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Collagen-induced Platelet Shape Change Is Not Affected by Positive Feedback Pathway Inhibitors and cAMP-elevating Agents*

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Shape change is the earliest response of platelets to stimuli; it is mainly dependent upon Ca^{2+}/calmodulin interaction subsequent to Ca^{2+} mobilization and is mediated by myosin light chain kinase (MLCK) activation. It has been recently suggested that collagen itself is not able to elicit platelet shape change in the absence of ADP and thromboxane A_2 costimulation but is capable of inducing MLCK activation. Since we hypothesize that the morphological changes of the few platelets that adhere to collagen might not be revealed by turbidimetry, the aim of this study was to assess platelet shape change using transmission electron microscopy, in the absence of the amplificatory feedback pathways of ADP and thromboxane A_2. Our results demonstrated that only the platelets in contact with insoluble collagen fibers underwent a typical shape change, whereas those further away remained quiescent. Moreover, since cAMP enhances Ca^{2+} mobilization in response to collagen, in the present study, we also investigated whether cAMP is involved in the inhibition of collagen-induced platelet shape change and MLC phosphorylation. Platelets were thus treated with iloprost (28 nM) prior to stimulation. Electron microscopy studies demonstrated that iloprost did not modify collagen-induced shape change, whereas immunoblotting studies showed a slight inhibition of MLC phosphorylation in the presence of enhanced cAMP levels. We can thus conclude that collagen is able to cause platelet shape change through activation of Ca^{2+}/calmodulin-dependent MLCK, without the involvement of amplificatory pathways. Enhanced cytosolic cAMP levels do not inhibit collagen-induced platelet shape change but exert a weak inhibitory action on MLCK.

Collagen appears to be the most thrombogenic constituent of the vascular subendothelium since it not only causes platelet adhesion but is also a powerful agonist capable of eliciting full platelet activation and aggregation. Because of the abundance of collagen fibers in the extracellular matrix, platelet collagen receptors can be considered as potential risk factors for thrombosis (1). Platelets interact with collagen through two major receptors, namely integrin $\alpha2\beta1$ and glycoprotein VI (GPVI).[1]

The $\alpha2\beta1$ receptor plays a primary role in supporting platelet adhesion to collagen, whereas GPVI is deeply involved in generating intracellular signaling. Indeed, GPVI activation stimulates tyrosine phosphorylation of many of the proteins in the GPVI-FcR γ-chain cascade, including Syk and phospholipase C $\gamma2$. Activation of phospholipase C $\gamma2$ results in the generation of inositol trisphosphate and elevation of intracellular Ca^{2+} (2–4). This biochemical cascade leads to the following sequence of responses: change from the discoid to the spheroid shape (shape change), secretion of the granule content, and aggregation. Shape change is thus the earliest response to a stimulus; it requires the reorganization of the cytoskeletal structure including formation of new actin filaments, disappearance of the marginal band of microtubules, and centralization of granules (5, 6), morphological events that precede pseudopodia extrusion. Agonist-induced shape change requires the elevation of cytosolic Ca^{2+} levels that regulates multiple Ca^{2+}-dependent enzymes such as Ca^{2+}/calmodulin-dependent myosin light chain (MLC) kinase (7), which, by phosphorylating MLC, is closely involved in the mechanism of platelet shape change.

However, the capability of collagen itself to elicit platelet shape change is still a matter of debate. Recently, Jarvis *et al.* (8), starting from the distinction of two responses induced by collagen, one primary and one secondary to ADP and TxA_2 release (9), demonstrated that collagen itself was unable to induce shape change since this response was prevented by the simultaneous blockade of ADP receptors $P2Y_1$ and $P2Y_{12}$ and the concomitant inhibition of the cyclooxygenase-dependent pathway. Furthermore, Mangin *et al.* (10), using scanning electron microscopy, showed the absence of extruded filopodia in a suspension of platelets treated with the selective $P2Y_1$ inhibitor MRS-2179 and aspirin.

