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Myristoylated alanine-rich C kinase substrate phosphorylation is involved in thrombin-induced serotonin release from platelets

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Summary. Stimulation of platelets by thrombin induces protein kinase C (PKC) activation, phosphorylation of pleckstrin, aggregation and serotonin release. Here, we demonstrate that, in human platelets, thrombin stimulation also induced phosphorylation of the myristoylated alanine-rich C kinase substrate (MARCKS) and serotonin release in intact and digitonin-permeabilized platelets. MARCKS is known to bind actin and cross-link actin filaments, and this is inhibited by PKC-evoked MARCKS phosphorylation. MARCKS phosphorylation and serotonin release in response to increasing concentrations of thrombin have a similar EC_{50} and time course and, in permeabilized platelets, peptide MPSD, with an amino acid sequence corresponding to the phosphorylation site domain of MARCKS, blocked both responses. However, pleckstrin and myosin light chain phosphorylations were not modified. Ala-MPSD, in which the four serine residues of MPSD were substituted by alanines was ineffective. The results suggest a role for MARCKS in platelet secretion. The fact that pleckstrin phosphorylation has a different time course and was not modified in the presence of MPSD when MARCKS phosphorylation and serotonin release were inhibited would suggest either that pleckstrin phosphorylation is unrelated to secretion or that it might only be involved upstream in the events leading to secretion.

Keywords: platelets, thrombin, MARCKS, serotonin, secretion.

Platelet activation consists of shape change, formation of pseudopodia, aggregation and secretion. Cytoskeleton rearrangement (Debus *et al*, 1981; Carroll *et al*, 1982; Cox *et al*, 1984), together with translocation of several accessory proteins (Tuszynski *et al*, 1982; Hynes, 1987; Bertagnolli & Beckerle, 1994) of the cytoskeleton, is involved in these changes. Cycles of actin polymerization-depolymerization take place in different areas (pseudopodia, etc.) during platelet activation. Platelets contain scinderin (Rodríguez Del Castillo *et al*, 1992) and gelsolin (Lind *et al*, 1982, 1987), which are Ca^{2+}-dependent F-actin-severing proteins that control the dynamic of actin networks. It has been suggested that actin polymerizes during platelet activation and that the content of secretory granules is released to the cell exterior. However, platelets still release serotonin when aggregation is inhibited (Nishikawa *et al*, 1980) and experiments from our laboratory showing that recombinant scinderin potentiates Ca^{2+}-evoked release of serotonin (Marcu *et al*, 1996) suggest that, as in other secretory systems (i.e. chromaffin cell), F-actin disassembly is required for secretion. Moreover, another set of published experiments, also from our laboratory, suggest that serotonin release in response to phorbol ester (PMA) stimulation might also be due to a decrease in F-actin cross-linking as a result of protein kinase C (PKC) activation (Elzagallaai *et al*, 2000). Stimulation of platelets by either PMA or thrombin is accompanied by phosphorylation of pleckstrin, a major PKC substrate (Haslam & Davidson, 1984; Brooks *et al*, 1990; Toker *et al*, 1995; Dalla Via *et al*, 1996; Kamiguti *et al*, 1997; Rotondo *et al*, 1997; Sloan & Haslam, 1997). It has also been suggested that pleckstrin is involved in platelet secretion, because inhibition of PKC decreases both pleckstrin phosphorylation and secretion (Rotondo *et al*, 1997; Sloan & Haslam, 1997). It seems that thrombin's effects on platelets are mediated, at least in part, by activation of PKC. Another PKC substrate is MARCKS (myristoylated alanine-rich C kinase substrate), a protein that is phosphorylated in response to PMA activation of PKC (Aderem, 1992a), and we have recently shown that PMA activation of platelet PKC induces phosphorylation of MARCKS (Elzagallaai *et al*, 2000). MARCKS can bind actin and cross-link actin

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filaments, and phosphorylation of MARCKS by PKC inhibits its ability to cross-link these filaments (Hartwig *et al*, 1992).

Therefore, because PKC seems to be involved in thrombin-evoked serotonin release (Haslam & Davidson, 1984; Hashimoto *et al*, 1994; Rotondo *et al*, 1997), it became important to determine under more physiological conditions whether or not MARCKS and its phosphorylation play a role in thrombin-evoked platelet secretion. In this study, we present evidence that thrombin induces phosphorylation of MARCKS in intact as well as permeabilized platelets. A parallel increase in serotonin release was observed in both platelet preparations, and MARCKS phosphorylation and serotonin release were similarly inhibited by a peptide with an amino acid sequence corresponding to the phosphorylation site domain of MARCKS. The results indicated that thrombin-induced phosphorylation of MARCKS is involved in serotonin release.

MATERIALS AND METHODS

Materials. Peptides that correspond to the phosphorylation site domain of MARCKS (MPSD, KKKKKRFSFKKSFKLSGFSFKKNKK) and to the same domain with the serine residues replaced by alanine residues (Ala-MPSD, KKKKKRFAFKKAFKLAGFAFKKNKK) were custom-made by Research Genetics (Huntsville, AL, USA). Digitonin and adenosine 5′-triphosphate (ATP, disodium salt) were obtained from Sigma (Oakville, Canada). Thrombin was purchased from Chrono-log (Hovertown, PA, USA), [^{3}H]-serotonin ([^{3}H]-5-HT) from DuPont (Boston, MA, USA) and carrier-free [^{32}P]-orthophosphate from Amersham (Oakville, Canada). Antibodies used were as follows: (i) mouse monoclonal IgG against the C-terminal domain of human MARCKS and rabbit polyclonal IgG against amino acids 641–673 of C-terminus of PKC (recognizes α, β and γ isoforms of PKC at 82 KDa) (Upstate Biotechnology, Lake Placid, NY, USA); (ii) goat polyclonal against the C-terminus of human MARCKS and goat polyclonal against the N-terminus of human MARCKS (Santa Cruz Biotechnology, Santa Cruz, CA, USA); (iii) mouse monoclonal against the N-terminus of human pleckstrin (Tranduction Laboratories, Lexington, KY, USA); (iv) HRP-conjugated goat anti-mouse IgG (BioRad, Mississauga, Canada); (v) horseradish peroxidase (HRP)-conjugated affinity-purified F(ab′)$_2$ fragment rabbit anti-goat F(ab′)$_2$ fragment-specific (Jackson Immuno Research, West Grove, PA, USA); (vi) HRP-conjugated goat anti-rabbit IgG (Jackson Immuno Research); (vii) HRP-conjugated F(ab′)$_2$ fragment donkey anti-mouse IgG (Jackson Immuno Research); and (viii) CY^3-conjugated affinity-purified F(ab′)$_2$ fragment donkey anti-mouse IgG (Jackson Immuno Research). Rhodamine phalloidin was from Molecular Probes (Eugene, OR, USA).

