Dual Effect of Thrombin on Voltage-Dependent Ca\(^{2+}\) Channels of Portal Vein Smooth Muscle Cells

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Thrombin induces a number of physiological responses in several types of cells. To determine the action of thrombin in the vein, the electrophysiological and mechanical effects of thrombin were studied in rat portal vein smooth muscle cells. Ca\(^{2+}\) channel currents were recorded using the whole-cell patch-clamp technique. Thrombin had both inhibitory and stimulatory effects on the Ca\(^{2+}\) channel current. The inhibitory effect was reversed on washout of thrombin, whereas the stimulatory effect was maintained after thrombin was removed. Thrombin (1 unit/ml) produced a reversible decrease of 27.3 ± 3.3% (n = 12) in the current amplitude and a sustained increase of 71.2 ± 12.9% (n = 20). The thrombin-induced inhibition of Ca\(^{2+}\) channel current was blocked by the thrombin inhibitor hirudin and by the protease inhibitor leupeptin. The stimulatory effect of thrombin was inhibited by hirudin, by intracellular application of guanosine 5’-O-(β-thio)diphosphate, and by antiphosphatidylinositide antibodies but not by pertussis toxin. The thrombin-induced enhancement of the Ca\(^{2+}\) channel current amplitude was not observed when the current was previously stimulated by phorbol 12,13-dibutyrate. This suggests that the inhibitory effect of thrombin was related to its proteolytic activity and that the stimulatory effect involved activation of a pertussis toxin–insensitive GTP-binding protein, phosphatidylinositide hydrolysis, and protein kinase C activation. Both thrombin effects occurred in the same concentration range (0.001–10 units/ml). The thrombin-induced contraction of portal vein strips was completely inhibited by isradipine, and thrombin did not produce an increase in cytosolic [Ca\(^{2+}\)]\(_i\), measured by indo-1 fluorescence in cells clamped at −50 mV, sufficient to activate Ca\(^{2+}\)-dependent chloride current. Thus, the effects of thrombin in portal vein smooth muscle are mainly dependent on Ca\(^{2+}\) entry through voltage-dependent Ca\(^{2+}\) channels. (Circulation Research 1993;72:1317–1325)

**Key Words** • thrombin • calcium channels • vascular smooth muscle cells • patch clamp

The proteolytic enzyme thrombin, catalyzing the formation of insoluble fibrin, plays a major role in blood coagulation but also induces a number of physiological responses in various tissues. These responses include phosphoinositide turnover,\(^1\) \(^3\) Ca\(^{2+}\) mobilization,\(^4\) inhibition of adenylate cyclase,\(^5\) release of arachidonic acid,\(^6\) stimulation of the Na\(^+\)-H\(^+\) antiport,\(^7\) and mitogenesis.\(^8\) These actions of thrombin have been studied primarily in platelets but have also been described in fibroblasts and endothelial cells.\(^9\) In addition, thrombin stimulates the breakdown of phosphoinositides, prolongs the action potential duration, and stimulates L-type Ca\(^{2+}\) channels in ventricular myocytes.\(^10\)\(^\)-\(^13\)

Although thrombin is present in high concentrations at sites of clots and may have important postclotting effects on adjacent vascular tissue, little is known about the action of thrombin on vascular smooth muscle. It has been reported that thrombin causes an endothelium-dependent relaxation of arteries\(^14\)\(^,\)\(^15\) and produces a contraction of veins that is only partially inhibited by endothelium removal.\(^14\) Recently, thrombin has been shown to increase the intracellular inositol 1,4,5-trisphosphate (InsP\(_3\)) level and free [Ca\(^{2+}\)]\(_i\) ([Ca\(^{2+}\)]\(_i\)) and to produce change in pH\(_i\) in cultured smooth muscle cells from rat aorta.\(^16\)\(^,\)\(^17\)

The aim of the present study was to characterize the electrophysiological effects of thrombin on vascular smooth muscle cells isolated from rat portal vein and to identify the mechanisms that potentially could underlie thrombin actions.