These data seem to be in disagreement with those of Smith *et al.* (9), who, even in the presence of feedback inhibitors, observed an increase in the phosphorylation of MLC, the preferential pathway leading to platelet shape change following collagen stimulation. Since the morphological changes of the few platelets that adhere to collagen might not be revealed by turbidimetry and since granule centralization cannot be observed by scanning electron microscopy, the aim of this study was to use transmission electron microscopy to assess the earliest phases of platelet shape change under experimental conditions that inhibit the positive, amplificatory feedback pathways of ADP and TxA_2.

cAMP is thought to affect nearly all aspects of platelet activation, including shape change (11, 12). Indeed, it has been demonstrated that cAMP inhibits platelet shape change and MLC phosphorylation following U46619 or convulxin stimulation, mainly acting on the Ca^{2+}/calmodulin-dependent path-

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[1] The abbreviations used are: GPVI, glycoprotein VI; MLC, myosin light chain; MLCK, myosin light chain kinase; CPZ, chlorpromazine, PKA, cAMP-dependent protein kinase; TxA_2, thromboxane A_2; PKI, protein kinase inhibitor; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; KT-5720, (8*R**,9*S**,11*S**)-(-)-9-hydroxy-9-*n*-hexyloxy-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7*b*, 11*a*-triazadibenzo(*a*,*g*)-cycloocta(*c*,*d*,*e*)trinden-1-one.

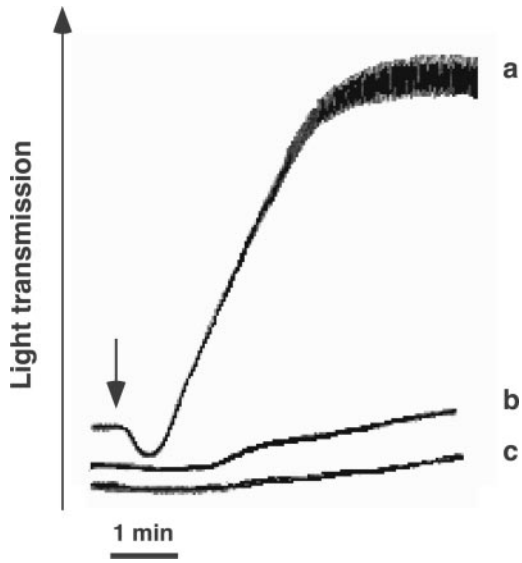

FIG. 1. **The effect of positive feedback inhibitors and iloprost on collagen-induced platelet shape change.** Platelet shape change was measured as described. The ordinate represents the changes in light transmission. Aspirinated platelets were treated with either vehicle (Me_2SO) (*a*) or ADP receptors antagonists AR-C66096 (100 nM for 1 min) and MRS-2179 (100 μM), both in the absence (*b*) and in the presence (*c*) of iloprost (28 nM for 3 min). Collagen was used at a concentration of 5 μg/ml in *a* and of 25 μg/ml in *b* and *c*. The traces are representative of four experiments performed.

way (11, 13). However, whether this inhibitory action is dependent upon the reduction of Ca^{2+} mobilization (14, 15) or on a PKA-dependent decrease in myosin kinase activity (16) has never been elucidated in platelets.

Collagen appears to be the ideal tool to address this unanswered question since it has been demonstrated that, contrary to that seen with all known platelet agonists, increased levels of cAMP enhance collagen-induced Ca^{2+} mobilization (9, 17). Therefore, in the present study, we sought to investigate how cAMP influences the collagen-generated intracellular signaling that, through Ca^{2+}-calmodulin interaction and subsequent MLC phosphorylation, leads to platelet shape change.

MATERIALS AND METHODS

Platelet Preparation—Blood samples from informed healthy volunteers, who denied having taken any drug in the 2 weeks before blood sampling, were collected in citric acid/citrate/dextrose (18)-containing tubes. Informed consent was obtained from all subjects participating in the study.

Platelet-rich plasma was obtained after centrifugation (180 × *g* for 15 min) and further centrifuged (800 × *g* for 20 min) to concentrate the platelets (6 × 10^8 cells/ml). The concentrated platelets were incubated for 15 min at 37 °C with 1 mM aspirin (Sigma) and, for Ca^{2+}-mobilization studies, Fura-2 AM (3 μM at 37 °C for 30 min plus at room temperature for 15 min) and then washed twice in Tyrode's buffer (137 mM NaCl, 2.68 mM KCl, 0.42 mM NaH_2PO_4, 1.7 mM $MgCl_2$) containing 10 mM HEPES (pH 6.5) and resuspended in Tyrode's buffer (pH 7.35) containing 0.2% bovine serum albumin, fraction V (BSA-V) and 0.1% glucose (all from Sigma).