Source of platelets. Platelet-rich plasma was obtained from the blood bank of Ottawa Red Cross and centrifuged at 800 ***g*** for 15 min to obtain a platelet sediment.

Platelet permeabilization and labelling of serotonin stores. The platelet sediment was resuspended in Ca^{2+}-free Locke's solution (NaCl, 154 mmol/l; KCl, 2·6 mmol/l; K_2HPO_4, 2·14 mmol/l; KH_2PO_4, 0·85 mmol/l; $MgCl_2$, 1·2 mmol/l; glucose, 10 mmol/l; and EGTA, 2·0 mmol/l; pH 7·2). After a wash with Locke's solution, the platelet concentration was adjusted to $7{\cdot}5 \times 10^8$/ml. Platelets were then incubated at 37°C for 90 min with 0·6 nmol of [^{3}H]-5-HT/ml (specific activity, 939·8 GBq/mmol; DuPont, Boston, MA, USA). After incubation, the [^{3}H]-5-HT-labelled platelets were washed by incubation with six changes of 1 ml of Ca^{2+}-free Locke's solution over a 60-min period before the experiments were commenced. [^{3}H]-5-HT-labelled platelets were permeabilized by treatment for 5 min with 15 μmol/l digitonin in K^+-glutamate buffer ($MgCl_2$, 12·5 mmol/l; K^+-glutamate, 160 mmol/l; EGTA, 2·5 mmol/l; EDTA, 2·5 mmol/l; ATP, 5 mmol/l; HEPES, 20 mmol/l; pH 7·4) (Elzagallaai *et al*, 2000). After permeabilization, platelets were centrifuged at 900 ***g*** for 2 min (4°C) and then resuspended in K^+-glutamate buffer. Ca^{2+} concentrations required to give appropriate pCa values were calculated as previously described (Fabiato & Fabiato, 1979; Marcu *et al*, 1996). The K^+-glutamate buffer used in the experiments has a pCa value of less than 9. The degree of permeabilization was determined using rhodamine phalloidin, a probe for filamentous actin (Elzagallaai *et al*, 2000). Intact and permeabilized platelets were centrifuged onto polylysine-coated glass slides using a bench-top cytospin centrifuge (cytofuge 2, Stat Spin, Norwood, MA, USA). Platelets were then fixed in 3·7% formaldehyde for 20 min and stained with rhodamine phalloidin (Molecular Probes; 1:200 dilution) for 15 min at room temperature, washed three times with phosphate-buffered saline (PBS; 130 mmol/l NaCl, 100 mmol/l Na-phosphate; pH 7·2) and mounted in 50% glycerol/PBS (Elzagallaai *et al*, 2000). Platelet preparations were examined using incident fluorescent light, pictures were taken and images processed as described below. The percentage of rhodamine phalloidin-positive cells (permeabilized platelets) was then determined from the prints.

Platelet aggregation. Platelet aggregation was measured using Whole Blood Dual Channel Lumi-aggregometer (Chrono-log). Aliquots (0·5 ml) of intact platelets (suspended in either Ca^{2+}-free Locke's solution or regular Locke's solution) and permeabilized platelets were suspended in K^+-glutamate buffer and placed in a siliconized glass cuvette positioned in the sample chamber and kept at 37°C under constant stirring using siliconized stirring bars.

Serotonin-release studies. Samples (100 μl) containing $7{\cdot}5 \times 10^8$ [^{3}H]-5-HT-labelled platelets/ml of either permeabilized platelets suspended in K^+-glutamate buffer or intact platelets suspended in Ca^{2+}-free Locke's solution were incubated in the absence or presence of different secretagogues for the indicated periods of time. Release experiments were terminated by the addition of an equal volume of 6% glutaraldehyde in 0·1 mol/l phosphate buffer (pH 7·4) and the total [^{3}H]-5-HT platelet content was determined as previously described (Elzagallaai *et al*, 2000). [^{3}H]-5-HT output was expressed as a percentage of platelet total content, after subtraction of values for spontaneous release.

[32P]-Pi-labelling of platelets. Platelets ($7{\cdot}5 \times 10^8$/ml) were suspended in a phosphate-free solution (buffer P) of the following composition (NaCl, 145 mmol/l; KCl, 5 mmol/l;

$MgSO_4$, 1 mmol/l; glucose, 10 mmol/l; HEPES, 25 mmol/l; EGTA, 0·5 mmol/l; pH 7·3) to give a platelet concentration of 5×10^8 platelets/ml. Platelets were incubated for 60 min in buffer P containing 5·55 MBq of carrier-free [^{32}P]-Pi/ml (Amersham). Platelets were then sedimented by centrifugation at 800 ***g*** for 2 min and washed twice with the same buffer.

Protein phosphorylation studies. Intact or digitonin-permeabilized platelets previously labelled with [^{32}P]-Pi were used in the experiments. When total proteins (heat-stable and heat-sensitive) were studied, incubation was terminated by the addition of an equal volume of twice-concentrated Laemmli's loading buffer (Tris-HCl, 125 mmol/l; glycerol, 20%; sodium dodecyl sulphate, 4%; 2β mercaptoethanol, 10%; bromophenol blue, 0·05%, pH 6·8), followed by incubation at 95°C for 7 min. When heat-stable proteins were studied, incubation was stopped by the addition of twice-concentrated RIPA (radio immunoprecipitation assay) buffer (NaCl, 140 mmol/l; KCl, 2·6 mmol/l; K_2HPO_4, 10 mmol/l; KH_2PO_4, 1·8 mmol/l; NP-40, 1%; sodium deoxycholate, 0·5%) containing 1 μg of aprotinin/ml, 1 μg of leupeptin/ml, 1 mmol/l PMSF, 1 mmol/l $NaVO_4$, 1 mmol/l NaF and 50 mmol/l benzamidine, followed by boiling for 10 min. Boiled platelet extracts were then centrifuged at 16 000 ***g*** for 2 min. Supernatants thus obtained were mixed with equal volumes of twice-concentrated Laemmli's loading buffer. The preparations were then heated to 95°C for 7 min.