**Materials and Methods**

**Cell Preparation**

Wistar rats (150 g) were stunned and then killed by cervical dislocation. The portal vein was cut into several pieces, incubated for 10 minutes in low-calcium (40 \(\mu\)M) physiological saline solution (PSS; composition is given below), and then incubated in low-calcium PSS containing 1.4 mg/ml collagenase, 0.5 mg/ml pronase, and 1 mg/ml bovine serum albumin at 37°C for 20 minutes. After this time, the solution was removed, and the pieces of vein were incubated again in a fresh enzyme solution at 37°C for 20 minutes. Tissues were then placed in enzyme-free solution and triturated using a
fire-polished Pasteur pipette to release cells. Cells were stored on glass coverslips at 4°C in PSS containing 0.8 mM calcium and used on the same day or maintained in short-term primary culture in medium M199 (Flow Laboratories, Inc., McLean, Va.) containing 10% fetal bovine serum, 2 mM glucose, 20 units/ml penicillin, and 20 μg/ml streptomycin (GIBCO Laboratories, Grand Island, N.Y.), kept in an incubator gassed with 95% O2–5% CO2 at 37°C, and used within 40 hours. The electrophysiological properties (Ca2+ channel current, effect of agonists) and [Ca2+]i of cells just after the dispersal procedure were similar to those of cells from the same batch maintained in short-term primary culture.

Membrane Current and Measurement of Cytosolic [Ca2+]i

Voltage-clamp and membrane-current recordings were made with standard patch-clamp techniques20 by use of a patch-clamp amplifier (model EPC-7, List Biological Laboratories, Inc., Campbell, Calif.). Whole-cell membrane currents were recorded with borosilicate patch pipettes of 1–4 MΩ resistance. The series resistances (5–8 MΩ) were not corrected. The liquid junction potentials were corrected with an offset circuit before each experiment. Current recordings were filtered with an eight-pole Bessel filter (–3 dB at 1 kHz) and sampled at 4 kHz for analysis by an IBM-PC computer (pCLAMP system, Axon Instruments, Burlingame, Calif.). Ca2+ channel currents were digitally corrected for capacitative and leakage currents. Ca2+ channel currents were evoked by depolarizations from a holding potential of −60 mV and thus corresponded to a mixture of fast and slow current components.19

Measurement of [Ca2+]i was carried out as described previously.20,21 Briefly, 50 μM indo-1 was added to the pipette solution and entered the cells after establishment of the whole-cell recording mode. The cell studied was illuminated at 360 nm. Emitted light from a window slightly larger than the cell was collected simultaneously at 405 and 480 nm by two photomultipliers (model P1, Nikon France, Champigny sur marne, France). Voltage signals at each wavelength were stored in an IBM-PC computer for subsequent analysis. Cytosolic [Ca2+]i ([Ca2+]i) was estimated from the 405/480 ratio22 by use of a calibration for indo-1 determined within cells.21,22

Noradrenaline (NA) and thrombin were applied to the recorded cell by pressure ejection from a glass pipette for the period indicated on the recordings. No change in the holding current and in Ca2+ channel amplitude was observed during ejection of the PSS. All experiments were done at 30°C.

Results

Effects of Thrombin on Mechanical Activity

Application of thrombin (1 unit/ml) on longitudinal strips of portal vein induced a slow increase in tension accompanied by an enhancement of the amplitude and frequency of spontaneous activity (Figure 1A, tracing a). When thrombin was removed, the basal tension slowly returned to its initial level. The effects on spontaneous contractions were much more maintained. The amplitude of contractions went on increasing after thrombin was washed out. After more than 1 hour, the amplitude and the frequency of spontaneous contractions were still stimulated. The effect of thrombin was also studied on the muscular strip already stimulated by NA (Figure 1A, tracing b). NA (3 μM) produced a large contraction consisting of a phasic component followed by a sustained phase, mainly dependent on calcium entry through voltage-dependent Ca2+ channels, which were stimulated through a protein kinase C (PKC) pathway.21,26 Thrombin (1 unit/ml) partially inhibited (approximately 25%) the maintained component of the NA-induced contraction. This effect was rapidly reversed when thrombin was removed. This observation suggests that, when the vein was already contracted by other agonists, the effect of thrombin was a partial relaxation.