All platelet preparations were further treated with the $P2Y_{12}$ ADP

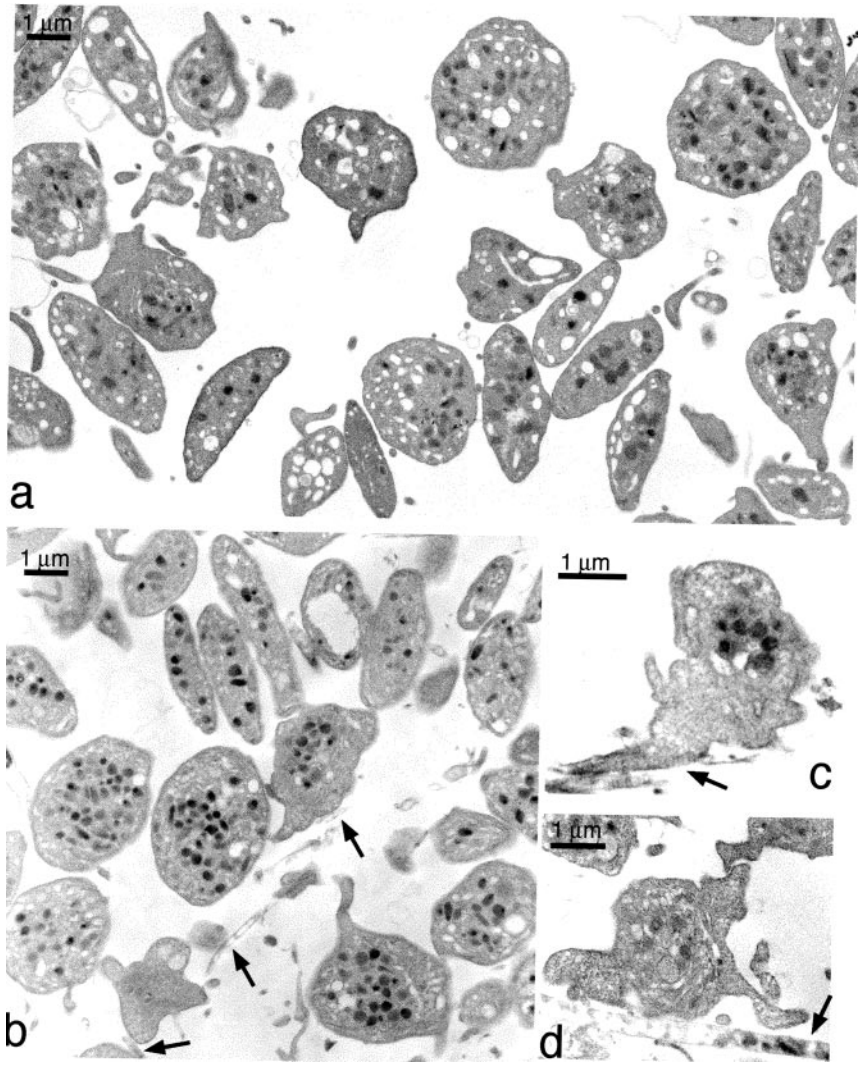

FIG. 2. **Collagen-induced platelet shape-change.** Transmission electron microscopy of aspirinated platelets treated with ADP receptor antagonists AR-C66096 (100 nM for 1 min) and MRS-2179 (100 μM) under resting conditions (*a*) and after activation with 25 μg/ml collagen, fixed 20 s after exposure (*b*), is shown. Centralization of secretory granules and pseudopodia formation are clearly evident in platelets in close contact with collagen fibers. *c* and *d* represent higher magnifications. *Arrows* indicate collagen fibers.

