of calcium signaling on coagulation factor VIIa-induced proliferation and migration of the SW620 colon cancer cell line

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Abstract. Tissue factor (TF)/VIIa/protease-activated receptor 2 (PAR2) has been shown to trigger the ERK1/2 signaling pathway. This was shown to be closely associated with the proliferation and migration of SW620 colon cancer cells; however, the detailed mechanisms remain unclear. The aim of the present study was to elucidate the effects of calcium signaling on the proliferation and migration of SW620 cells induced by coagulation factor VIIa. The results demonstrated that VIIa and PAR2 agonist PAR2-AP increased $[Ca^{2+}]_i$ in SW620 cells. In addition, VIIa-and PAR2-AP-induced ERK1/2 activation was inhibited by thapsigargin (TG)-induced depletion of intracellular Ca^{2+} stores and EGTA-mediated removal of extracellular Ca^{2+}. It was also identified that VIIa and PAR2-AP-induced proliferation and migration of SW620 cells was modulated by EGTA and TG. Taken together, the present results indicate that VIIa triggers calcium signaling in SW620 cells, in a TF-dependent manner, which is critical for VIIa-induced ERK1/2 activation in SW620 cells. These results suggested that calcium signaling had a vital role in the proliferation and migration of SW620 cells.

Introduction

Tissue factor (TF) triggers the blood coagulation cascade through binding to plasma factor VII/VIIa (1). TF has numerous functions in addition to its role in coagulation, including mediating tumor growth, invasion and metastasis (2). Protease-activated receptors (PARs) are members of the G-protein coupled receptor family typically characterized by seven-transmembrane domains. At present, four PAR members (PAR1, PAR2, PAR3 and PAR4) have been identified and characterized in human and mice (3). Several studies have demonstrated that PAR1, PAR3 and PAR4 may be activated by thrombin, resulting in platelet activation and blood coagulation. By contrast, PAR2 is resistant to thrombin but is activated by other proteases (including trypsin, coagulation factor VIIa and Xa) (4). In our previous study, the expression of TF, PAR1 and PAR2, and their functions were evaluated in various tumor cell lines, as well as in colorectal tissue specimens. It was observed that TF and PAR2 were both highly expressed in the SW620 colon cancer cell line and in numerous colorectal cancer specimens. PAR2 agonist peptide (PAR2-AP), a peptide derivative of PAR2 (amino acid sequence SLIGKV-NH_2), directly binds to PAR2 and irreversibly inhibits its function. It is a valuable tool in PAR2-associated studies. It was shown that PAR2-AP and coagulation factor VIIa may enhance the proliferation and migration of SW620 cells and promote the secretion of interleukin-8 (IL-8). In addition, the stimulatory effects of VIIa were inhibited by anti-TF and anti-PAR2 antibodies, but not by the anti-PAR1 antibody (5). More importantly VIIa, activated PAR2 and promoted the proliferation and migration of SW620 cells, in a TF-dependent manner. In SW620 cells, the TF/VIIa complex mediates the activation of PAR2 and shapes the tumor microenvironment through the induction of certain cytokines and the activation of the TF/VIIa/PAR2-axis (6,7).

Calcium ions (Ca^{2+}) are an important second messenger that regulate various cellular processes, particularly tumorigenesis and tumor progression, including tumor metastasis, invasion and angiogenesis (8). Ca^{2+} is highly regulated to achieve precise mediation of cell signaling pathways responding to various stimuli. It has been reported that both PAR1 and PAR2 stimulation induced the transient increase of $[Ca^{2+}]_i$ in DLD-1 colon cancer cells (9). Following the activation of the TF/VIIa/PAR2 axis, changes in the Ca^{2+} contents were observed in SW620 cells using the Ca^{2+} fluorescent indicator fluo-4 acetoxymethylester (fluo-4/AM),

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together with laser scanning confocal microscope, implying the involvement of Ca^{2+}-mediated signaling pathways in this process.