Electrophoresis, immunoblotting, autoradiography and densitometric analysis. All protein samples were analysed using monodimensional 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Doucet & Trifaró (1988). For Western blot analysis, proteins were electrotransferred to nitrocellulose membranes (pore size: 0·45 μm, Bio-Rad, Mississauga, Canada) and immunoblotting was performed as described previously (Marcu *et al*, 1998) with antibodies against different antigens (MARCKS, PKC and pleckstrin). Nitrocellulose membranes obtained from the phosphorylation experiments were exposed to Hyperfilm-ECL (Amersham). The intensity of the autoradiograph bands were analysed using SCION IMAGE BETA 2 software (Scion Corporation, Frederick, MD, USA). The areas under the peaks were integrated using the same program and results were expressed in arbitrary units.

Fluorescence microscopy.. Platelets were centrifuged at 1000 ***g*** for 10 min onto polylysine-coated glass slides using a bench-top cytospin centrifuge (cytofuge 2, Stat Spin) and were immediately fixed in 3·7% formaldehyde in PBS for 20 min. Preparations were washed several times with PBS, permeabilized with 1% Triton X-100 for 3 min, washed again with PBS, and incubated with 1% bovine serum albumin (BSA) and 1% donkey preimmune serum in PBS for 1 h at room temperature to block non-specific binding sites. Platelets were then washed with PBS and incubated with either non-specific mouse IgG (control, 1:250 dilution) or human MARCKS mouse monoclonal antibody (1:250 dilution) for 1 h at room temperature. All preparations were then washed three times with PBS and incubated for 1 h with (secondary antibody) affinity-purified CY^3-conjugated donkey Fab_2 fragment raised against mouse IgG (1:200 dilution). Preparations were then washed with PBS and mounted in Slowfade buffer containing 50% glycerol (Molecular Probes). Preparations were examined using incident fluorescent light under a Zeiss Axoplan microscope equipped with a HBO 50 mercury lamp and an oil immersion objective (100×; 1·3 aperture). Pictures were taken with a Sony digital camera and the images saved using a Northern Eclipse software (Empix, Mississauga, Canada). Images were then digitally imported into ADOBE PHOTOSHOP software for further analysis. Images were printed on Epson quality paper using an Epson Stylus Photo EX colour printer (Epson America, Torrance, CA, USA).

RESULTS

Effect of thrombin stimulation on platelet serotonin release and MARCKS phosphorylation in the presence or absence of extracellular calcium

It is known that thrombin stimulation of platelets increases the phosphorylation of pleckstrin, an effect mediated through PKC activation (Haslam & Davidson, 1984; Yamada *et al*, 1987; Walker & Watson, 1993; Hashimoto *et al*, 1994, 1997; Freedman *et al*, 1996; Sloan & Haslam, 1997). It is also quite possible that thrombin stimulation might lead to an increase in the phosphorylation of MARCKS, another PKC substrate. The presence of MARCKS in platelets and its phosphorylation in response to PMA have been recently demonstrated (Elzagallaai *et al*, 2000). Therefore, platelets previously labelled with [^{32}P]-Pi were incubated with thrombin (1 U/ml) and aggregation was monitored as indicated in *Materials and methods*. At the point of maximal aggregation, heated and unheated protein extracts were prepared from platelets and separated by SDS-PAGE. Autoradiography and scannings of one such experiment are shown in Fig 1A. As expected, thrombin stimulation significantly increased the level of phosphorylation of pleckstrin ($P < 0·01$, $n = 5$) and myosin light chain (MLC; $P < 0·01$, $n = 5$). Moreover, the phosphorylation of MARCKS was increased ($P < 0·01$, $n = 5$), suggesting that MARCKS might mediate some of the thrombin-induced effects in platelets. Figure 1C shows cumulative data from five different phosphorylation experiments. The level of phosphorylation of pleckstrin, MLC and MARCKS induced by thrombin stimulation was similar in the presence or absence of the extracellular cation (Fig 1A), a condition in which platelet aggregation is inhibited. Similarly, [^{3}H]-5-HT release from [^{3}H]-5-HT prelabelled platelets in response to increasing concentrations of thrombin was similar in the presence or absence of extracellular Ca^{2+} (Fig 2B), indicating that, in the absence of platelet aggregation, the platelet release reaction is fully operative. Furthermore, the fact that removal of extracellular Ca^{2+} did not modify either protein phosphorylation or serotonin release in response to thrombin stimulation would suggest that these processes used Ca^{2+} from intracellular stores.