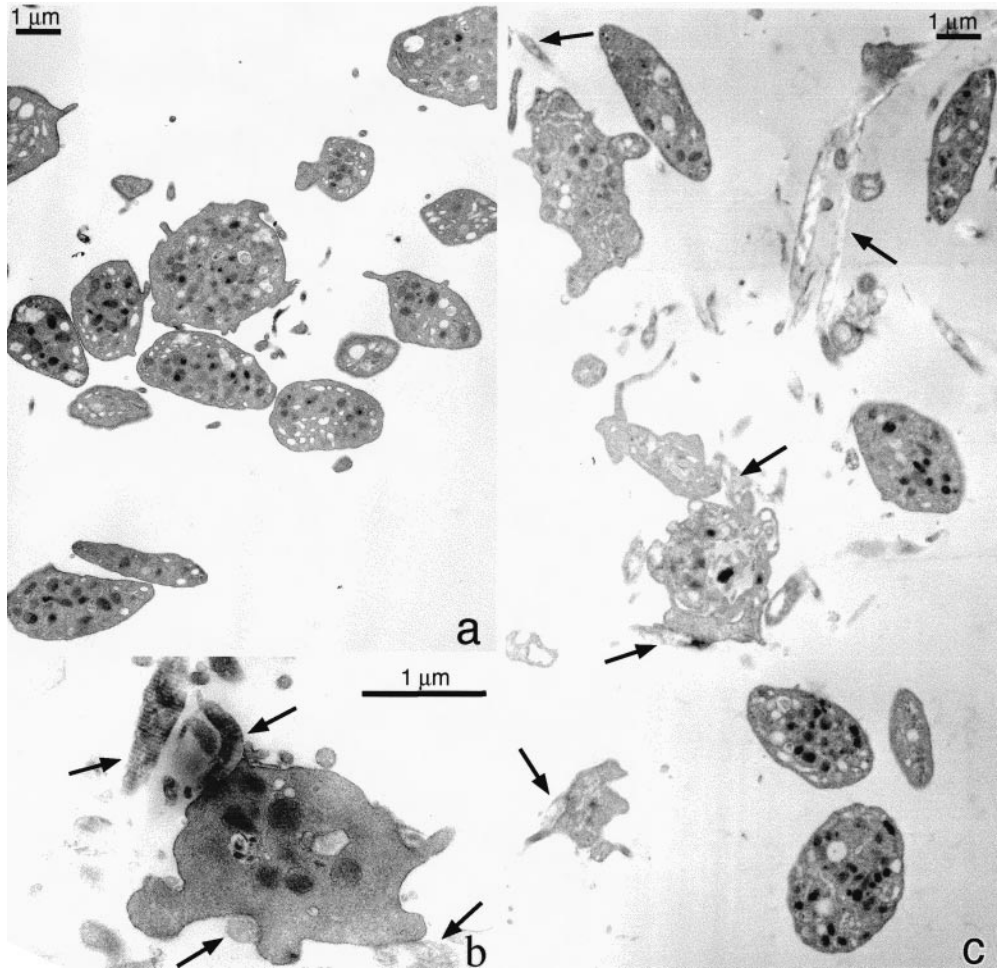

FIG. 3. **The effect of iloprost on collagen-induced platelet shape change.** Transmission electron microscopy of aspirinated, iloprost-treated (28 nM for 3 min) platelets in which the amplificatory pathways were inhibited as in Fig. 2 and activated for 20 s by 25 μg/ml collagen (*c*). The morphological changes observed after stimulation parallel those observed in Fig. 2. Iloprost treatment does not alter the morphology observed under resting conditions. (*a*), *b* shows a higher magnification. *Arrows* point to collagen fibers.

receptor antagonist AR-C66096 (100 nM for 1 min; generous gift of AstraZeneca, B. Humphries, Charnwood, Loughborough, UK) and with the $P2Y_1$ ADP receptor antagonist MRS-2179 (100 μM, Sigma) before the addition of the agonists. To enhance cAMP levels, platelets were treated with iloprost (28 nM for 3 min at 37 °C; generous gift of Italfarmaco S.p.a., Milan, Italy). *N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7, 60 μM) and a naphthalenesulfonamide derivative, chlorpromazine (CPZ, 60 μM), both from Sigma, were used to inhibit Ca^{2+}/calmodulin pathway (19, 20). Cell-permeant (8*R**,9*S**,11*S**)-(-)-9-hydroxy-9-*n*-hexyloxy-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7*b*,11*a*-triazadibenzo(*a*,*g*)-cycloocta(*c*,*d*,*e*-)trinden-1-one (KT-5720, 3 μM for 10 min at 37 °C) and myristoylated PKI peptide-14,22 (5 μM for 10 min at 37 °C), both from Calbiochem, were used as selective cAMP-protein kinase inhibitors (21). Platelet stimulation was achieved by means of collagen type I (25 μg/ml; Mascia Brunelli, Italy), with the thromboxane synthetic analogue U46619 (1 μM, Sigma) or with convulxin (5 ng/ml; Latoxan, Rosans, France), purified according to Polgar *et al.* (22).