Materials and methods

Materials. Leibovitz's L-15 medium, fetal bovine serum (FBS) and trypsin were obtained from Gibco-BRL (Grand Island, NY, USA). Recombinant human VIIa was obtained from NovoNordisk (Maaloev, Denmark). PAR2-AP (SLIGKV-NH_2) were synthesized by Proteintech Group Inc. (Wuhan, China). Anti-phospho-ERK1/2 and anti-ERK1/2 antibodies were purchased from KangChen Biotech Inc. (Shanghai, China). Fluo-4/AM was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Thapsigargin (TG) was purchased from Sigma (St. Louis, MO, USA). EGTA was obtained from Biosharp Co. (Anhui, China). Cycle Test™ plus DNA Reagent kit was purchased from R&D Systems, Inc. (Minneapolis, MN, USA).

Cell culture and treatment. The SW620 human colon cancer cell line was obtained from the Shanghai Institute for Biological Sciences (Shanghai, China). The cells were maintained in Leibovitz's L-15 medium supplemented with 10% FBS and incubated at 37˚C in a humidified atmosphere containing 5% CO_2 (Forma Scientific Inc., Marietta, OH, USA). All of the experimental data were obtained with the cells passaged between 3 and 10 generations.

In the western blot analysis, MTT assay, flow cytometry and Transwell assay, the SW620 cells were divided into six groups, including (i) the control group; (ii) the VIIa group (VIIa concentration, 10 nm); (iii) thePAR2-AP group (PAR2-AP concentration, 100 μM); (iv) EGTA + TG group; (v) the EGTA + TG + VIIa group; (vi) the EGTA + TG + PAR2-AP group.

Determination of change in Ca^{2+} using fluorescence indicator fluo-4. The Ca^{2+} changes were measured in single cells using fluo-4, a fluorescence indicator for free Ca^{2+}. The cells were grown on MATTEK coverglass (MatTek Corporation, Ashland, MA, USA) and washed with HEPES buffer containing Ca^{2+}. Next, the cells were incubated for 30 min at 37˚C in L-15 medium containing 5 μM fluo-4/AM (Invitrogen Life Technologies). Following loading, the cells were washed and incubated in L-15 medium prior to detection for maintaining the live cells. A laser scanning confocal microscope (Lecia TCS SPII; Leica Co., Ltd, Wetzlar, Germany) was used to detect the fluorescence signal. Excitation and emission wavelengths were set to 488 nm and 505 nm, respectively. Following the treatments, continuous observation for intracellular Ca^{2+} changes in cells was performed (magnification, x400).

Western blotting. To detect the levels of phospho-ERK1/2 and total-ERK1/2, SW620 cells ($1.5x10^6$) were seeded into six-well plates and deprived of serum for 16 h. The cells were then treated as mentioned previously, according to group. The reagent concentrations and time periods for treatments were based on our previous study (7). The cells were then collected and lysed. The cell lysate samples (100 μg) were denatured by heating at 95˚C for 5 min, separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% non-fat milk in tris-buffered saline (TBS; pH 7.6) containing 0.1% Tween-20 (TBST) for 1 h at room temperature (RT). Following three washes with TBST, the membranes were probed with anti-phospho-ERK1/2 (1:800) and anti-total-ERK1/2 (1:600) overnight at 4˚C. Following this, the membranes were washed three times with TBST and incubated for 1 h at RT with horseradish peroxidase-conjugated secondary antibodies (1:3,000; Jingmei Biotech Co., Ltd., Shenzhen, China). The signals were developed with enhanced chemiluminescence western blotting detection reagent (GE Healthcare, Little Chalfont, UK) and detected using a Bio-Rad Fluor-S Multi Imager (Typhoon 9400; Amersham, Uppsala, Sweden).

MTT assay. The proliferation ability of SW620 cells under different conditions was measured using an MTT assay. The SW620 cells were seeded at $1x10^4$/well into a 96-well plate. The cells were treated with PAR2-AP (100 μM) or VIIa (10 nm) for 24 h. Cells of group iv-vi were pretreated with EGTA and TG for 30 min. A total of 20 μl of MTT solution [(5 mg/ml in phosphate-buffered saline (PBS)] was added to the culture medium and incubated for 4 h at 37˚C. Next, the reaction was terminated by adding 150 μl of dimethyl sulfoxide (DMSO), followed by gentle agitation for 10 min until the crystals were completely dissolved. The absorbance of the reaction solution was measured at 490 nm with a multi-function reader to determine the cell proliferation.