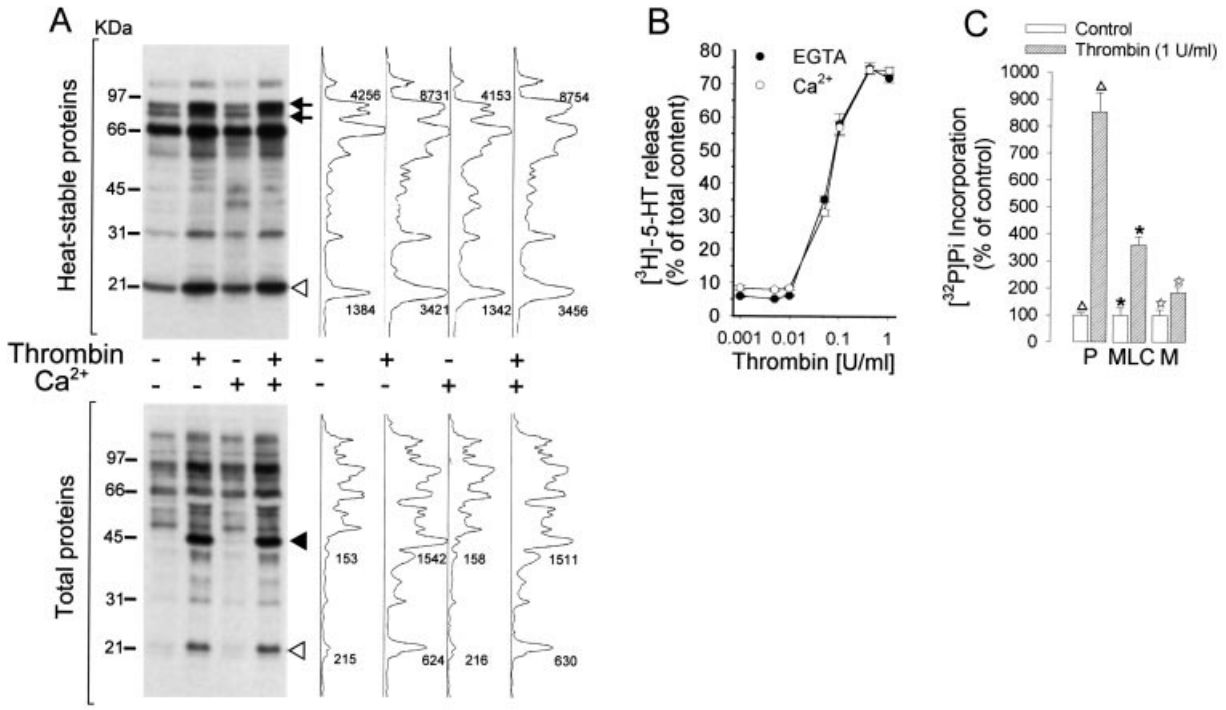

Fig. 1. Effects of extracellular Ca^{2+} on thrombin-induced protein phosphorylation and [^{3}H]-5-HT release from intact platelets. (A) Intact platelets labelled with [^{32}P]-Pi were suspended either in Ca^{2+}-free (0·5 mmol/l EGTA) Locke's solution or in the same buffer containing 2·2 mmol/l free Ca^{2+} and incubated, as indicated, for 3 min with either vehicle or 1 U of thrombin/ml. At the end of the incubation period, heat-stable platelet extracts (top) and total platelet extracts (bottom) were prepared and subjected to SDS-PAGE, followed by autoradiography. Arrows, closed arrowheads and open arrowheads indicate the position of MARCKS, pleckstrin and myosin light chain (MLC) respectively. On the right, densitometric scannings of the autoradiographs are shown. The numbers beside the peaks represent arbitrary units obtained from computer integration of peak areas. (B) [^{3}H]-5-HT-labelled platelets were suspended either in Ca^{2+}-free Locke's solution (closed circles) or regular Locke's solution containing 2·2 mmol/l free Ca^{2+} (open circles) and incubated for 3 min with different concentration of thrombin. At the end of this stimulation period, the [^{3}H]-5-HT content was measured in platelets, as described in *Materials and methods*. [^{3}H]-5-HT outputs were expressed as a percentage of the total content. Values represent mean ± SEM of results obtained from eight different preparations. (C) Cumulative data on thrombin-induced phosphorylation of pleckstrin, MLC and MARCKS. Experiments were performed as described in (A) except that platelets were incubated in Ca^{2+}-free Locke's solution. Values are arbitrary units expressed as a percentage of the control (unstimulated platelets). Bars represent the mean ± SEM of [^{32}P]-Pi incorporation obtained from five different experiments for each condition tested (△, $P < 0{\cdot}01$; *, $P < 0{\cdot}01$; ☆, $P < 0{\cdot}01$).

Similar thrombin concentration-dependence for MARCKS phosphorylation and serotonin release

Experiments were performed to simultaneously measure serotonin release, pleckstrin and MARCKS phosphorylation in platelets stimulated with increasing concentrations (0·001–1 U/ml) of thrombin. Tests were carried out in the absence of Ca^{2+} and one such phosphorylation experiment is shown in Fig 2A. Solid arrowheads indicate the first protein band for each protein (pleckstrin or MARCKS) showing a significant ($P < 0{\cdot}01$) increase in phosphorylation. Figure 2B shows that MARCKS phosphorylation and serotonin release curves were almost identical (EC_{50} of 0·09 and 0·1 thrombin units for serotonin and MARCKS respectively), whereas the pleckstrin phosphorylation curve was shifted to the left. In other words, it was necessary to reach a concentration of thrombin of 0·05 U/ml to observe significant ($P < 0{\cdot}01$) and parallel increases in MARCKS phosphorylation ($n = 5$) and serotonin release ($n = 8$), whereas concentrations equal to or lower than 0·01 U/ml significantly ($P < 0{\cdot}01$, $n = 5$) increased pleckstrin phosphorylation (Fig 2B).

Effects of MPSD and Ala-MPSD peptides on protein phosphorylation and serotonin release from permeabilized pletelets induced by thrombin stimulation

The closed correlation between MARCKS phosphorylation and serotonin release in response to thrombin prompted us to investigate the possible role of phospho-MARCKS in serotonin release. Therefore, experiments were carried out to test the effect of two MARCKS-related peptides. MPSD is a 25-amino-acid peptide with a sequence corresponding to the phosphorylation site domain of MARCKS, which is also the site of calmodulin (CaM) and actin binding (Fig. 3A). Ala-MPSD was the second peptide tested in which the four serine residues of MPSD were substituted by alanines (Fig. 3A). Peptides were to be tested in permeabilized platelets, a preparation fully characterized in our laboratory in terms of degree of permeabilization, protein and serotonin

