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# Thrombin-induced $Ca^{2+}$ Mobilization in Vascular Smooth Muscle Utilizes a Slowly Ribosylating Pertussis Toxin-sensitive G Protein

## EVIDENCE FOR THE INVOLVEMENT OF A G PROTEIN IN INOSITOL TRISPHOSPHATE-DEPENDENT $Ca^{2+}$ RELEASE*

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**The role of pertussis toxin (PT)-sensitive and -insensitive guanine nucleotide-binding proteins (G proteins) in the stimulation of $Ca^{2+}$ mobilization by thrombin was investigated in cultured rat aortic smooth muscle cells. Characterization using immunoblotting with specific antisera indicated the presence in isolated membranes of the $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_s$, $G\beta_{35}$, and $G\beta_{36}$ protein subunits as well as a lower molecular weight species of unknown identity. To assess the importance of G proteins in the coupling of thrombin receptors to $Ca^{2+}$ mobilization, we investigated the effect of PT on $Ca^{2+}$ responses using fluorescence spectroscopy and the $Ca^{2+}$ indicator dye Fura-2. Pretreatment of cells for 2 h with PT (1 μg/ml), which produced 91.3% ADP-ribosylation of PT-sensitive G proteins, did not affect the magnitude of thrombin-induced release of $Ca^{2+}$ from internal stores, suggesting that the residual 8.7% of PT-sensitive G proteins, or PT-insensitive mechanisms, was responsible for $Ca^{2+}$ release. However, after an 18-h pretreatment with PT, which produced ADP-ribosylation of the total complement of PT-sensitive G proteins, the thrombin-induced peak $Ca^{2+}$ response was inhibited by approximately 72%, suggesting that the major fraction of the $Ca^{2+}$ response was mediated by a slowly ribosylating component. The delayed effect of the toxin was not caused by down-regulation of the β-subunit of G proteins because quantitative immunoblots showed that levels of the β-subunit remained constant throughout the period of PT pretreatment. It was also not caused by a reduction in the size of the thrombin-releasable $Ca^{2+}$ pool because $Ca^{2+}$ release induced by agents that release $Ca^{2+}$ directly from internal stores, 2,5-di-*tert*-butylhydroquinone or thapsigargin, was not affected. In addition, the delayed effect of PT could not be explained in terms of differences in thrombin-induced [$^3$H]inositol trisphosphate ($IP_3$) formation because the level of inhibition of $IP_3$ formation after a 2-h PT treatment was similar to that present after an 18-h pretreatment.**

**The results indicate that a slowly ribosylating PT-sensitive species is the major G protein pathway that couples thrombin-receptor activation to $Ca^{2+}$ mobilization. This G protein appears to be involved not in the mechanisms that generate $IP_3$ but rather possibly in coupling at the level of the intracellular $Ca^{2+}$ store.**

α-Thrombin elicits a wide variety of important responses in vascular cells. In endothelial cells, it increases the intracellular $Ca^{2+}$ concentration $[Ca^{2+}]_i$[1] (1, 2), increases the expression of mRNA for platelet-derived growth factor BB (3), and stimulates the release of endothelial-derived relaxing factor (4). In the absence of endothelium, α-thrombin is a potent vasoconstrictor agent (5) and in some instances may also be mitogenic (6). It stimulates many growth-related signals in vascular smooth muscle including $Na^+/H^+$ exchange (6–8), mRNA for the protooncogene c-*fos* (8), and protein synthesis (6). It also activates phospholipase C with the generation of diacylglycerol and inositol 1,4,5-trisphosphate ($IP_3$) (6). The last substance is closely associated with an early elevation in $[Ca^{2+}]_i$ (9). Many of the above mentioned responses to α-thrombin appear dependent on this early elevation in $[Ca^{2+}]_i$.

A rise in $[Ca^{2+}]_i$ is the primary trigger for many cellular processes in vascular smooth muscle. An elevation in $Ca^{2+}$ triggers cell contraction via activation of myosin light chain kinase (10). $Ca^{2+}$ has also been demonstrated to stimulate directly the expression of c-*fos* mRNA in vascular smooth muscle (11, 12). In other cell types $Ca^{2+}$ has been demonstrated to modulate gene expression leading to increases in the levels of c-*fos* mRNA (13, 14), c-*myc* mRNA (15, 16), and repression of c-*jun* mRNA (14). A rise in $Ca^{2+}$ has also been observed to precede the onset of anaphase in Swiss 3T3 cells, suggesting that transient elevations in $Ca^{2+}$ are important during particular stages of the cell cycle (17).

There is increasing evidence to suggest that a variety of guanine nucleotide-binding proteins (G proteins) are involved in signal transduction (18). In smooth muscle, G proteins have been suggested to be involved in the coupling of receptors to phospholipase C (19), modulation of the $Ca^{2+}$ sensitivity of

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[1] The abbreviations used are: $[Ca^{2+}]_i$, intracellular $Ca^{2+}$ concentration; $IP_3$, inositol 1,4,5-trisphosphate; G protein, guanine nucleotide-binding protein; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PT, pertussis toxin; *t*BuBHQ, 2,5-di-*tert*-butylhydroquinone; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; HPLC, high performance liquid chromatography; TBS, Tris-buffered saline; GppNHp, guanyl-5′-yl imidodiphosphate.

the contractile mechanism (20, 21), release of $Ca^{2+}$ from the sarcoplasmic reticulum (22, 23), and in the translocation of $Ca^{2+}$ between intracellular pools (24). However, little is known of the nature and identity of the G proteins present in vascular smooth muscle. Attempts to characterize them using pertussis toxin (PT) have been complicated by the variable effects of the toxin in different species and/or vascular beds. For example, responses are markedly inhibited in the dog mesenteric vessel (25) and in cultured cells from the rat aorta (26–28), partially inhibited in cultured cells from the rabbit renal artery (29), and are not affected in pig coronary arteries (30) or in a vascular smooth muscle cell line (31). Because G proteins were not characterized in these studies it is not known whether the apparent discrepancies of the effect of PT are the consequence of variability in the nature of the G protein subunits present in vascular smooth muscle from the different vessels.

In the present study we examined the importance of G proteins in $\alpha$-thrombin-stimulated $Ca^{2+}$ mobilization in cultured aortic smooth muscle. We initially characterize the major PT-sensitive G proteins present in vascular smooth muscle. We then demonstrate that a slowly ribosylating G protein, which represents less than 9% of the total pool of ribosylated G proteins, is involved in the mobilization of $Ca^{2+}$ by $\alpha$-thrombin. Importantly, this G protein component does not appear to play a role in the generation of $IP_3$, and this, together with its slow time course of ribosylation, leads us to suggest that this G protein is directly involved in $Ca^{2+}$ release from the sarcoplasmic reticulum. Our evidence indicating the existence of a slowly ribosylating G protein helps to clarify the apparent controversy as to the role of PT-sensitive G proteins through which some vasoconstrictors elevate $Ca^{2+}$ in vascular smooth muscle (26, 30–32).