Determination of cell cycle distribution by flow cytometry. The SW620 cells were seeded into six-well plates ($1.0x10^6$/well) and maintained in serum-free medium for 12 h. The cells were then treated with VIIa (10 nm) or PAR2-AP (100 μM) for 12 h. Cells of group iv-vi were pretreated with EGTA and TG for 30 min. The cells were then washed with PBS prior to adding the reaction solution in the dark. Following filtering through a nylon mesh to remove the cell clusters, the cell cycle distribution was analyzed by flow cytometry.

Cell migration assay. Cell migration was investigated using an Transwell assay. The SW620 cells were harvested by brief exposure to trypsin/EDTA, followed by neutralization with L-15 culture medium. The cells were washed and resuspended in L-15 with 0.1% bovine serum albumin (BSA). Cells of group iv-vi ($1.0x10^5$ in 100 μl) were incubated with EGTA and TG for 30 min at 37˚C. The cells were then placed in the upper compartment of the migration chamber. In the lower compartment, 600 μl L-15 medium with 0.1% BSA, VIIa (10 nm) and PAR2-AP (100 μM) were added. Following 10 h incubation, the cells on the lower side of membrane were fixed with methanol and stained with Giemsa (Solarbio Science & Technology Co., Ltd., Beijing, China). The cells were counted in five randomly selected fields using a light microscope (magnification, x200). For each triplicate, the numbers of cells in ten high-power fields were determined.

Statistical analyses. The data are expressed as the mean ± standard error of the mean. The statistical significance was

Table I. Effects of EGTA + TG on the cell cycle distribution of SW620 cells stimulated by VIIa or PAR2-AP.

	%		
Group	G0/G1	S	G2/M
Control	76.64±4.04	21.04±2.46	2.33±4.04
VIIa	64.24±1.17	32.02±1.59[a]	3.73±3.30
PAR2-AP	68.56±3.63	28.97±2.89[a]	3.18±1.83
EGTA+TG	75.52±0.63	20.13±1.76	4.35±3.77
EGTA+TG +VIIa	77.56±1.42	18.71±1.17[b]	3.67±4.33
EGTA+TG+PAR_2-AP	77.52±1.48	19.29±0.85[c]	3.18±1.83

SW620 cells ($1.0x10^6$) were pretreated with or without EGTA + TG for 30 min and stimulated by VIIa (10 nm) or PAR2-AP (100 μM) for an additional 12 h. The cell cycle distribution of SW620 cells was analyzed by flow cytometry using propidium iodide. The proportions of cells in G0/G1, S and G2/M phases were determined. The data are expressed as the mean ± standard error of the mean of triplicate. [a]P<0.05 vs. the control; [b]P<0.05 vs. the VIIa treatment alone; [c]P<0.05 vs. the PAR2-AP treatment alone. PAR2, protease-activated receptor 2; AP, agonist peptide; TG, thapsigargin.