In the presence of the Ca2+ channel blocker isradipine (0.1 μM), application of thrombin (1 unit/ml) did not produce any change in tension (Figure 1B), whereas NA (1 μM) was still able to produce a transient contraction corresponding to calcium release from the intracellular store.27
FIGURE 1. Tracings showing the effects of thrombin (Thr) on mechanical activity. NA, noradrenaline. Thr (1 unit/ml) was added to the 2 mM calcium-containing physiological saline solution under control conditions (panel A, tracing a), during NA (3 μM)-induced contraction (panel A, tracing b), and in the presence of 0.1 μM isradipine (panel B). In the presence of isradipine, NA (1 μM) but not Thr induced a transient contraction.

Effects of Thrombin on Ca\(^{2+}\) Channel Current

Effects of thrombin were studied on Ca\(^{2+}\) channels by using 5 mM barium as the charge carrier. Experiments were started after perfusion of the cells with a barium-containing PSS for at least 30 minutes. The Ca\(^{2+}\) channel currents were evoked by repetitive depolarizations from a holding potential of −60 mV at a frequency of 0.05 Hz. Under these conditions, within 20 minutes, the decrease of the Ca\(^{2+}\) channel current due to the rundown phenomenon was weak (11.2±1.8%, n=13). The first thrombin application was done at least 4 minutes after establishment of the whole-cell configuration, and zero time on the figures had no specific meaning. Application of thrombin (1 unit/ml) produced an increase in the amplitude of the Ca\(^{2+}\) channel current evoked by depolarization to 0 mV from a holding potential of −60 mV (Figure 2A). The current amplitude reached a stable value after 2–2.5 minutes of thrombin application. Surprisingly, the current amplitude started again to increase when thrombin was removed, and a new steady state was reached 1–2 minutes after thrombin washout; the maximal increase measured at this time was between 25% and 260% of the control current, depending on the cells, with a mean value of 71.2±12.9% (n=20). This stimulatory action of thrombin on the Ca\(^{2+}\) channel could not be attributed to a nonspecific binding to α₁-adrenoceptors, whose activation led to an increase in Ca\(^{2+}\) channel current since it was unchanged in the presence of 0.1 μM prazosin. A second application of thrombin (1 unit/ml) on the stimulated current produced a decrease in the current amplitude ranging between 9% and 54% with a mean value of 27.3±3.3% (n=12). The percentages of increase and decrease given here were calculated from cells in which both thrombin effects were observed at the first application. These two effects were abolished when thrombin (1 unit/ml) was applied in the presence of hirudin (2 units/ml, Figure 2B), which is known to specifically antagonize the action of thrombin by binding to it. As shown in Figure 2A, the current amplitudes reached during both applications of thrombin were similar. Since the effect of the second application of thrombin was only a reversible inhibition of the stimulated current, it is possible that the effect observed during the first application was the result of an inhibition of the Ca\(^{2+}\) channel current, which was reversed upon washout of thrombin, and a maintained increase in the Ca\(^{2+}\) channel current amplitude, which persisted after thrombin was removed. Thus, the increase in the current amplitude occurring after the termination of the first thrombin application could correspond to the wash-out of the inhibitory effect. This hypothesis agreed with the maintained stimulation of spontaneous contractions and with the reversible inhibition of the NA-induced contraction produced by thrombin application on strips.
of the portal vein (Figure 1). This hypothesis is also in agreement with the fact that, depending on the cell batches, thrombin-induced enhancement or thrombin-induced inhibition of the Ca\(^{2+}\) channel current was observed alone and suggests that thrombin action involves at least two different mechanisms.