## EXPERIMENTAL PROCEDURES

*Materials*—$\alpha$-Thrombin (from rat plasma), phenylmethylsulfonyl fluoride, Ponceau S, and Hepes were obtained from Sigma. 2,5-Di-*tert*-butylhydroquinone (*t*BuBHQ) was obtained from Fluka (Buchs, Switzerland). Thapsigargin was obtained from LC Services (Woburn, MA). Ionomycin was from Calbiochem, and lanthanum chloride was from BDH Chemicals Ltd., Melbourne, Australia. Teric G12A9 was obtained from ICI Biochemicals, Sydney, Australia. Coomassie Brilliant Blue protein assay was purchased from Pierce Chemical Co. Dulbecco's modified Eagle's medium, M199, fetal calf serum, and bovine serum albumin (BSA) were purchased from ICN; Dulbecco's phosphate-buffered saline, Monomed, and all other cell culture products were from the Commonwealth Serum Laboratories, Melbourne, Australia. Fura-2/AM was purchased from Molecular Probes, Eugene, OR.

PT was purchased either from Sigma or from the Institute of Bioorganic Chemistry of the Ukrainian Academy of Sciences or was kindly provided by Dr. Vitaly O. Rybin (USSR Cardiology Center). All three PT preparations gave identical results. U-49 (anti-$\beta_{36}$) antipeptide antiserum was a generous gift of Drs. Alfred G. Gilman and Susan Mumby; A-10 (anti-$\alpha_0$), A-54 (anti-$\alpha_{i2}$), A-56 (anti-$\alpha_{i3}$), 584 (anti-$\alpha_s$), and GC-2 (anti-$\beta_{35}$) antipeptide antisera were kind gifts of Dr. Janet Robishaw (University of Texas Health Science Centre).

*Smooth Muscle Cell Culture*—Aortic smooth muscle cells from adult WKY rats were isolated by enzymatic digestion as described previously (33). Experiments were performed on multiply passaged smooth muscle cells. The cells were seeded onto glass coverslips and grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Cells reached confluence after 2 days, and then the serum in the medium was replaced with 0.46% BSA to induce cell quiescence. Cells were used 24–48 h later. Treatment with PT was performed by replacing the growth medium with Dulbecco's modified Eagle's medium containing the BSA in the presence or absence of PT (1 $\mu$g/ml) and incubating cells for the times indicated.

*Determination of Inositol Phosphates*—Cells were seeded into 24-well plates (2.0 $cm^2$/well) in M199 plus 10% fetal calf serum. At confluence the medium was replaced with 0.5 ml of M119 plus 4% Monomed and 5 $\mu$Ci of *myo*-[$^3$H]inositol (Amersham Corp.). After 48 h the cells were washed (four times) with Dulbecco's phosphate-buffered saline to remove unincorporated *myo*-[$^3$H]inositol and incubated in 1 ml of M199 plus 4% Monomed and 20 mM LiCl for 15 min at 37 °C in a 5% $CO_2$ incubator. Experiments were conducted by adding microliter quantities of agonists to the cells for the times indicated. The reaction was terminated by rapid addition of 1 ml of stop solution to each well (1% SDS in 30 mM EDTA). Inositol polyphosphates were separated using Dowex AG 1-X8 ion exchange resin as described elsewhere (34). Chromatography conditions were validated by running $^3$H-labeled inositol monophosphate, bisphosphate, and $IP_3$ standards. Fractions (6 ml) collected were mixed with Insta-gel (14 ml) (Packard Instrument Co., Downer's Grove, IL) and counted in a liquid scintillation spectrometer.

*Intracellular $Ca^{2+}$ Measurement*—The cell monolayers on coverslips were loaded with Fura-2 (35) by incubation in 1 ml of Dulbecco's modified Eagle's medium containing 2 $\mu$M Fura-2/AM for 30 min at 37 °C. The Fura-2/AM was added as a 2-$\mu$l aliquot of 0.1% solution in dimethyl sulfoxide during the final 30 min of PT pretreatment. There was no difference in the loading of Fura-2 in PT-pretreated or control cells. At the end of the loading period coverslips were washed in physiological salt solution (3 × 2 ml) and kept in the dark at room temperature until use; the cells were used within 1 h of loading. The composition of physiological salt solution used in the experiments was (in mM): 150 NaCl, 5 KCl, 1.8 $CaCl_2$, 1 $MgSO_4$, 10 Hepes, 10 glucose, pH 7.4, at 37 °C. Immediately before the experiment coverslips were rapidly rinsed in physiological salt solution to remove any residual possibly leaked dye and inserted into a standard cuvette containing physiological salt solution. The cuvette with its stirring device was placed into the thermostatically controlled chamber at 37 °C in a SPEX dual wavelength 1681 fluorolog spectrometer. Excitation wavelengths were 340 nm and 380 nm, and emitted light collected at 505 nm. Readings were collected using the dM3000 software (Spex Industries, Inc., Edison, NJ). Slit widths were set at 1.5 mm on both excitation monochromators and 2.5 mm on the emission side. A 450-watt xenon arc lamp was used as the light source. Integration time was 0.1 s at each wavelength, and the time increment was 0.3 s. Determination of cell autofluorescence was performed on each coverslip after the experiment by the addition of 2 mM $MnCl_2$ and 2 $\mu$M ionomycin to quench intracellularly located dye, and this level was subtracted from each wavelength reading before $Ca^{2+}$ levels were calculated. Typically, autofluorescence readings under basal conditions represented approximately 30% of each fluorescence reading. All drugs were added by direct injection of either 10- or 20-$\mu$l volumes of stock solutions into the cuvette. The fluorescence readings at 340 nm were divided by those at 380 nm to give a ratio that was then processed according to the equation (35) $[Ca^{2+}]_i = K_d \cdot ((R - R_{min})/(R_{max} - R)) \cdot (S_{f2}/S_{b2})$. $[Ca^{2+}]_i$ is the intracellular free calcium ion concentration, the $K_d$ for Fura-2 was assumed to be 224 nM (35), $R_{max}$ and $R_{min}$ are the maximal and minimal fluorescence ratios of Fura-2 acid in solution containing saturating $Ca^{2+}$ (1 mM), or $Ca^{2+}$-free and EGTA (1 mM) and were 31.2 and 0.66, respectively. $S_{f2}$ and $S_{b2}$ are the fluorescence values at 380 nm in the absence and in the presence of saturating $[Ca^{2+}]_i$, respectively. $S_{f2}/S_{b2} = 11.66$ in our system.