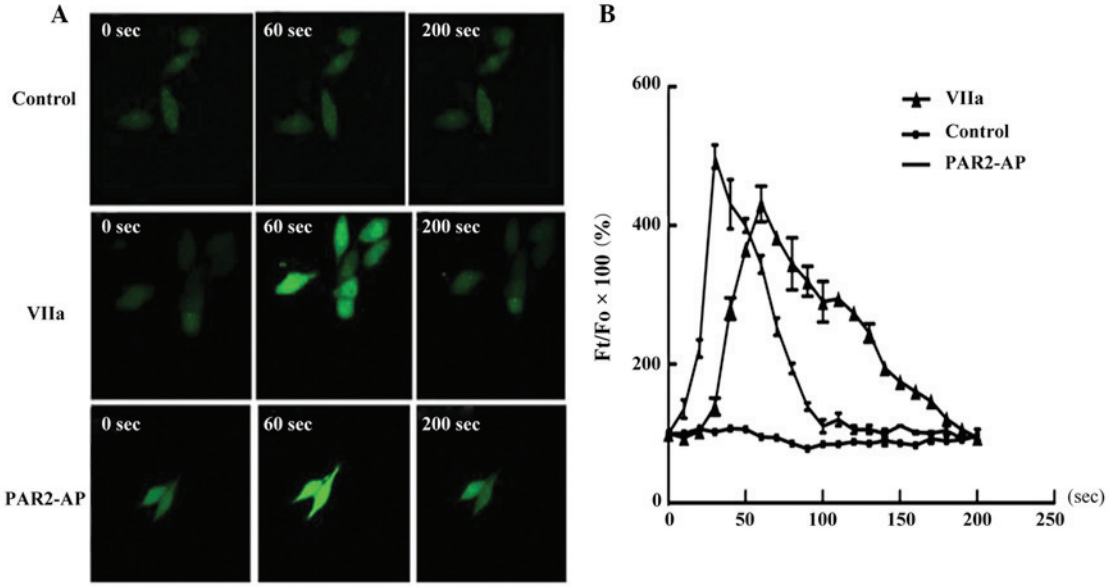

Figure 1. Ca^{2+} responses in SW620 cells induced by the activation of PAR-2 using VIIa (10 nm) or PAR2-AP (100 μM). SW620 cells grown on MATTEK coverglass were loaded with fluo-4-AM. For fluorescence signal detection, an inverted confocal laser scanning microscope was used. The excitation wavelength was set at 488 nm. (A) Fluorescence images of SW620 cells preloaded with fluo-4-AM dye and stimulated by VIIa (10 nm) or PAR2-AP (100 μM). A fast and transient green fluorescence increase was observed (magnification, x400). (B) The time course of Ca^{2+} response induced by VIIa (10 nm) or PAR2-AP in SW620 cells preloaded with fluo-4-AM. The data are expressed as the mean ± standard error of the mean of five independent experiments. PARP2, protease-activated receptor 2; TG, thapsigargin.

calculated by analysis of variance using SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

VIIa and PAR2-AP increases the $[Ca^{2+}]_i$ in SW620 cells. PAR2-AP (100 μm, selected based on our previous study) was used in the experiments as a positive control. As demonstrated in Fig. 1A, VIIa and PAR2-AP adminisration resulted in a rapid increase of $[Ca^{2+}]_i$ in the SW620 cells, which demonstrated a fast and transient increase in green fluorescence, which reached the peak phase at 60 and 30 sec, respectively, and was followed by a delayed decay phase (Fig. 1B). By contrast, the control group exhibited no significant changes. These results demonstrated that VIIa and PAR2-AP stimulation induced the transient increase in $[Ca^{2+}]_i$ and activated calcium signaling.

Calcium signaling is involved in VIIa-induced ERK1/2 activation in SW620 cells. Western blotting was used to investigate whether calcium signaling was involved in VIIa-induced ERK1/2 phosphorylation in SW620 cells. As demonstrated in Fig. 2, VIIa induced phosphorylation of