The voltage dependence of the Ca\(^{2+}\) channel current was not modified by thrombin. Under control conditions, the current–voltage relation of the Ca\(^{2+}\) channel current showed an apparent threshold at \(-43.7\pm1.7\) mV (\(n=8\)), a maximum at 0.62±3.1 mV (\(n=8\)), and an apparent reversal potential at 73.4±1.9 mV (\(n=8\)) (Figures 3A and 4A). To study the inhibitory effect of thrombin, the current–voltage relations were made during the second response to thrombin or during the first response, when only the inhibition was observed. A typical curve showed that the application of thrombin (1 unit/ml) reduced the Ca\(^{2+}\) channel current at any given voltage pulse without any change in the voltage threshold, the potential for the maximal current, or the apparent reversal potential (Figure 3A). As shown in Figure 3B, the inhibition of the Ca\(^{2+}\) channel current did not significantly alter the steady-state inactivation curve. The midpoint of the curve was \(-43.2\pm1.7\) mV (\(n=4\)) in control conditions and \(-45.7\pm1.1\) mV (\(n=4\), \(p>0.3\)) in the presence of 1 unit/ml thrombin. Similarly, the stimulatory effect of thrombin (1 unit/ml) did not change the voltage threshold, the potential for the maximal current, or the apparent reversal potential of the current–voltage relation (Figure 4A). Figure 4B shows the mean peak conductance curves against voltage obtained from the Ca\(^{2+}\) channel current in control conditions and from current stimulated by 1 unit/ml thrombin. The potentials corresponding to half activation were not significantly different: \(-9.1\pm1.3\) mV (control, \(n=5\)) and \(-13.7\pm2.8\) mV (thrombin, \(n=5\), \(p>0.3\)). Thus, the mechanism by which thrombin in-
In the application of solution, Ca\textsuperscript{2+} the or had tracings current expressed hypothesis, this due of Ca\textsuperscript{2+} induced amplitude, peptin in thrombin in the presence and absence of GDP-β-S on the same cell. Thus, the effect of GDP-β-S was only studied in cell batches in which both inhibitory and stimulatory actions of thrombin on Ca\textsuperscript{2+} channel current were observed in more than 90% of the cells; the results were compared with those obtained under control conditions in the same cell batch. With 1 mM GDP-β-S in the pipette solution, application of 1 unit/ml thrombin failed to produce an increase in peak inward current elicited by depolarizing pulses to 0 mV from a holding potential of −60 mV but still induced a reversible inhibition (Figure 5B). The second application of thrombin produced an inhibition of the Ca\textsuperscript{2+} channel current similar to that obtained during the first application. This thrombin-induced inhibition of the Ca\textsuperscript{2+} channel current in the presence of internal GDP-β-S (24.2±7.4%, n=4) was not significantly different (p>0.5) from that obtained during the second thrombin application under control conditions in the same cell batch (20.2±6.6%, n=4). These observations favor the idea that the enhancement but not the inhibition of Ca\textsuperscript{2+} channel current induced by thrombin involves the activation of a G protein. This G protein is not sensitive to PTX, because, in the presence 0.3 µg/ml PTX in the pipette solution, the enhancement of the Ca\textsuperscript{2+} channel current induced by thrombin (1 unit/ml) was not significantly different from that observed in control conditions (75.7±13.2% [n=3] with PTX compared with 67.5±15.1% [n=3, p>0.5] under control conditions in the same batch of cells; data not shown).

Previous studies in portal vein smooth muscle cells have demonstrated that voltage-dependent Ca\textsuperscript{2+} channels were modulated through PKC activation. To determine whether thrombin acted via PKC activation, thrombin was applied in the presence of PDBu, a phorbol ester known to directly activate PKC. This experiment was performed in cell batches in which thrombin induced both inhibition and stimulation of the Ca\textsuperscript{2+} channel current in at least 90% of the cells tested under control conditions. As illustrated in Figure 6A, when the peak inward current elicited by depolarizing pulses to 0 mV from a holding potential of −60 mV was maximally stimulated by 0.1 µM PDBu (90.4±9.6%, n=28), a further application of thrombin (1 unit/ml) did not produce an additional increase in current amplitude but induced an inhibition that was maintained as long as thrombin was present. A second application of thrombin (1 unit/ml) reproduced the same effect. This thrombin-induced inhibition of the Ca\textsuperscript{2+} channel current stimulated by PDBu (28.0±4.1%, n=6) was similar to that obtained during the second application of thrombin under control conditions on cells from the same batch (30.8±7.3%, n=3, p>0.5). The inhibition of the thrombin-induced enhancement of Ca\textsuperscript{2+} channel current when the Ca\textsuperscript{2+} channels were previously stimulated by PDBu suggests that these two agents may share a common