*Electrophoresis*—SDS-polyacrylamide gel electrophoresis was run through 12% gels according to Laemmli (36). Gels were stained with Coomassie Brilliant Blue, dried, and autoradiographed. For quantitative determinations, pieces of gels containing labeled bands were excised and added to water for measurement of Cherenkov radiation.

Electrophoretic blotting onto nitrocellulose was carried out following the procedure of Towbin *et al.* (37) in a Mini-Transblot apparatus (Bio-Rad) overnight at 45–60 V (usually 150–250 mA). Transfers were run at 100 V for the first 2 h (current limit, 0.48 A) in a cold room using a Bio-Ice cooling unit, and then the voltage was set to 50–60 V (current limit, 0.25 A) for overnight electrophoresis. Transfer buffer was fortified with 0.0025–0.005% SDS for increased transfer of samples. Various amounts of membrane protein were blotted to ensure a linear relationship between the amount of antigen and the amount of bound radioactive antibody. Proteins on blots were visualized with Ponceau S before blocking blots in TBS (Tris-buffered saline, 10 mM Tris-HCl, pH 7.5, 250 mM NaCl) containing 1% BSA and 0.05% Tween 20 (TBS-Tween-BSA) for 1 h. The Western blots were incubated sequentially with antipeptide antisera against selected G protein subunits followed by $^{125}$I-labeled anti-rabbit IgG (sheep or goat). All antibodies in TBS-Tween-BSA plus 0.01% $NaN_3$ were of appropriate dilution to saturate binding sites. Blots were washed between incubation with TBS-Tween and finally (before drying) with

TBS alone. Dried blots were autoradiographed with an intensifying screen using Kodak XAR film.

*Membrane Preparations*—The aortic smooth muscle cell cultures were washed three times with ice-cold Dulbecco's phosphate-buffered saline ($Ca^{2+}$ and $Mg^{2+}$ free), and cells were scraped from dishes into Dulbecco's phosphate-buffered saline using a rubber policeman. After centrifuging cells at ~ 3,000 × *g* for 10 min, pellets were homogenized in a motor-driven glass-Teflon homogenizer in the medium consisting of 25 mM Na-Hepes (pH 7.5), 1 mM EDTA, and 100 μM phenylmethylsulfonyl fluoride. Homogenates were centrifuged in an Eppendorf microcentrifuge (13,000 rpm) for 30 min, resuspended, and washed again in the same manner. Resulting pellets were reconstituted in a small volume of homogenization buffer but with 10 μM phenylmethylsulfonyl fluoride. After resuspension, membranes were aliquoted and frozen in liquid nitrogen. These preparations were designated as "crude smooth muscle cell membranes" and used in subsequent ADP-ribosylation and immunoblotting experiments.

Membranes were resuspended at protein concentrations within the range 0.5–2.5 mg/ml. Protein concentration was determined using the Coomassie Brilliant Blue protein assay with BSA as a standard.

*ADP-ribosylation*—Membranes from control and PT-treated smooth muscle cells were ADP-ribosylated *in vitro* to estimate residual PT substrate(s) using [$^{32}$P]NAD. ADP-ribosylation was performed as described previously (38) with minor modifications. Briefly, membranes (5–15 μg of protein) were incubated at 37 °C for 60–90 min in 40 μl of medium containing 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 2 mM $MgCl_2$, 10 mM dithiothreithol, 10 mM thymidine, 1 mM ATP, 0.1 mM GTP, 0.1 mM NADP, 0.1% Lubrol PX, 10 μM [*adenylate*-$^{32}$P]NAD (specific activity 4.7 Ci/mmol), and 1 μg/ml PT. For these studies, PT was activated by preincubation with 5 mM ATP and 50 mM dithiothreitol for 15 min at 37 °C. Under these conditions all available PT substrates became labeled, and no significant degradation of NAD was observed. The reaction was terminated by adding concentrated (4 ×) SDS-polyacrylamide gel electrophoresis sample buffer and immediate boiling.

Lubrol PX was usually added to membranes because it causes considerable enhancement of PT-dependent ADP-ribosylation (more than 2.5-fold in these membranes). In some cases, Teric G12A9 was used instead of Lubrol in the same concentration and with the same results. To ensure that differences in labeling of PT substrates were not a result of alterations in their physical availability for toxin, ADP-ribosylation rate, or NAD degradation, we carried out time courses of ADP-ribosylation of smooth muscle cell membranes *in vitro*. Labeling reached its maximal values within 15 min and did not change up to 1.5 h (data not shown). ADP-ribosylation reactions were tested for NAD survival during the incubation time utilizing TLC on cellulose plates in 50% ethanol, 0.3 M ammonium acetate, pH 5. Not less than 90% NAD survived in this medium with smooth muscle cell membranes up to 1.5 h (not shown).

*Statistical Analysis*—Statistical analysis in Fig. 9 was performed using two-way analysis of variance, and results were compared using the standard error of the difference. The averaging of multiple $[Ca^{2+}]_i$ traces was performed using a standard spreadsheet which calculated, at each time increment, the arithmetic mean of individual $Ca^{2+}$ values taken from multiple experiments. Thus, all $Ca^{2+}$ traces depicted in the figures represent the mean of at least five similar traces from separate experiments.

## RESULTS

### *Characterization of G Protein Subunits*

Because G protein subunits have not been characterized previously in vascular smooth muscle we initially examined the nature of the PT-sensitive G proteins present in the plasma membranes of these cells. Membranes were prepared from primary cultured cells, exponentially growing cells, and confluent subcultured cells and G proteins assessed using immunoblotting with specific antisera (Fig. 1).

The α-subunits, $G\alpha_{i2}$ and $G\alpha_{i3}$ are PT sensitive because of the presence of a consensus sequence containing a cysteine residue (39), the acceptor site for ADP-ribosylation (40). These $G\alpha_i$ subunits were found to be present in all three cell preparations in approximately equal amounts as judged by their intensity on autoradiographic film. Vascular smooth muscle cells were also found to possess the $G\alpha_s$ protein which

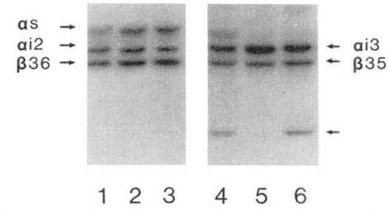


FIG. 1. **Characterization by immunoblotting of G protein subunits present in vascular smooth muscle cells in culture.** Membranes prepared from primary (*lanes 1* and *4*), exponentially growing (*lanes 2* and *5*), or confluent subcultured (*lanes 3* and *6*) cells were probed with antisera raised against $G\alpha_s$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\beta_{35}$, $G\beta_{36}$ protein subunits. 10 μg of protein was loaded in each case. *Lanes 1–3* were probed sequentially with antibodies to $\alpha_s$, $\alpha_{i2}$, and $\beta_{36}$. *Lanes 4–6* were probed with antibodies to $\alpha_{i3}$ and $\beta_{35}$. All *lanes* were then probed with a $^{125}$I-labeled anti-rabbit IgG.

is known to couple to adenylate cyclase (18). Interestingly, primary cultured smooth muscle cells contained significantly less $G\alpha_s$ than the exponentially growing and confluent synthetic cultures. No detectable levels of the PT-sensitive α-subunit found most abundantly in neural tissue, $\alpha_0$, were found in smooth muscle cell membranes because no immunoreactivity was observed on blots probed with the anti-$\alpha_0$-specific antisera A-10 (not shown).