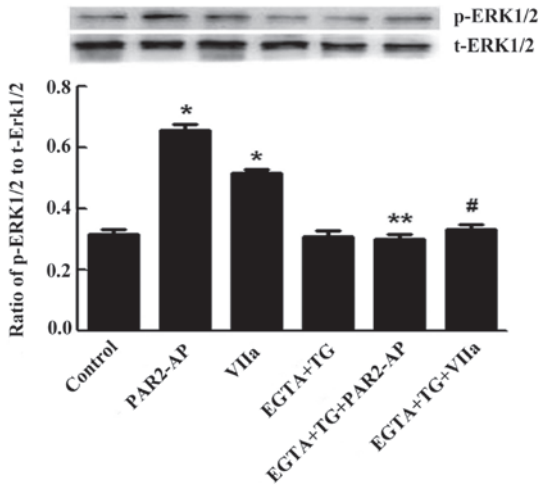

Figure 2. EGTA and TG reduced VIIa-induced p-ERK1/2 protein expression in SW620 cells. The SW620 cells were pre-treated for 5 min with TG (1 μM) to deplete the intracellular Ca^{2+} stores, EGTA (1 mm) to remove extracellular Ca^{2+}, and stimulated with VIIa (10 nm) or PAR2-AP (100 μM) for an additional 30 min. The whole cell lysates were then prepared. The levels of p-ERK1/2 and t-ERK1/2 were measured by western blotting. The expression level of p-ERK1/2 was normalized to t-ERK1/2. The data are expressed as the means ± standard error fo the mean of three independent experiments. i) Control; ii) PAR2-AP; iii) VIIa; iv) EGTA + TG; v) EGTA + TG + PAR2-AP; vi) EGTA + TG + VIIa. *P<0.05, vs. the control; $^{\#}$P<0.05, vs. the VIIa treatment alone; **P<0.05 vs. the PAR2-AP treatment alone. ERK1/2, extracellular signal regulated kinase 1/2; TG, thapsigargin; t, total; p, phosphorylated.

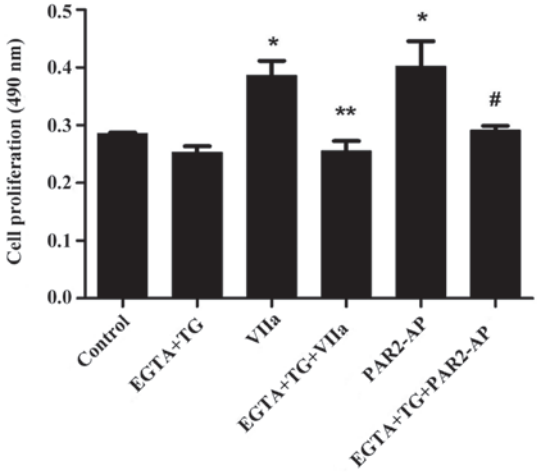

Figure 3. EGTA and TG reduced VIIa-induced cell proliferation of SW620 cells. The SW620 cells were pre-treated with EGTA and TG for 30 min prior to changing to fresh culture medium. The cells were then stimulated by VIIa (10 nm) or PAR2-AP (100 μM) for an additional 24 h. The cell proliferation was analyzed by MTT assay. The data are expressed as the means ± standard error fo the mean of three independent experiments. *P<0.05, vs. the control, **P<0.05, vs. the VIIa treatment alone; $^{\#}$P<0.05, vs. the PAR2-AP treatment alone. PARP2, protease-activated receptor 2; TG, thapsigargin.

extracellular signal regulated kinase (ERK)1/2 (p-ERK1/2; P<0.05 vs. the untreated cells), which is similar to the effect of PAR2-AP. These results demonstrated that both PAR2-AP and VIIa activate ERK1/2 phosphorylation in SW620 cells. In order to further elucidate whether VIIa-induced ERK1/2 activation is mediated by calcium signaling, TG (depletion of intracellular Ca^{2+} stores) and EGTA (removal of extracellular Ca^{2+}) were used to block calcium signaling. As demonstrated in Fig. 2, the effect of VIIa-induced ERK1/2 activation was markedly attenuated upon pretreatment with TG and EGTA (P<0.05 vs. VIIa treatment alone). A similar effect was also observed in the case of PAR2-AP. These data indicate that VIIa-induced ERK1/2 activation is mediated by calcium signaling in SW620 cells.

Calcium signaling is involved in VIIa-induced proliferation of SW620 cells. As demonstrated in Fig. 3, the cell proliferation was significantly enhanced by the stimulation of VIIa or PAR2-AP (P<0.05 vs. the control). However, the enhancing effects of VIIa and PAR2-AP on cell proliferation were markedly blocked by TG and EGTA (P<0.05 vs. VIIa or PAR2-AP treatment alone). These results suggest that calcium signaling may be involved in VIIa-induced proliferation of SW620 cells.