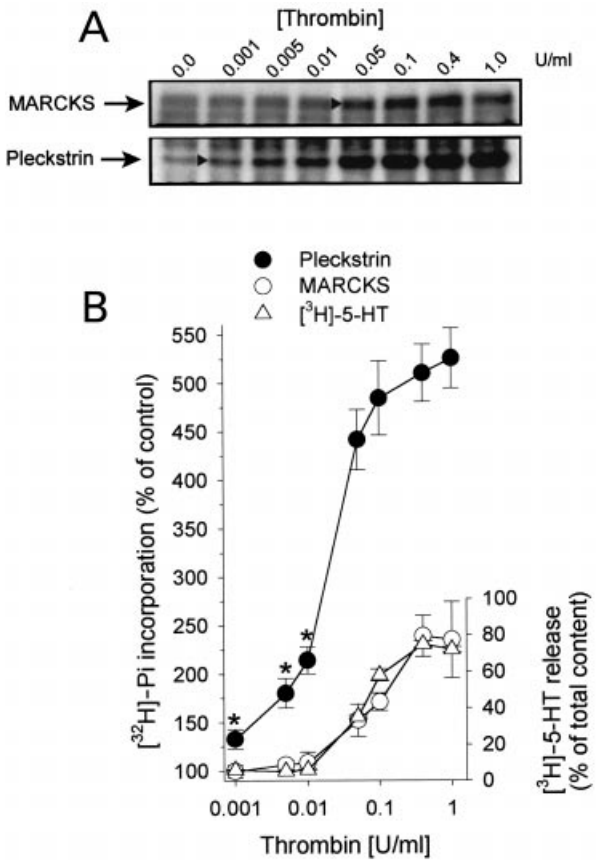

Fig. 2. Thrombin concentration-dependent responses. Platelets were labelled with either [^{32}P]-Pi or [^{3}H]-5-HT, as indicated in *Materials and methods*, and stimulated for 3 min with increasing concentrations (0·001–1 U/ml) of thrombin in Ca^{2+}-free Locke's solution. [^{3}H]-5-HT release and protein phosphorylation were measured as described in *Materials and methods*. (A) Autoradiography of SDS-PAGE gels of heat-stable platelet extracts (MARCKS) and whole platelet extracts (pleckstrin). Concentrations of thrombin used are indicated at the top of the figure. Arrowheads indicate the smallest concentration of thrombin producing a significant increase in the phosphorylation of each protein. (B) Thrombin concentration-dependent curves for [^{3}H]-5-HT release, and pleckstrin and MARCKS phosphorylation. Values represent the mean ± SEM of five different platelet preparations (*, $P < 0{\cdot}01$).

leakages (Elzagallaai *et al*, 2000). However, the response to thrombin has not been studied in this preparation. A good level of permeabilization was obtained in this preparation for up to 20 min and no MARCKS was detected in the medium (Elzagallaai *et al*, 2000). Permeabilized platelets do not aggregate in response to thrombin, even in the presence of Ca^{2+} in the medium (Fig 4). However, permeabilized platelets released serotonin in response to thrombin stimulation, although the response was approximately 60% ($P < 0{\cdot}01$, $n = 8$) of that observed in intact platelets (Fig 3B). The reduced release was not as a result of the presence of chelators in the K^{+}-glutamate buffer used in the permeabilized platelets experiments, as chelator (EGTA + EDTA) levels of 0·2 or 5 mmol/l yielded similar results (Fig 3B). When permeabilized platelets were stimulated for 3 min with 1 U of thrombin/ml in the presence of 10 μmol/l MPSD, there was a 40% inhibition ($P < 0{\cdot}01$, $n = 8$) of the secretory response (Fig 3C). However, no inhibition was observed when thrombin stimulation was in the presence of 10 μmol/l Ala-MPSD (Fig 3C). This would suggest that the PKC–MARCKS pathway is involved, at least in part, in thrombin-induced release of serotonin. Similar experiments were performed on platelets previously labelled with [^{32}P]-Pi. Platelets were stimulated for 3 min with 1 U of thrombin/ml in the absence or presence of either MPSD or Ala-MPSD. Total proteins and heat-stable proteins were then separated using 10% SDS-PAGE. A representative autoradiograph of four different experiments and its corresponding scannings are depicted in Fig 5A and B. Thrombin stimulation significantly ($P < 0{\cdot}01$, $n = 4$) increased the phosphorylation of MARCKS and MLC, as well as two other (p31 and p66) unidentified heat-stable polypeptides (open triangles, Fig 5A). In the presence of 10 μmol/l MPSD, there was a significant ($P < 0{\cdot}01$, $n = 4$) inhibition of thrombin-induced phosphorylation of MARCKS (Fig 5A). However, there was no inhibition of phosphorylation of MLC, p31 or p66 (Fig 5A) in response to thrombin stimulation (open arrowhead and open triangles in Fig 5A). Moreover, when 10 μmol/l Ala-MPSD was present in the incubation medium, there was no inhibition of thrombin-induced MARCKS phosphorylation (Fig 5A). The autoradiographs of gels of total unheated proteins showed a significant ($P < 0{\cdot}01$, $n = 4$) increase in the phosphorylation of pleckstrin upon thrombin stimulation (Fig 5B). This, as well as the increase in MLC phosphorylation, were not modified in the presence of either MPSD or Ala-MPSD (Fig 5B) at concentrations at which MPSD inhibits MARCKS phosphorylation and serotonin release. Figure 5C shows cumulative data obtained from densitometric analysis of autoradiographs from four different experiments.

DISCUSSION

The results of the present experiments show that MARCKS is phosphorylated in response to platelet stimulation by thrombin. Phosphorylation of MARCKS in response to thrombin has also been observed in pulmonary artery endothelial cells (Zhao & Davis, 1996). The response of platelet MARCKS phosphorylation and serotonin release increased in parallel to increasing concentrations of thrombin. These effects were similar either in the presence or absence of extracellular Ca^{2+}, suggesting that Ca^{2+} release from intracellular stores might mediate these effects. Furthermore, thrombin treatment is able to release intracellular Ca^{2+} even when platelets have been previously treated with thapsigargin, an inhibitor of ATP-dependent Ca^{2+} pump (Authi *et al*, 1993). Pleckstrin, another PKC substrate was, as previously demonstrated (Brooks *et al*, 1990; Toker *et al*, 1995; Dalla Via *et al*, 1996; Kamiguti *et al*, 1997; Rotondo *et al*, 1997; Sloan & Haslam, 1997; Elzagallaai *et al*, 2000), phosphorylated in response to thrombin stimulation and the level of phosphorylation was