Two β-subunits of G proteins, $G\beta_{35}$ and $G\beta_{36}$, which are approximately 10% different in amino acid sequence (41, 42) and coded for by different genes (42), could also be detected with antisera raised against specific peptides. Both β-subunits were present in approximately equal amounts in all three preparations. A lower molecular weight species was also observed on blots probed with antisera against the $\alpha_{i3}$- and $\beta_{35}$-subunits. The nature of this protein is unknown at present; however, it showed differential expression in that it was detected in primary and confluent cultures but not in the exponentially growing cells.

These results indicate that vascular smooth muscle cells possess multiple G proteins which may serve a variety of functions.

### *Assessment of Intracellular $Ca^{2+}$ Mobilization by α-Thrombin*

Estimation of intracellular $Ca^{2+}$ mobilization is normally carried out by measuring $Ca^{2+}$ elevations in nominally $Ca^{2+}$-free media or in media containing EGTA in excess of $Ca^{2+}$. A possible drawback to these strategies is that removing extracellular $Ca^{2+}$ has the potential to lower $Ca^{2+}$ in the internal stores. To investigate whether this was a problem in vascular smooth muscle, we compared $Ca^{2+}$ elevations with α-thrombin in the presence of external $Ca^{2+}$, in the presence of lanthanum ions to block $Ca^{2+}$ entry, and in the presence of EGTA to chelate extracellular $Ca^{2+}$.

The rise in $Ca^{2+}$ induced by α-thrombin was found to be comprised of an initial transient peak, caused by $Ca^{2+}$ release from internal stores, and a sustained phase, presumably caused by $Ca^{2+}$ entry. Fig. 2 shows that the response in the presence of lanthanum (200 μM) precisely matches the initial transient peak observed in the control response with no evidence of a sustained phase. In contrast, treatment with EGTA (1 mM) produced first a slow decline in base line followed by a markedly attenuated $Ca^{2+}$ release. This effect of EGTA is consistent with it causing a slow "leeching out" of $Ca^{2+}$ from agonist-sensitive internal pools. Therefore, it was decided to employ $Ca^{2+}$ channel blockade with lanthanum as a means to quantitate internal $Ca^{2+}$ release.

### *PT and α-Thrombin-induced $Ca^{2+}$ Mobilization*

*Short-term PT Treatment*—PT was used to examine the contribution of G proteins in the intracellular $Ca^{2+}$ response

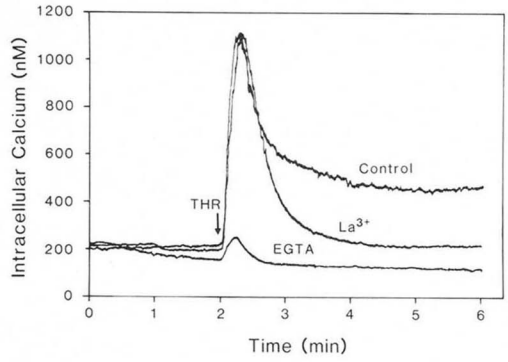


FIG. 2. **Effect of lanthanum and EGTA on the α-thrombin (*THR*)-induced rise in cytoplasmic free $Ca^{2+}$ concentration in vascular smooth muscle cells.** The cytoplasmic $Ca^{2+}$ concentration in monolayers of smooth muscle cells was monitored using fura-2 and dual-wavelength fluorescence spectroscopy as described under "Experimental Procedures." For the lanthanum treatment, LaCl (200 μM) was added at 60 s. For the EGTA treatment coverslips were transferred into buffer containing EGTA (1 mM) and no added $Ca^{2+}$ approximately 30 s prior to the start of the experiment. Each *trace* is the mean of three similar experiments.

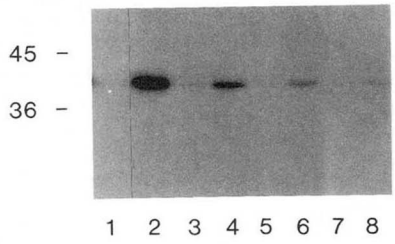


FIG. 3. **Time course of PT-induced ADP-ribosylation in vascular smooth muscle cells.** SDS-polyacrylamide gel electrophoresis autoradiogram showing the reduction in the ability of PT to ADP-ribosylate G proteins in membranes prepared from intact cells pretreated for increasing periods of time with PT (1 μg/ml). ADP-ribosylation was determined using PT-catalyzed incorporation of [$^{32}$P]NAD as described under "Experimental Procedures." *Lanes 1* and *2*, *3* and *4*, *5* and *6*, and *7* and *8* were pretreated with PT for 0, 1, 2, and 4 h, respectively. *Lanes 1*, *3*, *5*, and *7* represent membranes incubated *in vitro* with [$^{32}$P]NAD in the absence of PT, and *lanes 2*, *4*, *6*, and *8* are membranes incubated *in vitro* with [$^{32}$P]NAD in the presence of PT. Molecular weight standards are shown on the *left*.

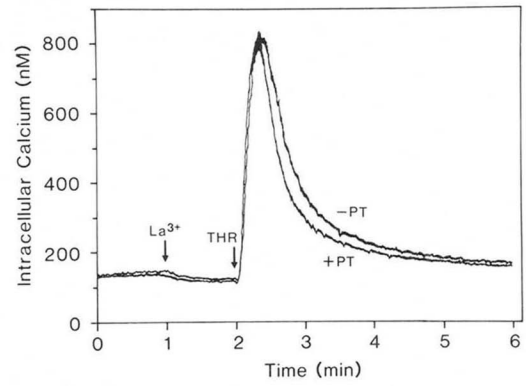


FIG. 4. **Effect of short term PT treatment on α-thrombin (*THR*)-induced $Ca^{2+}$ mobilization in vascular smooth muscle cells.** The effect of α-thrombin (2 units/ml) on the release of intracellular $Ca^{2+}$ was tested on cell monolayers pretreated for 2 h in the presence or absence of PT (1 μg/ml). The ability of α-thrombin to release $Ca^{2+}$ from intracellular stores was determined by using lanthanum ions (200 μM). α-Thrombin and lanthanum were added at the times indicated. PT pretreatment (2 h) was performed as described under "Experimental Procedures."