Figure 5. Graphs showing the effect of thrombin (Thr) on the Ca\textsuperscript{2+} channel current evoked by depolarizations to 0 mV from a holding potential of −60 mV in the presence of leupeptin or S\textsuperscript{5}-O-(β-thio)diphosphate (GDP-β-S). Panel A: In the presence of leupeptin (100 µg/ml) in the external solution, application of Thr (5 units/ml) induced only a maintained increase in current amplitude. The second Thr application had no effect. Panel B: In the presence of GDP-β-S (1 mM) in the pipette solution, Thr (1 unit/ml) produced only a reversible inhibition of the current. Currents are expressed as a fraction of control values (I/I_o). Insets are current tracings corresponding to the letters on the curves.
mechanism of action. Thus, it could be supposed that diacylglycerol (DAG), the endogenous activator of PKC, was produced during thrombin stimulation.

To test this hypothesis, effects of thrombin on Ca$^{2+}$ channel current were examined in the presence of anti-PtdIns Abs in the intracellular solution. This experiment was done in cell batches in which thrombin induced both inhibition and stimulation of the Ca$^{2+}$ channel current in more than 90% of the cells under control conditions. We have recently shown that the inclusion of this anti-PtdIns Ab in the pipette solution prevented the production of DAG from phosphatidylinositides during $\alpha_2$-adrenergic stimulation. When the cell was injected with the anti-PtdIns Ab (12 $\mu$g immunoglobulin G/ml), thrombin (1 unit/ml) induced only a decrease (27.5±2.8%, n=6) in the inward current amplitude, which returned to its initial value when thrombin was removed, whereas PDBu (0.1 $\mu$M) was still able to produce an increase in the Ca$^{2+}$ channel current (Figure 6B). This decrease was similar to that obtained during the second application of thrombin in the same batch of cells under control conditions (28.0±4.4%, n=4, p>0.5). This effect is consistent with an involvement of phosphatidylinositides in the intracellular transduction mechanism responsible for the thrombin-induced enhancement of the Ca$^{2+}$ channel current. In addition, the involvement of a phosphorylation (maybe through PKC activation) was supported by the strong inhibition of the thrombin-induced enhancement of the Ca$^{2+}$ channel current when the pipette solution did not contain ATP (Figure 7). Under these conditions, only an inhibition of the Ca$^{2+}$ channel current (22.0±5.2% [n=3, p>0.5] compared with 24.7±5.2% [n=3] under control conditions) could be obtained during thrombin applications.

Concentration Dependence of Thrombin Action on Ca$^{2+}$ Channel Current

The inhibitory and stimulatory effects of increasing concentrations of thrombin were measured on Ca$^{2+}$ channel current obtained at 0 mV from a holding potential of −60 mV. The inhibitory effect was measured from the second response to thrombin or from cells that showed only an inhibition of the Ca$^{2+}$ channel current in the presence of thrombin. A maximal inhibition (approximately 30% of control current) was obtained in the presence of thrombin concentrations greater than 0.1 unit/ml (Figure 8A). The thrombin concentration required to produce 50% of the maximal inhibition (IC$_{50}$) was estimated to be 0.0061 unit/ml, because the mean value for −log IC$_{50}$ and the standard error obtained from the fit of the data were 2.21±0.06.