Cyclic AMP Extraction and Measurement—Gel-filtered platelets were incubated for 2 min at 37 °C with iloprost (28 nM or 3 μM) and activated with the agonists. The reaction was stopped after 3 min by adding trichloroacetic acid (final concentration 10%). After removal of trichloroacetic acid with water-saturated diethyl ether, cAMP concentration of resuspended dried aqueous layer extract was determined using cAMP enzyme immunoassay system (Amersham Biosciences).

Changes in Intracellular Calcium Concentration—The fluorescent changes in intracellular calcium concentration were studied in Fura 2-loaded platelets (Molecular Probes, Eugene, OR) and monitored in a Kontron SFM 25 spectrofluorimeter thermostatically regulated at 37 °C, under continuous stirring. Excitation and emission wavelengths were 340 and 510 nm, respectively. Intracellular free calcium concentration was calibrated according to Grynkiewicz *et al.* (23). Data are reported as ΔnM and percentages of change in intracellular calcium concentrations (%Δ) induced by agonists.

Stimulation was performed with collagen (25 μg/ml), convulxin (5 ng/ml), and U46619 (1 μM) to test cAMP inhibitory effects on our platelet preparation. If the Δnm of Ca^{2+} obtained in response to U46619 plus iloprost was higher than 10, the platelet preparation was discharged.

Platelet Shape Change—In vitro platelet shape change was evaluated in a lumi-aggregometer (Chrono-log Corp. Havertown, PA) using siliconized glass cuvettes at 37 °C under continuous stirring. Once the full function of the platelet preparation was tested in response to collagen (25 μg/ml) and convulxin (5 ng/ml), platelets were treated with RGDS (120 μg/ml) which prevents fibrinogen binding to the integrin αIIbβ3 but allows shape change to occur. The extent of the shape change (magnitude) was measured as a decrease in light transmission.

Electron Microscopy—For conventional thin section electron microscopy, platelets were treated as described (24). 450-μl aliquots of platelet suspension were fixed in the aggregometer cuvettes with 0.1% glutaraldehyde and kept at 37 °C for 15 min. The platelets were then spun at 15,000 × *g* for 5 min and fixed in 2% glutaraldehyde for 3 h. After fixation, cells were washed in phosphate-buffered saline, postfixed with 1% OsO_4 in veronal acetate buffer (pH 7.4) for 1 h at 25 °C, stained with 0.1% tannic acid in the same buffer for 30 min at 25 °C and with uranyl acetate (5 mg/ml) for 1 h at 25 °C, dehydrated in acetone, and embedded in Epon 812. Thin sections were finally stained with uranyl acetate and lead hydroxide and examined with a Philips electron microscope.

Myosin Light Chain Phosphorylation—MLC phosphorylation has been analyzed according to Daniel and Sellers (25). Briefly, aspirinated platelets were resuspended in Tyrode's buffer at a concentration of 2×10^9 cells/ml. After 30 s from agonist stimulation, the reaction was stopped by the addition of 6.6 N $HClO_4$. The pellets were centrifuged at 10,000 × *g* for 2 min and washed twice; the resulting pellets were