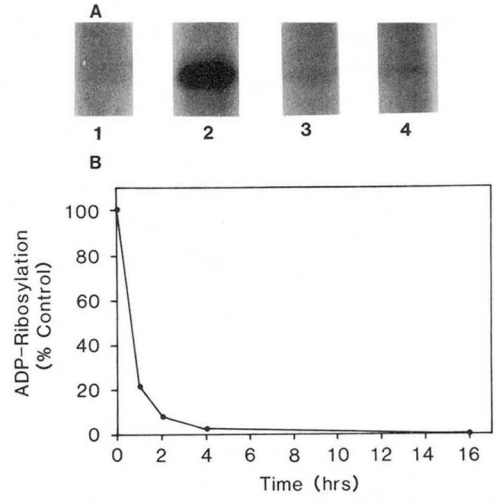


FIG. 5. **Residual nonribosylated PT-sensitive G proteins in membranes of vascular smooth muscle cells preexposed to PT for 18 h.** *A*, cells were incubated in culture for 18 h in the absence (*lanes 1* and *2*) or presence (*lanes 3* and *4*) of PT (1 μg/ml), and membranes were prepared as described under "Experimental Procedures." Membranes were incubated with [$^{32}$P]NAD in the absence (*lanes 1* and *3*) and presence (*lanes 2* and *4*) of PT in the ribosylation mixture. 5.9 μg of membrane protein was used in all *lanes*. *B*, the time course of ADP-ribosylation as assessed by counting the [$^{32}$P]NAD radioactivity present in excised bands. Ribosylation is expressed as a percentage of control cells not exposed to PT. The *curve* shown is typical of three separate experiments.

to α-thrombin. First, the time course of PT-mediated ADP-ribosylation of the G proteins in smooth muscle cells was assessed by incubating intact cells for various periods of time with PT (1 μg/ml), preparing membranes from these cells, and testing the subsequent ability of PT to incorporate $^{32}$P-labeled NAD. PT produced a rapid effect in intact cells; the proportion of PT-sensitive G protein left unribosylated after 1, 2, and 4 h of pretreatment was 21.6, 8.7, and 2.6% of that present in untreated cells, respectively (Fig. 3). No ADP-ribosylation of species with molecular mass lower than 36 kDa was observed (not shown).

Because the amount of PT-induced ADP-ribosylation was greater than 90% at 2 h, we tested the effect of this period of PT pretreatment on α-thrombin-induced $Ca^{2+}$ mobilization. Treatment of cells for 2 h with PT did not affect the $Ca^{2+}$ response to a maximal concentration of α-thrombin (2 units/ml) (Fig. 4). This raised the possibility that the residual component (approximately 8.7% of total) of PT-sensitive G protein was sufficient to couple α-thrombin receptors to $Ca^{2+}$ release.

*Long Term PT Treatment*—To obtain ADP-ribosylation of the total amount of PT-sensitive G proteins present in vascular smooth muscle, long term (18 h) treatment was used. Fig. 5*A* shows that treatment of cells with PT for 18 h inactivated all PT-sensitive G proteins, within the limits of detection. In addition, measurement of radioactivity present in excised bands revealed no incorporation of [$^{32}$P]NAD in membranes pretreated for 18 h (Fig. 5*B*). The effect of long term pretreatment with PT on $Ca^{2+}$ release is shown in Fig. 6. Long term treatment was found to reduce markedly α-thrombin-induced $Ca^{2+}$ release, indicating that the residual 8.7% of PT-sensitive G proteins left after a 2-h PT pretreatment accounts for most of the $Ca^{2+}$ response. In addition, despite the fact that long term PT treatment inactivated all PT-sensitive G proteins present, 28% of the $Ca^{2+}$ response to α-thrombin remained, indicating that PT-insensitive G proteins or G protein-independent mechanisms may be involved in mediating this residual component of $Ca^{2+}$ release from

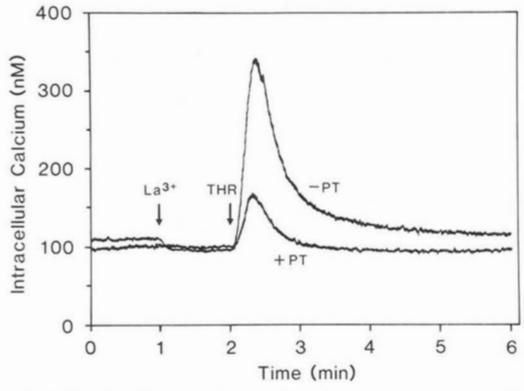


FIG. 6. **Effect of long term PT treatment on α-thrombin (*THR*)-induced $Ca^{2+}$ mobilization in vascular smooth muscle cells.** The effect of α-thrombin (2 units/ml) on the release of intracellular $Ca^{2+}$ was tested on cell monolayers pretreated for 18 h in the presence or absence of PT (1 μg/ml). α-Thrombin and lanthanum were added at the times indicated. PT pretreatment was performed as described under "Experimental Procedures."

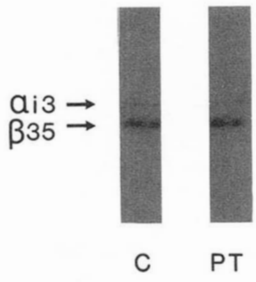


FIG. 7. **$G\beta_{35}$- and $G\alpha_{i3}$-subunits present in membranes of vascular smooth muscle cells before and 18 h after exposure to PT.** Autoradiogram of an immunoblot using specific antisera raised against $\alpha_{i3}$ and $\beta_{35}$ G protein subunits. The *left lane* represents membranes prepared from control (*C*) cells; the *right lane* was from cells treated with PT (1 μg/ml) for 18 h. Exactly 15 μg of protein was loaded in each case.

internal stores. It is also interesting to note here that no marked effects were observed on $Ca^{2+}$ entry, as assessed by comparing the sustained phases of the $Ca^{2+}$ elevations in the absence of lanthanum (data not shown).