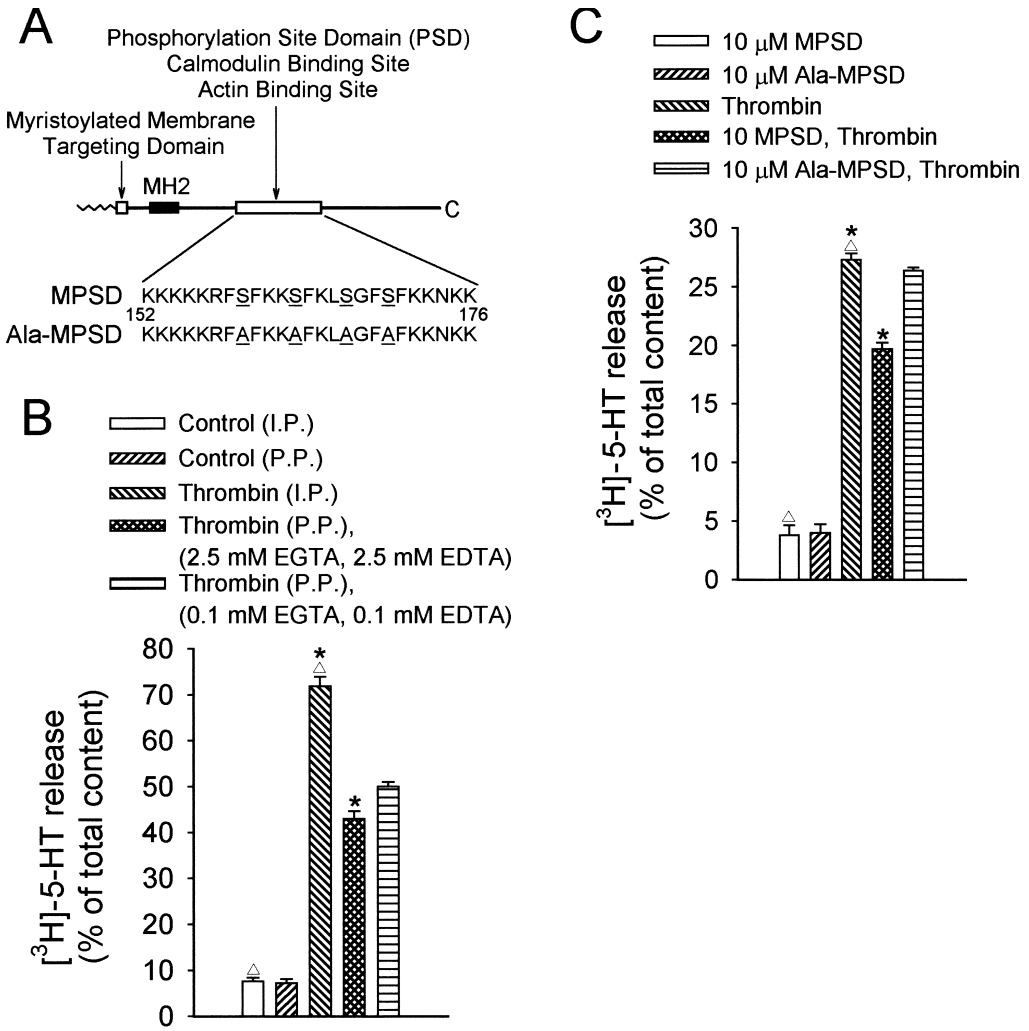

Fig. 3. Effects of peptides MPSD and Ala-MPSD, and digitonin permeabilization, on thrombin-induced [^{3}H]-5-HT release from platelets. (A) Diagram of the structure of MARCKS and amino acid sequences of peptides MPSD and Ala-MPSD. MPSD has a 25-amino-acid sequence corresponding to the phosphorylation site domain of MARCKS. In Ala-MPSD, the four serine residues of MPSD were substituted by alanines. (B) [^{3}H]-5-HT-labelled platelets were used. Intact platelets (I.P.) were suspended in Ca^{2+}-free Locke's solution, whereas digitonin-permeabilized platelets (P.P.) were suspended in K^+-glutamate buffer containing different concentrations of EGTA and EDTA. Platelets were then incubated in the same buffers for 3 min in the presence of either vehicle or thrombin (1 U/ml). At the end of the incubation period, [^{3}H]-5-HT was measured in the medium and in platelets. [^{3}H]-5-HT outputs were expressed as a percentage of the total content. Each bar represent the mean ± SEM of results obtained from eight different preparations (△, $P < 0{\cdot}01$; *, $P < 0{\cdot}01$). (C) [^{3}H]-5-HT-labelled patelets were permeabilized for 5 min with 15 μmol/l digitonin in K^+-glutamate buffer in the absence or presence of 10 μmol/l of either MPSD peptide or Ala-MPSD peptide. Platelets were recovered by centrifugation and resuspended in same medium for 3 min in absence or presence of 1 U thrombin/ml. At the end of this stimulation period, [^{3}H]-5-HT content was measured in the medium and in platelets, as described in *Materials and methods*. [^{3}H]-5-HT outputs were expressed as a percentage of the total content. Each bar represents the mean ± SEM obtained from eight different preparations (△, $P < 0{\cdot}01$; *, $P < 0{\cdot}01$).

again similar in the presence or absence of extracellular Ca^{2+}. The experiments described here clearly show that the pattern of phosphorylation of pleckstrin in response to increasing concentrations of thrombin was different from that shown by MARCKS. Thrombin, in concentrations between 0·001 and 0·01 U/ml, only induced phosphorylation of pleckstrin, suggesting some difference in the pathways that trigger the phosphorylation of the two proteins. Furthermore, the phosphorylation curve of MARCKS was almost identical to the serotonin release curve, indicating a functional relationship between these two events, although there are probably many steps between MARCKS phosphorylation and secretion.

The suggestion that pleckstrin is involved in secretion in response to PKC activation comes from a number of publications all showing an increase or decrease in both pleckstrin phosphorylation and the platelet release reaction during either stimulation or inhibition of PKC activity

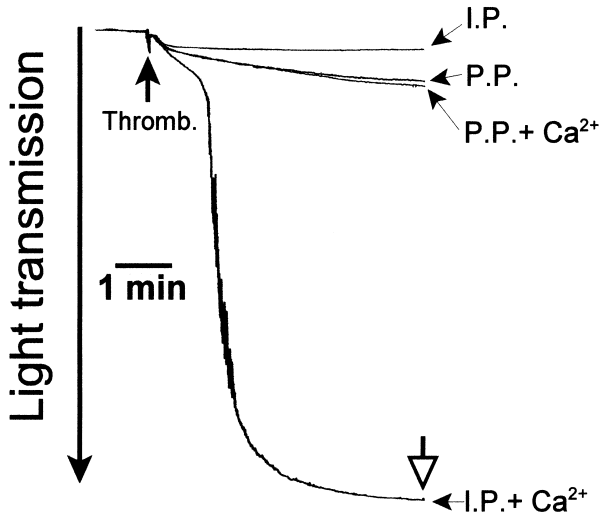

Fig. 4. Effects of digitonin permeabilization on thrombin-induced platelet aggregation. Intact platelets (I.P.) were resuspended in regular Locke's or in Ca^{2+}-free Locke's solution whereas digitonin-permeabilized platelets (P.P.) were resuspended in K^+-glutamate buffer, either in the presence or absence of 10 μmol/l free Ca^{2+}. Platelet aliquots (500 μl; $7{\cdot}5 \times 10^8$ platelets/ml) were placed in siliconized glass cuvettes under constant stirring. Platelet preparations were then challenged with 1 U of thrombin/ml and aggregation was monitored as described in *Materials and methods*. Aggregation was only observed in I.P. when Ca^{2+} was present.