To reduce variability, the stimulatory effect of thrombin was expressed as a fraction of the total increase in the Ca$^{2+}$ channel current induced by a sequential application of thrombin and 0.1 $\mu$M PDBu. The measurement of the effect of thrombin was made when the current reached a steady state after thrombin was removed. The maximal stimulation (approximately 80% of the PDBu effect) was obtained with 0.1–10 units/ml thrombin, and the concentration giving 50% of the maximal effect (EC$_{50}$) was 0.0053 unit/ml (Figure 8B, −log EC$_{50}$=2.27±0.11). These results indicate that the
opposite effects of thrombin on Ca$^{2+}$ channel current occurred in the same range of concentrations, but the stimulatory effect was always higher than the inhibitory effect so that, in all the range of effective concentrations, the result of thrombin action, in cells in which both effects were observed, was a stimulation of the Ca$^{2+}$ channel current.

**Measurements of [Ca$^{2+}$]$_i$**

[Ca$^{2+}$]$_i$ was measured in cells clamped at -50 mV in the 2 mM Ca$^{2+}$-containing PSS in the presence of 50 μM D600. The resting [Ca$^{2+}$]$_i$ was 67±2 nM (n=24). Application of thrombin (10 units/ml) induced small oscillations in [Ca$^{2+}$]: the maximal [Ca$^{2+}$]$_i$ reached was 102±6 nM (n=9) (Figure 9A), and no change in the membrane current was observed. Under the same conditions, stimulation of the cell with NA (1 μM) produced a two-phase increase in [Ca$^{2+}$]$_i$ (Figure 9B); the

**Discussion**

In the present work, we have studied the effect of thrombin on the electrical and mechanical properties of venous smooth muscle. We report for the first time the action of thrombin on the voltage-dependent Ca$^{2+}$ channel of vascular smooth muscle cells. The thrombin concentrations used (0.001–10 units/ml, corresponding to 0.01–100 nM) are in the range observed during blood clotting, supporting potential physiological significance.37

Thrombin induced a maintained contraction of portal vein strips that was completely abolished by isradipine. This suggests that the effects of thrombin are mainly dependent on Ca$^{2+}$ entry through voltage-dependent Ca$^{2+}$ channels; however, other actions of thrombin, such as a direct effect on the contractile process, could also contribute to the mechanical response.

Whole-cell clamp experiments showed that thrombin had both a stimulatory and an inhibitory effect on Ca$^{2+}$ channels. However, in cells in which both effects were
observed, the result of thrombin actions was always an enhancement of the Ca\textsuperscript{2+} channel current. Such a dual effect led to the proposal that either two thrombin receptors or one receptor coupled to distinct signal transduction mechanisms is present in venous smooth muscle cells. The similarity in the dose–response curves for thrombin-induced inhibition and thrombin-induced enhancement of Ca\textsuperscript{2+} channel current favors the hypothesis that both effects are mediated by one thrombin receptor. However, we cannot exclude that both thrombin effects might be related to the existence of various active thrombin forms, such as α- and γ-thrombin. The results of our experiments with leupeptin seem to indicate that thrombin receptor occupancy alone is sufficient to trigger the stimulation of the Ca\textsuperscript{2+} channel current, whereas both receptor occupancy and thrombin-induced proteolysis are required for the inhibition of Ca\textsuperscript{2+} channel current. We have not yet determined the exact mechanism by which thrombin decreases the Ca\textsuperscript{2+} channel current and why this effect is rapidly reversed, but our results seem to indicate that a G protein is not involved.

On the contrary, the stimulatory effect of thrombin on Ca\textsuperscript{2+} channel current was inhibited by GDP-β-S. In addition, the thrombin-induced enhancement of the Ca\textsuperscript{2+} channel current was nonadditive with that produced by PDBu (thrombin had no effect when the current was already stimulated by PDBu) and was blocked by the anti-PtdIns Ab. This suggests that the stimulation of Ca\textsuperscript{2+} channels was due to PKC activation; thrombin receptor occupancy might activate a PTX-insensitive G protein–mediated stimulation of phospholipase C, leading to production of DAG, which activated PKC. However, thrombin did not seem to be able to maximally activate PKC, because, even after stimulation of the current with a maximal concentration of thrombin, PDBu could still increase the current. Another possibility would be that thrombin could activate a different kinase that phosphorylates the same amino acid residues as PKC.