### *PT and Gβ-subunits*

It was shown recently that long term PT treatment of cells can produce down-regulation of the levels of the β subunit of G proteins, thereby causing nonspecific inhibition of all G proteins present (43). We tested this by measuring levels of $G\beta_{35}$ and $\alpha_{i3}$ in membranes prepared from control and long term PT-treated cells, using immunoblotting with specific antisera. No marked alteration in the level of either the $\beta_{35}$- or the $\alpha_{i3}$-subunit was detected (Fig. 7). Thus, the reduction in α-thrombin-mediated $Ca^{2+}$ release by long term PT pretreatment in these cells was caused by a specific inhibition of PT-sensitive G protein subunits rather than a reduction in β-subunit levels.

### *PT and $Ca^{2+}$ Stores*

Recent reports have implicated the involvement of GTP-binding proteins in communication between distinct intracellular $Ca^{2+}$ pools (24). To test the possibility that PT was affecting the size of the agonist-sensitive $Ca^{2+}$ pool, the effects of PT were tested on $Ca^{2+}$ release induced by two agents that act independently of $IP_3$ generation. *t*BuBHQ, which has been shown to release $Ca^{2+}$ selectively from the $IP_3$-sensitive $Ca^{2+}$ pool (44), caused a rapid increase in $[Ca^{2+}]_i$ in the presence of $La^{3+}$, which was not affected to any great extent by short term exposure to PT (Fig. 8*A*). Similarly, thapsigargin, an inhibitor of $Ca^{2+}$ uptake into internal stores (45), produced a slower increase in $Ca^{2+}$ levels but was also unaffected by PT treatment (Fig. 8*B*). Intracellular $Ca^{2+}$ release induced by either agent was also not affected by long term (18 h) PT treatment (data not shown). These results provide evidence to suggest that PT was not affecting the size of the agonist-sensitive $Ca^{2+}$ pool in vascular smooth muscle.

### *PT and α-Thrombin-induced [$^3$H]$IP_3$ Formation*

It was possible that the marked inhibition of $Ca^{2+}$ release in long term PT-treated but not short term PT-treated cells was caused by a greater effect of PT on $IP_3$ formation at 18 h. However, no correlation between the inhibition of $IP_3$ formation and the inhibition of $Ca^{2+}$ release was observed. Fig. 9 shows that treatment of cells for 2 h with PT (which produces 91.3% ADP-ribosylation of PT-sensitive G proteins) significantly attenuated ($p < 0.05$) α-thrombin-induced $IP_3$ formation both in the presence or absence (not shown) of $La^{3+}$ (200 μM). However, although an 18-h PT pretreatment produced total ADP-ribosylation, no further inhibition of $IP_3$ formation was seen (*i.e.* the extent of inhibition after 18 h was not significantly greater than after a 2-h pretreatment; $p > 0.10$) despite there being a marked effect on $Ca^{2+}$ release. Similar results were obtained when we measured the products of phosphoinositide hydrolysis using HPLC. For these experiments we measured inositol 4-monophosphate and inositol 1,3,4-trisphosphate, two products that accumulate in the presence of lithium and which are good indicators of flux through the phosphorylation and dephosphorylation pathways of $IP_3$

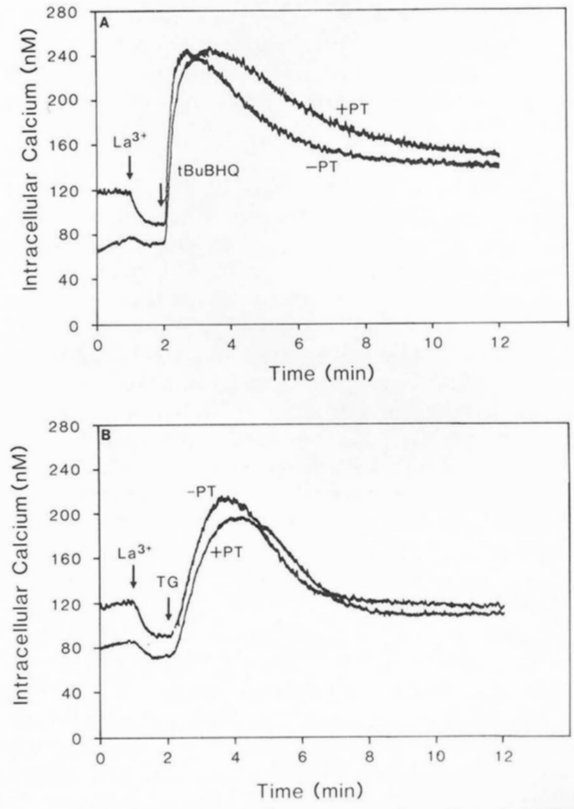


FIG. 8. **Effect of PT on $Ca^{2+}$ release induced by *t*BuBHQ (20 μM) (*A*) and thapsigargin (2 μM) (*B*) in vascular smooth muscle cells.** Cells were incubated with or without PT (1 μg/ml) for 2 h, and Fura-2/AM was added for the final 30 min. All *traces* are means of data from two similar experiments. $La^{2+}$ and the $Ca^{2+}$ mobilizing agents were added as indicated.

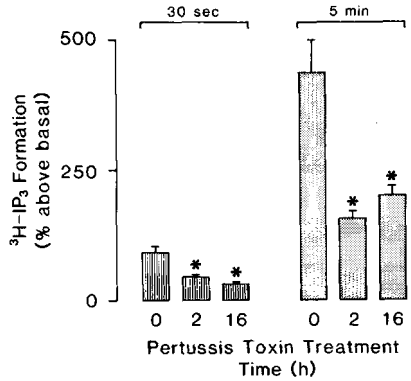


FIG. 9. **Effect of PT on α-thrombin-induced $IP_3$ formation in vascular smooth muscle cells.** Cells were exposed to PT (1 μg/ml) for 0, 2, or 18 h before measurement of [$^3$H]$IP_3$ formation stimulated by α-thrombin over 30-s (*striped histograms*) or 5-min (*dotted histograms*) periods. Results shown are the mean of three separate experiments conducted in triplicate. *Error bars* represent S.E. *Asterisks* indicate statistically significant differences from cells not treated with PT ($p < 0.05$).

metabolism. We found very little difference between the level of inhibition of either inositol 4-monophosphate or inositol 1,3,4-trisphosphate accumulation by 2- or 18-h PT treatment of cells. The accumulation of inositol 4-monophosphate in response to thrombin (which increased 4.8-fold over basal) was inhibited by 66.6 and 71.5%, and the accumulation of inositol 1,3,4-trisphosphate (17.7-fold increase over basal) was inhibited by 87.4 and 88.4% by 2- and 18-h PT treatment, respectively. These results indicate that first, α-thrombin receptor coupling to phospholipase C appears to occur via PT-sensitive and PT-insensitive mechanisms; and second, inhibition of $Ca^{2+}$ release after 18-h but not after 2-h pretreatment is not caused by increased inhibition of phospholipase C, and therefore the residual 8.7% of PT-sensitive G proteins does not appear to be involved in coupling to this enzyme.