Calcium signaling is involved in VIIa-induced differentiation of SW620 cells. As demonstrated in Table I and Fig. 4, the percentage of SW620 cells in S phase was significantly increased by the treatment with VIIa or PAR2-AP (P<0.05 vs. the control). These data coincide with our previous results (10). However, the enhancing effects of VIIa and PAR2-AP on cell differentiation were markedly inhibited by TG and EGTA (P<0.05 vs. VIIa or PAR2-AP treatment alone). Taken together, these results suggest that VIIa enhances differentiation of SW620 cell via calcium signaling.

Calcium signaling is involved in VIIa-induced migration of SW620 cells. As demonstrated in Fig. 5A and B, the migratory ability of SW620 cells was significantly enhanced by the stimulation of VIIa or PAR2-AP (P<0.05 vs. the control). However, the effects of VIIa and PAR2-AP on cell migration were blocked by thapsigargin and EGTA (P<0.05 vs. VIIa or PAR2-AP treatment alone). These results suggest that VIIa induces SW620 cell migration via calcium signaling.

Discussion

The TF/VIIa complex has been found to induce tumorigenesis by both coagulation and non-coagulation reactions (11). In the coagulation reaction, TF/VIIa complex promotes thrombin generation and the secretion of vascular endothelial growth factor, as well as a change in the properties of the extracellular matrix, which is favorable to tumor cell adhesion and angiogenesis (11). In the case of non-coagulation, TF/VIIa complex accelerates tumor growth, angiogenesis, invasion and metastasis through two mechanisms, one depending on the activation of PAR-2, the other depending on the TF cytoplasmic domain and the phosphorylation of protein kinase C (13-15).

Intracellular free Ca^{2+} is an important secondary messenger and Ca^{2+} homeostasis in the cell is maintained by Ca^{2+} buffering and the membrane Ca^{2+}-transport system (16). When cells are stimulated, $[Ca^{2+}]_i$ rapidly increases and then returns to normal. Ca^{2+} has an important role in numerous pathological and toxicological processes (17), and is involved in tumor metastasis, invasion and angiogenesis (8). In the present study, the correlation between calcium signaling and the TF/VIIa/PAR2 axis was investigated, and the effects of