Alternatively, InsP\textsubscript{3}, the other major intracellular second messenger formed stoichiometrically by this pathway, might release Ca\textsuperscript{2+} from intracellular stores as it was described for α\textsubscript{2A}-adrenoceptor stimulation, which involved the same intracellular transduction mechanism.\textsuperscript{26,34} In portal vein myocytes, the Ca\textsuperscript{2+} released by InsP\textsubscript{3} during NA stimulation produced a transient increase in [Ca\textsuperscript{2+}], and induced activation of a Ca\textsuperscript{2+}-dependent chloride current.\textsuperscript{26} In fact, thrombin was not able to produce a transient increase in [Ca\textsuperscript{2+}], or to activate the Ca\textsuperscript{2+}-dependent chloride current.\textsuperscript{26} In the presence of blocker of voltage-dependent Ca\textsuperscript{2+} channels, the thrombin-induced contraction was completely inhibited, whereas NA was still able to produce a transient contraction. In this regard, venous smooth muscle was different from arterial smooth muscle, in which thrombin induced a transient increase in [Ca\textsuperscript{2+}], by InsP\textsubscript{3}–induced Ca\textsuperscript{2+} release\textsuperscript{6,12} and produced a contraction resistant to Ca\textsuperscript{2+} channel blockers.\textsuperscript{38} The presence of only a few thrombin receptors on the portal vein membrane activating a few G proteins and then leading to the formation of relatively low concentrations of intracellular second messengers may explain these results.

However, thrombin (1 unit/ml) and NA (10 μM)\textsuperscript{26} stimulate the Ca\textsuperscript{2+} channel current to nearly the same extent, and although the amplitude of the thrombin-induced contraction was lower than that of the NA-induced contraction, the effect of thrombin on the spontaneous activity was more maintained than that of NA. Our results suggest that, like NA-induced stimulation,\textsuperscript{26} thrombin-induced stimulation of the Ca\textsuperscript{2+} channel current involved DAG production. Thus, it is possible that thrombin caused DAG production by more than one mechanism, even if the action of the anti-PtdIns Abs suggested that the phosphatidylinositides were the only substrates of DAG production, since anti-PtdIns Abs were bound to phosphatidylinositol and also, with a lower affinity, to phosphatidylinositol monophosphate and phosphatidylinositol diphosphate but did not bind phosphatidycholine and phosphatidyethanolamine.\textsuperscript{34} This hypothesis is supported by the ability of thrombin to produce in platelets sequential activation of a phosphatidylinositol diphosphate–specific phospholipase C, followed by activation of a phosphatidylinositol monophosphate– and phosphatidylinositol-specific phospholipase C.\textsuperscript{39,40} In addition, it has been recently reported that the first phase of the multiphasic generation of DAG in thrombin-activated platelets was due to phospholipase C–mediated hydrolysis of phosphatidylinositol diphosphate, whereas the subsequent phases were not derived from phosphatidylinositol diphosphate.\textsuperscript{41} In light of these studies, it should not be surprising to find a dissociation between responses depending on DAG and InsP\textsubscript{3}. If such mechanisms for DAG production were stimulated by thrombin in portal vein smooth muscle cells, thrombin could induce formation of as much DAG as NA but with different kinetics; this could explain the difference in the time course of the NA- and the thrombin-induced stimulation of Ca\textsuperscript{2+} channels.

The observations we report concerning the thrombin effects on electrophysiological and mechanical properties of venous smooth muscle are novel and provide the basis for both conclusions and speculation about thrombin physiological and physiopathological roles. Under normal conditions, when the integrity of the endothelium is maintained, it is unlikely that thrombin comes into direct contact with vascular smooth muscle cells. However, endothelium damage and/or the setting of a thrombus are likely to bring myocytes into direct contact with clotting factors. Under these circumstances, it is of interest to speculate that thrombin may act as a plasma-derived humoral mediator of electrophysiological events. Because of its dual effect on voltage-dependent Ca\textsuperscript{2+} channels, the action of thrombin might be different: in resting myocytes, the effect of thrombin would be a contraction; on the contrary, in myocytes already contracted by other agonists (such as NA), the action of thrombin would be a partial relaxation.

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