## DISCUSSION

Our observations on the effects of PT on α-thrombin-induced $Ca^{2+}$ mobilization in intact cells suggest that a slowly ribosylating G protein is directly involved in $IP_3$-mediated $Ca^{2+}$ release from intracellular pools. Evidence supporting this conclusion is based on the findings that: (*a*) a small component representing less than 9% of the total PT-sensitive G proteins is ADP-ribosylated only after long term exposure to PT; (*b*) whereas α-thrombin-stimulated $IP_3$ formation is maximally inhibited after a 2-h exposure to PT, α-thrombin-induced $Ca^{2+}$ release is reduced only after long term PT exposure; and (*c*) PT has no effect on $Ca^{2+}$ release induced by agents acting independently of $IP_3$. In these studies we also demonstrate that multiple G proteins are present in vascular smooth muscle and that coupling to either phospholipase C or $Ca^{2+}$ release occurs via both PT-sensitive and -insensitive mechanisms.

A possible explanation for our results is that there may be marked differences in the kinetics of ADP-ribosylation of the individual $G\alpha_i$-subunits so that one of either $G\alpha_{i2}$ or $G\alpha_{i3}$ is significantly ADP-ribosylated only after long term PT treatment and is therefore able to elicit full $Ca^{2+}$ responses in short term PT-treated cells. In separate experiments we set out to determine which α-subunits were ADP-ribosylated and the extent to which each was ribosylated after PT treatment of cells for 2 h. We found that it was possible to observe a doublet in the 41-kDa region, which corresponds to separation of $G\alpha_{i2}$ and $G\alpha_{i3}$, if membrane protein was run on large electrophoresis gels. Both bands of this doublet were significantly ADP-ribosylated by 2 h of PT treatment of intact cells (data not shown). In addition, two-dimensional electrophoresis experiments, which resulted in better separation of $G\alpha_{i2}$- and $G\alpha_{i3}$-subunits, produced identical results. Thus, although we have not performed a detailed time course experiment we can report that there appears to be no major difference in the extent of ADP-ribosylation of $G\alpha_{i2}$ and $G\alpha_{i3}$ after a 2-h PT treatment of cells, and therefore it does not provide an explanation for the marked differences observed on $Ca^{2+}$ mobilization between the two periods of PT treatment.

Previous work has been inconclusive as to whether PT-sensitive G proteins are involved in agonist-dependent responses in vascular smooth muscle. For instance, endothelin-stimulated inositol polyphosphate production is partially inhibited in cells cultured from the rabbit renal artery (29) whereas endothelin-stimulated $IP_3$ production in the A-10 vascular smooth muscle cell line is not affected (31). In rat aortic smooth muscle cells, agonist-induced $Ca^{2+}$ elevations have been shown to be sensitive to PT although by different degrees (26–28). α-Thrombin-induced alterations in $pH_i$ have also been shown to be inhibited by PT in these cells (7). In studies on whole vessels, contractions are inhibited by PT in dog mesenteric artery (25) but not in the pig coronary artery (30). Electrophysiological studies of $Ca^{2+}$ channels in guinea pig mesenteric artery have also shown a lack of effect of PT even though the channels appear to be dependent on a G protein (32). The differences in the sensitivity to PT could be explained by the different treatment periods used in each study. For instance, noradrenaline-stimulated increases in quin-2 fluorescence in rat aortic smooth muscle cells were sensitive to PT only if cells were exposed for periods in excess of 9 h (26). In the present study we show that although short term exposure to PT produces a maximal effect on $IP_3$ formation, inhibition of $Ca^{2+}$ release is seen only after long term exposure, indicting that these two systems may involve different G proteins that follow different time courses of PT-catalyzed ADP-ribosylation.

Several factors led us to suggest that this G protein exerts its action at the level of the sarcoplasmic reticulum. The pathway requires long term exposure (>2 h) to PT before it is inactivated by ADP-ribosylation, which suggests that PT is not ribosylating a G protein on the plasma membrane but rather is being internalized and is ribosylating a cytosolic or microsomal protein. The time course of inactivation of $Ca^{2+}$ release is consistent with the time taken for internalization of extracellular molecules by endocytotic mechanisms in smooth muscle (46). Another finding that is consistent with the G protein not acting at the plasma membrane is that the pathway does not appear to be involved in the mechanisms which generate $IP_3$, *i.e.* phospholipase C activation. $IP_3$ formation is maximally inhibited after a 2-h exposure to PT, whereas $Ca^{2+}$ release is not affected after that short period of exposure. Thus, although the possibility that the G protein is plasma membrane bound cannot be excluded, the present findings suggest that PT is inactivating a component located deeper within the cell. Several reports have demonstrated GTP-binding proteins present within the cytosol (47). The major cytosolic G protein present in bovine smooth muscle is rhoA p21, a member of the ras p21-like superfamily of GTP-binding proteins (48). A G protein has also been postulated to modulate the sensitivity of the contractile mechanism in smooth muscle (20, 21). These proteins appear to be distinct from those that couple receptors to various effectors on the plasma membrane (47–49), and it is likely that they have important intracellular functions. In addition to these, the Gα-subunits of the more classical G proteins have been de-

tected in the cytosol and appear to be present as uncomplexed species (50). For instance, the PT-sensitive $\alpha_i$ has been detected in a hepatic microsomal fraction (51). Thus, it is not unreasonable to suggest that in vascular smooth muscle, one or more of these cytosolic proteins are involved in $Ca^{2+}$ release.

There is also increasing evidence to suggest that GTP-binding proteins are involved in modulating $Ca^{2+}$ release from intracellular stores (52). This concept originated from the findings that in permeabilized cells or in isolated subcellular fractions, GTP is a potent releasing agent on its own and also greatly enhances $Ca^{2+}$ release induced by $IP_3$ (53–56). The mechanism of GTP-stimulated $Ca^{2+}$ release is still unresolved; however, it has been suggested recently that small GTP-binding proteins may enhance $Ca^{2+}$ release by fusing small microsomal vesicles together (52) or by promoting the translocation of $Ca^{2+}$ from an $IP_3$-insensitive to an $IP_3$-sensitive calcium pool (24). In vascular smooth muscle, recent evidence has implicated a role for a GTP-binding protein in $Ca^{2+}$ release (22). In saponin-permeabilized rabbit mesenteric artery (23, 57) and primary cultured rat aortic smooth muscle cells (23) $IP_3$-dependent $Ca^{2+}$ release shows an absolute requirement for GTP. The nonhydrolyzable analogue $G_{pp}NH_p$, substitutes adequately for GTP, suggesting that GTP binding rather than hydrolysis is involved (57). Strong evidence that a G protein is involved in $IP_3$-dependent $Ca^{2+}$ release was obtained from the skinned artery preparation in which $IP_3$- but not caffeine-induced $Ca^{2+}$ release was inhibited by PT (23). Therefore, taken together with our present findings in intact cells it appears likely that a PT-sensitive G protein is directly involved in $IP_3$-dependent $Ca^{2+}$ release from the sarcoplasmic reticulum.