(Haslam & Davidson, 1984; Hashimoto *et al*, 1994; Toker *et al*, 1995; Rotondo *et al*, 1997; Sloan & Haslam, 1997). However, in these publications, no cause–effect relationship between the two parameters was demonstrated. An explanation for the different phosphorylation patterns observed for pleckstrin and MARCKS might be that different PKC isozymes are involved in these phosphorylations. If this was the case, the same isozyme should be responsible for both MARCKS phosphorylation and serotonin release because the two processes have a similar EC_{50} for thrombin. It has also been shown that activation of platelets by thrombin results in the translocation of PKC α, β and ζ isoforms (Baldassare *et al*, 1992). Other possibilities are that pleckstrin phosphorylation is either involved upstream of MARCKS in the cascade of events leading to serotonin release or that pleckstrin phosphorylation mediates platelet aggregation and the protein has a different threshold of phosphorylation for this process.

The experiments with permeabilized platelets described here further indicate a role for MARCKS in thrombin-evoked serotonin release. The digitonin-permeabilized platelets used in these experiments are a reliable preparation in terms of degree of permeabilization, stability of serotonin stores and the relatively small leakage of protein (Elzagallaai *et al*, 2000). As demonstrated earlier with other techniques of permeabilization (Haslam & Davidson, 1984; Knight *et al*, 1984), thrombin was effective in releasing serotonin from digitonin-permeabilized platelets, although the secretory response was smaller than that observed in intact platelets. Furthermore, digitonin-permeabilized platelets do not aggregate in response to thrombin and therefore this preparation offered the advantage of studying secretion in the absence of aggregation. Previous studies have also demonstrated that, when aggregation was inhibited by lack of stirring of platelet suspensions, serotonin was still secreted in response to thrombin (Nishikawa *et al*, 1980). Peptide MPSD with an amino acid sequence corresponding to the phosphorylation site domain of MARCKS inhibited both MARCKS phosphorylation and serotonin release, effects not observed with Ala-MPSD (a peptide in which the serine residues were substituted by alanine residues). MPSD not only inhibited the two responses but it also was phosphorylated in the process, suggesting that PKC activation was not impaired. Although previous work from our laboratory has demonstrated that PMA stimulation of permeabilized platelets increases MARCKS phosphorylation (Elzagallaai *et al*, 2000), the present experiments show that, under resting conditions, there is a basal level of MARCKS phosphorylation in intact platelets and that this level of phosphorylation is not affected by removal of extracellular Ca^{2+}.

In addition, we show here that thrombin stimulation produces similar increases in the phosphorylation of MARCKS in the absence or presence of extracellular Ca^{2+}, and in intact as well as permeabilized platelets. The similar increases observed in pleckstrin phosphorylation in response to thrombin in the presence or absence of MPSD also suggested that PKC was active. Moreover, an additional proof of the selectivity of MPSD inhibition was that MLC and other two unidentified heat-stable proteins (p31 and p66) were also phosphorylated in response to thrombin stimulation in presence of MPSD. Increased phosphorylation of pleckstrin and MLC in permeabilized platelets while MARCKS phosphorylation and serotonin release were inhibited by MPSD would suggest that phosphorylation of these two proteins is unrelated to the transduction pathway in which MARCKS is involved. One possibility is that these two proteins are involved in platelet-shape change and/or aggregation (Nishikawa *et al*, 1980; Ikebe & Reardon, 1990), steps that cannot be studied separately from serotonin release in intact platelets. In this regard, it is also known that thrombin receptor stimulation can induce platelet-shape change through activation of tyrosine kinase, a Ca^{2+} and integrin independent mechanism (Negrescu *et al*, 1995). It is also known that thrombin activates several transduction pathways through its G-protein-coupled receptor. Thus, as a result of phospholipase C (PLC) activation and phosphatidylinositol 4,5 bisphosphate (PIP_2) hydrolysis, two important second messengers (1,2 diacylglycerol, DAG and inositol 1,4,5 triphosphate, IP_3) are formed which activate PKC and mobilize intracellularly stored Ca^{2+} (Lapetina, 1990; Kroll & Sullivan, 1998). Novel phosphoinositide species are also generated in platelets in response to thrombin activation of phosphatidylinositol 3-kinase (PI 3-K) (Lapetina, 1990; Hartwig *et al*, 1995). The PIP_3 thus formed is not a substrate of PLC and has the property of uncapping actin filaments, an effect that favours actin polymerization (Hartwig *et al*, 1995). Wortmannin (Ui *et al*, 1995) and LY294002 (Vlahos *et al*, 1994), two inhibitors of PI 3-K, have been shown to inhibit platelet aggregation (Zhang & Rittenhouse, 1995) and secretion (Yatomi *et al*,