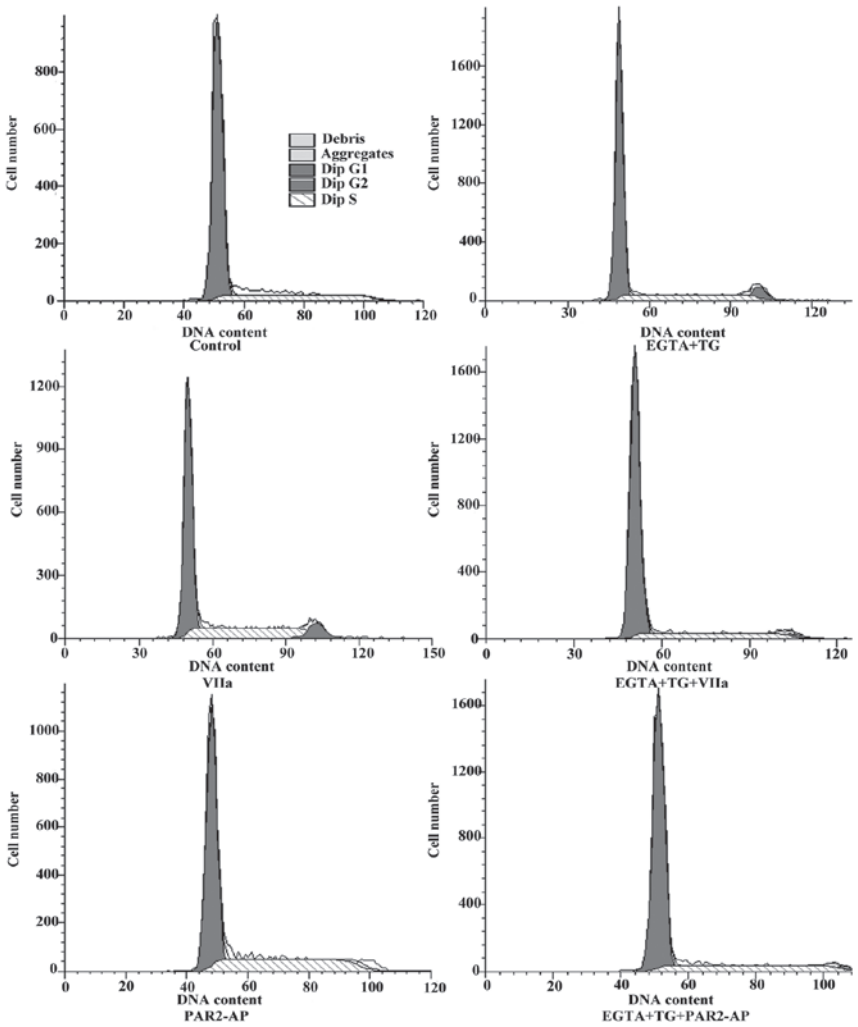

Figure 4. Effects of EGTA + TG on the cell cycle distribution of SW620 cells stimulated by VIIa or PAR2-AP. The SW620 cells ($1.0x10^6$) were pretreated with or without EGTA + TG for 30 min and stimulated by VIIa (10 nm) or PAR2-AP (100 μM) for an additional 12 h. The cell cycle distribution was analyzed by flow cytometry using propidium iodide. The proportions of cells in G0/G1, S and G2/M phases were determined. The data are expressed as the mean ± standard error fo the mean of three independent measurements. *P<0.05, vs. the control, **P<0.05, vs. the VIIa treatment alone; $^{\#}$P<0.05, vs. the PAR2-AP treatment alone. PAR2, protease-activated receptor 2; AP, agonist peptide; TG, thapsigargin.