It is possible that the G protein is directly coupled to the $Ca^{2+}$ channel on the sarcoplasmic reticulum. A wide variety of membrane ion channels are directly modulated by G proteins (58). Sequence and structural analysis has shown that the sarcoplasmic reticulum $Ca^{2+}$ channel activated by $IP_3$ comprises part of the $IP_3$ receptor protein (59). The $IP_3$ receptor includes a large cytoplasmic N terminus domain which contains the $IP_3$ recognition site (59) and putative regulatory domains (60). This region of the receptor contains sites for phosphorylation by cAMP-dependent protein kinase (61), protein kinase C, and $Ca^{2+}$/calmodulin-dependent protein kinase II (62). It is suggested in the present study that the $IP_3$ receptor also contains a recognition site for a regulatory G protein. An attractive hypothesis is that a G protein may be directly involved in modulating the affinity state of the $IP_3$ receptor (or perhaps the affinity state for intraluminal $Ca^{2+}$), which has been proposed to control $Ca^{2+}$ flux (63). The residual $Ca^{2+}$ release component after long term PT treatment may represent $IP_3$-mediated $Ca^{2+}$ release in the absence of a coupled G protein, and this G protein-independent basal $Ca^{2+}$ flux may account for the $IP_3$-mediated $Ca^{2+}$ release from purified $IP_3$ receptors inserted in lipid bilayers (64, 65) which lack a coupled G protein.

Alternatively, it is possible that PT is affecting communication between intracellular $Ca^{2+}$ pools. It has been suggested recently that translocation of $Ca^{2+}$ between $IP_3$-sensitive and $IP_3$-insensitive $Ca^{2+}$ pools in $DDT_1MF$-2 smooth muscle cells is activated by a class of small GTP-binding proteins (24, 56). Although these proteins appear to be distinct from the G proteins that interact with receptors on the plasma membrane (47–49), they may contain consensus sites for ADP-ribosylation by PT (40). We could not provide evidence to support this hypothesis; $Ca^{2+}$ elevations in response to *t*BuBHQ, a releaser of $Ca^{2+}$ from $IP_3$-sensitive pools (44), or thapsigargin, an endoplasmic reticulum $Ca^{2+}$ ATPase inhibitor (45), were not altered by PT treatment. This does not necessarily rule out a role for a PT-sensitive pathway mediating communication between pools if communication is controlled by an inositol phosphate because these compounds act independently of inositol polyphosphates. However, these results do provide an important control in that PT does not appear to affect the total amount of releasable $Ca^{2+}$. Another possibility worth noting here is that PT may be affecting the interaction of the $IP_3$-sensitive $Ca^{2+}$ pool with the plasma membrane. It was suggested recently that in rat liver the $IP_3$-sensitive organelle is anchored to the plasma membrane through actin microfilaments (66). If a G protein is also involved, then inactivation by long term PT treatment may cause a redistribution of these pools in the cell such that, because of its rapid metabolism, less of the $IP_3$ generated at the plasma membrane would reach its receptor, which would result in a reduced $Ca^{2+}$ response. A close association of $IP_3$-sensitive $Ca^{2+}$ pools to the plasma membrane is supported by our finding that PT inhibited greater than 50% of $IP_3$ formation without affecting $Ca^{2+}$ mobilization in response to thrombin. This may indicate that there is considerable "spare" $IP_3$ in stimulated vascular smooth muscle cells and that the amount remaining after PT treatment is adequate to mobilize $Ca^{2+}$ fully. This is not unexpected if the $Ca^{2+}$ pools that are most sensitive to $IP_3$ lie directly beneath the plasma membrane close to the sites of $IP_3$ formation. $IP_3$ in these regions could well be produced in excess of that which is required for activation of $Ca^{2+}$ release. The concept of spare G protein was used several years ago to explain why in hepatocytes, angiotensin II-induced inhibition of adenylate cyclase was not lost until greater than 90% of the G protein was ADP-ribosylated by PT (67). By analogy, it could be argued that the reason for why $Ca^{2+}$ release is not inhibited until greater than 91.3% of the G protein is ADP-ribosylated is the presence of spare $IP_3$. This could not be the case because the levels of $IP_3$ present in cells that contained 91.3 or 100% ADP-ribosylation were identical. Thus, we can only conclude that the residual G protein left unribosylated after a 2-h treatment is not involved in coupling to phospholipase C, and it is suggested that this represents a separate pool of G protein which regulates internal $Ca^{2+}$ release.

We have also demonstrated that vascular smooth muscle cells contain G proteins of the $G_s$ and $G_i$ subfamilies; however the presence of additional $\alpha$-subunits appears likely. The PT-sensitive G proteins probably belong to the $G\alpha_i$ subfamilies which contain consensus sequences for PT-catalyzed ADP-ribosylation (40), and recent evidence has indicated that several of these proteins can be activated by a single receptor (68). These G proteins have also been demonstrated to activate phospholipase C in other cells (69), and therefore it is likely that the PT-sensitive $G\alpha_{i2}$ and $G\alpha_{i3}$ subunits are involved in coupling $\alpha$-thrombin to phospholipase C activation in vascular smooth muscle. Our evidence also indicates that PT-insensitive G proteins interact with phospholipase C. Several PT-insensitive G proteins have also been identified in other cells, including $Gz\alpha$ (39), and more recently, Gq, which has been shown to activate phospholipase C (70). Determination of which of these proteins are activating phospholipase C in vascular smooth muscle cells requires investigation; however, it is clear from our data that these cells contain multiple G proteins which are able to couple thrombin receptor activation to $Ca^{2+}$ mobilization.

It is becoming evident that the coupling of receptors to various effector systems, or even to the one effector, involves multiple G proteins (68, 71). Thus, in vascular smooth muscle, $\alpha$-thrombin activates different G proteins which could subserve different cellular functions ranging from contraction (5)

to gene transcription (8) and mitogenesis (6). In the present study evidence has been provided indicating a new component in the signaling pathway which couples $\alpha$-thrombin receptor activation to $Ca^{2+}$ release. This pathway involves a PT-sensitive G protein that appears to be involved directly in $IP_3$-dependent $Ca^{2+}$ release. The G protein could therefore play a major role in mediating the propagative $Ca^{2+}$ release which occurs after exposure of cells to $\alpha$-thrombin (9).

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