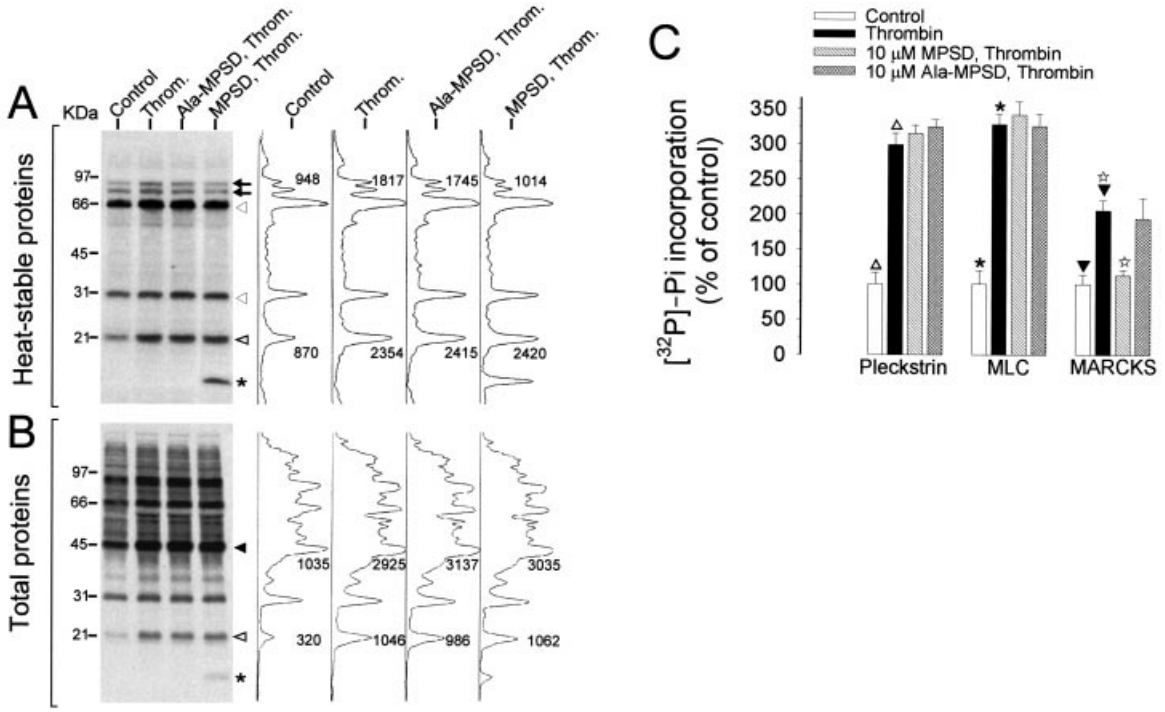

Fig. 5. Effects of MPSD and Ala-MPSD on the phosphorylation of MARCKS, pleckstrin and MLC induced by thrombin stimulation. Platlelets were labelled with [^{32}P]-Pi and permeabilized with 15 μmol/l digitonin in the absence or presence of 10 μmol/l of either Ala-MPSD (lane 3) or MPSD (lane 4), and subsequently stimulated with thrombin as described in the legend to Fig 3. At the end of the stimulation periods, heat-stable (A) and total (B) platelet extracts were prepared and their proteins separated using SDS-PAGE, as indicated in *Materials and methods*. Proteins were then electrotransferred to nitrocellulose membranes; these were exposed to hyperfilm and the autoradiographs thus obtained were scanned as indicated in *Materials and methods*. (A) and (B) show the autoradiography of two such experiments. Double arrows, closed arrowhead and open arrowheads show the position of MARCKS, pleckstrin and MLC respectively. Open triangles indicate the position of two other unidentified heat-stable proteins. The asterisk indicates the position of phosphorylated MPSD. The right of the figure shows densitometric scannings of the autoradiographs. The numbers beside the peaks are arbitrary units obtained from computer integration of peak areas. (C) Cumulative data on the effect of MPSD and Ala-MPSD on thrombin-induced protein phosphorylation in permeabilized platelets. Experiments were performed as described in A and B. Bars represent the mean ± SEM of [^{32}P]-Pi incorporation, expressed as a percentage of the control (absence of thrombin), obtained from four different experiments for each condition tested (△, $P < 0{\cdot}01$; *, $P < 0{\cdot}01$; ▼, $P < 0{\cdot}01$; ☆, $P < 0{\cdot}01$).

1992). In addition, thrombin also stimulates phospholipase A_2 (PLA_2) with release of arachidonic acid (AA) and formation of thromboxane A_2, a potent platelet agonist (Lapetina, 1990). Therefore, because all these transduction pathways are activated by thrombin, platelet aggregation and secretion are very complex processes.

Thus, the inhibition of the PKC–MARCKS pathway decreases thrombin-induced secretion by 40% as it still leaves other transduction pathways intact. The situation was quite different when PKC was directly stimulated by PMA, a condition in which the inhibition by MPSD was much larger (Elzagallaai *et al*, 2000). However, PMA treatment affects only a specific pathway and, consequently, it is not a physiological response (Elzagallaai *et al*, 2000). Therefore, only under physiological conditions (i.e. thrombin stimulation) can the importance and the real contribution to secretion of the PKC–MARCKS pathway be evaluated.

The observations presented in this study raised the question of how MARCKS is involved in platelet secretion. Early experiments from our laboratory with recombinant scinderin in permeabilized platelets indicated that it is not actin polymerization, as earlier suggested, but rather F-actin disassembly, perhaps at a specific site, that should be required for secretion (Marcu *et al*, 1996). These experiments demonstrated that recombinant scinderin (a Ca^{2+}-dependent F-actin-severing protein) potentiated Ca^{2+}-evoked serotonin release from platelets, an effect blocked by PIP_2 and by peptides with amino acid sequence corresponding to either of the two actin binding sites of scinderin, suggesting the need for F-actin disassembly in the platelet release reaction (Marcu *et al*, 1996). Cortical F-actin disassembly is also required for amine and hormone release from other secretory systems such as chromaffin cells (Vitale *et al*, 1991, 1995) and laptotropes (Carbajal & Vitale, 1997). Furthermore, cytochalasin E treatment has been shown to decrease actin polymerization, inhibiting platelet aggregation without affecting secretion (Lefebre *et al*, 1993). Additional work has demonstrated cortical F-actin rearrangement during the PKC activation that accompanied

neurotransmitter release upon cell stimulation (Vitale *et al*, 1995) and both effects were blocked by the same MPSD peptide used in the present studies (Rosé *et al*, 1997). Phosphorylation of MARCKS decreases its affinity for filamentous actin and phospho-MARCKS cannot cross-link actin filaments, a property only observed with dephospho-MARCKS (Aderem, 1992b; Hartwig *et al*, 1992). Therefore, PKC activation as a result of thrombin receptor stimulation increases MARCKS phosphorylation with a consequent decrease in actin filaments cross-linking which, in turn, would decrease the density of the actin network at a specific site. This would allow, as in other secretory systems (Trifaró & Vitale, 1993; Vitale *et al*, 1995; Carbajal & Vitale, 1997), the movement of secretory vesicles to release sites, increasing serotonin release. This hypothesis would also explain why the serotonin release reaction is not impaired when actin polymerization and aggregation are inhibited.

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