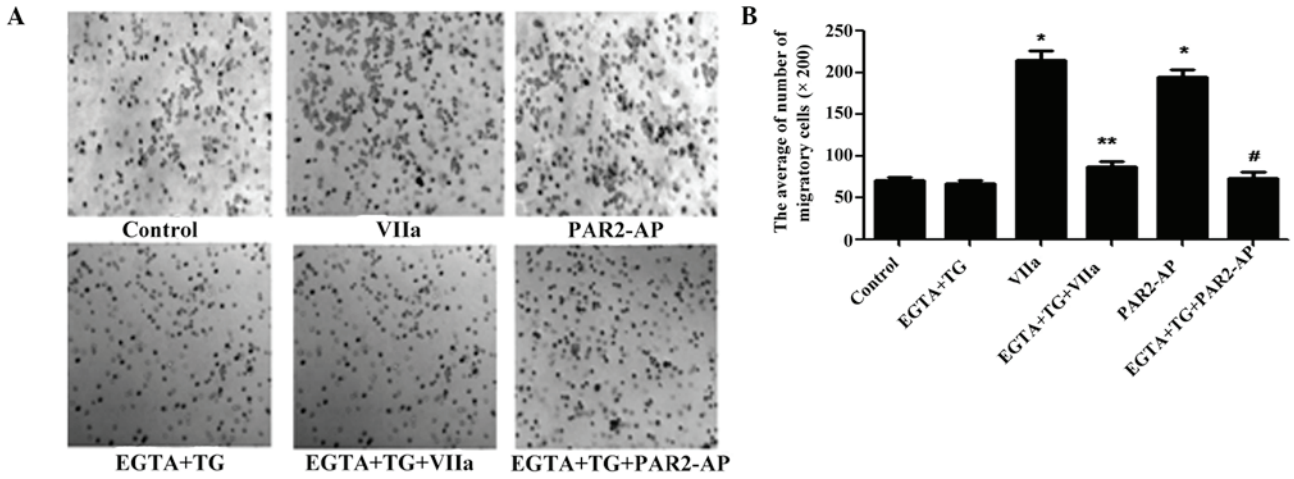

Figure 5. EGTA and TG reduced VIIa-induced migration of SW620 cells. The SW620 cells ($1.0x10^5$ in 100 μl) were incubated with EGTA and TG for 30 min at 37˚C prior to placing in the upper compartment of the migration chamber. Then VIIa (10 nm) or PAR2-AP (100 μM) was added to the lower compartments. Following 10 h of incubation, cells in lower side of membrane were fixed with methanol and stained with Giemsa. The cell migration potential was determined by modified Boyden Chambers. (A) Cells were visualized using light microscopy (magnification, x200) and (B) the average number of migratory cells was counted in five fields of triplicate experiments. *P<0.05, vs. the control, **P<0.05, vs. the VIIa treatment alone; $^{\#}$P<0.05, vs. the PAR2-AP treatment alone. PARP2, protease-activated receptor 2; TG, thapsigargin.

calcium signaling on TF/VIIa/PAR2 axis-mediated signaling pathways were examined.

Fluo-4/AM, a sensitive Ca^{2+} indicator, proportionally binds to intracellular free Ca^{2+}, and produces specific fluorescence signals upon excitation. It is an excellent indicator for monitoring the dynamic change of the $[Ca^{2+}]_i$ (18). In the present study, SW620 cells were treated with VIIa and a PAR-2 agonist. The changes of intracellular $[Ca^{2+}]_i$ were detected with fluo-4 using confocal laser scanning microscopy. The enhanced fluorescence intensity indicated the rapid release of intracellular Ca^{2+} following the activation of TF/VIIa/PAR2 axis. VIIa and PAR2-AP rapidly increased $[Ca^{2+}]_i$ in SW620 cells, which reached the peak phase at 60 sec and 30 sec, respectively, followed by a delayed decay phase (Fig. 1). These results suggested that the activation of TF/VIIa/PAR2 axis in SW620 cells caused a transient increase of intracellular $[Ca^{2+}]_i$.

Other studies have demonstrated that changes in Ca^{2+} concentrations have two different modes (instantaneous change and sine curve change), which correspond to different signaling pathways (19). The present study demonstrated that Ca^{2+} has a role in the regulation of colon epithelial cell growth (20). Previous, *in vivo* studies, have also demonstrated that a Ca^{2+}-rich diet is associated with a decreased incidence of colorectal cancer (21). However, the mechanism underlying calcium chemoprevention remains unclear, although it is hypothesized to be corelated with signaling pathways regulating the proliferation and differentiation of colon cells. It is well-estbalished that the extracellular Ca^{2+}-sensing receptor (CaR) expressed along the entire gastrointestinal tract is involved in maintaining a stable intracellular Ca^{2+} microenvironment. By contrast to normal gastrointestinal cells, the expression of CaR is markedly reduced or completely lost in differentiated colorectal carcinoma (19,20). It would be of interest to investigate whether CaR is involved in VIIa-induced proliferation and migration of SW620 cells.

The present study further investigated the correlation between calcium signaling and the TF/VIIa/PAR2/ERK axis. It was noted that ERK1/2 activation induced by TF/VIIa/PAR2 axis was markedly attenuated upon pretreatment with TG and EGTA. Therefore, TF/VIIa/PAR2 axis-dependent calcium signaling may be critical for ERK1/2 activation.

Our previous studies have demonstrated that the activation of TF/VIIa/PAR2 axis promotes colon cancer SW620 cell proliferation, differentiation and migration (5-7). The enhancing effects of TF/VIIa/PAR2 axis on cell proliferation, differentiation and migration were markedly inhibited by TG and EGTA. The ERK signaling pathway is one of the most important mitogen-activated protein kinase transduction pathways associated with cell proliferation, transformation and differentiation (22-23). Therefore, calcium signaling may be involved in the TF/VIIa/PAR2 axis-induced proliferation, differentiation and migration of SW620 cells.

In conclusion, the present results indicated that the activation of TF/VIIaPAR2 axis induced ERK1/2 activation via calcium signaling in SW620 cells, and enhanced SW620 cell proliferation, differentiation and migration. Therefore, calcium signaling had an important role in the proliferation, differentiation and migration of SW620 cells.

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