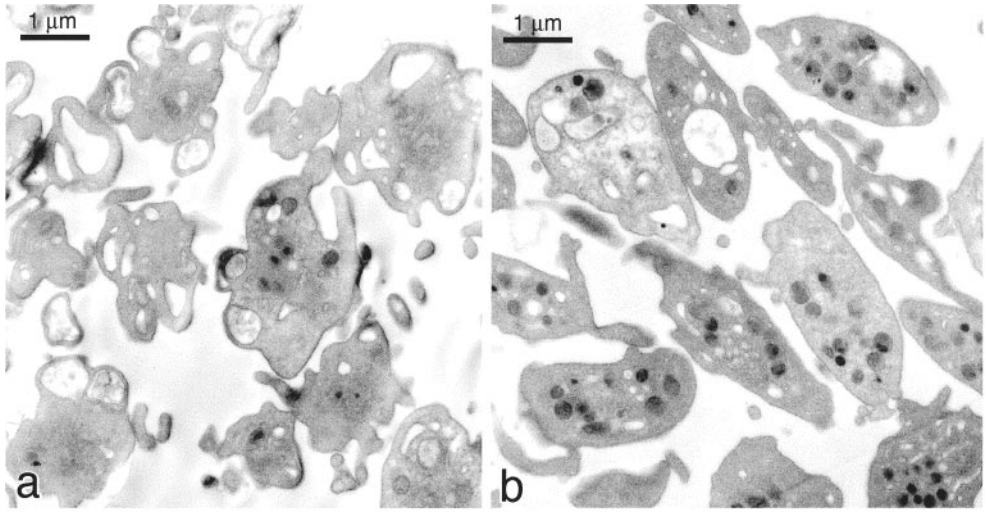

FIG. 4. **Convulxin-induced platelet shape-change.** Transmission electron microscopy of aspirinated platelets treated with ADP receptor antagonists AR-C66096 (100 nM for 1 min) and MRS-2179 (100 μM) after activation with 5 ng/ml convulxin both in the absence (*a*) and in the presence (*b*) of iloprost (28 nM for 3 min), fixed 20 s after exposure, is shown.

dissolved in 50 μl of sample buffer containing 8 M urea, 20 mM Tris, 122 mM glycine, 5 mM dithiothreitol (pH 8.6), to which 0.1% bromphenol blue dye was added. The suspended pellet was sonicated (PBI Briansonic 220) for 30 min. Gel electrophoresis was performed in a 10% polyacrylamide-urea minigel apparatus (Hoefer Scientific Instruments, San Francisco, CA). The running buffer used in the top chamber was composed of 20 mM Tris, 122 mM glycine at pH 8.6 containing 4 mM urea. The electrophoresis was stopped 1 h after the blue marker had come off. Proteins were transferred to Immobilion-P (Millipore) membranes and identified with anti-phospho-MLC monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) followed by horseradish peroxidase-conjugated secondary antibody and detected with ECL chemiluminescence reaction reagent (Amersham Biosciences) and Kodak x-ray film (X-OMAT AR). In some experiments, the membranes, once probed, were stripped of bound antibodies by incubation in buffer containing 100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7, for 30 min at 60 °C and then reprobed with an antibody against MLC.

RESULTS

Platelet Shape Change—Fig. 1 shows the changes in light transmission recorded after collagen (5 μg/ml) stimulation in the absence of cyclooxygenase and ADP receptor inhibitors and of RGDS (*trace a*). Once the full function of the platelet preparation was tested in response to collagen, platelets were treated with AR-C66096, MRS-2179, and RGDS. Under these experimental conditions, we were unable to detect any change in light transmission in response to collagen (25 μg/ml), both in the absence (*trace b*) and in the presence (*trace c*) of the cAMP-enhancing drug iloprost (28 nM for 3 min at 37 °C).

Thus, to verify whether platelet shape change really did not occur in the presence of positive feedback inhibitors when platelets were stimulated by collagen, we analyzed by transmission electron microscopy the morphological changes of a suspension of platelets activated for 20 s with collagen (25 μg/ml). Fig. 2 shows platelet morphology during the resting state (Fig. 2*a*). Following collagen stimulation, only those platelets that were close to the collagen fibers (*arrows*) underwent a change in shape, showing signs of cytoskeletal rearrangements, centralization of secretory granules, and pseudopodia formation, whereas all other platelets did not modify their morphology (*b–d*). When analyzed in iloprost-treated samples, platelet shape change paralleled that observed in untreated samples (Fig. 3, *a–c*).

The inhibitory effect of cAMP on convulxin-stimulated platelets was assessed in one of our previous studies (13) and documented by means of turbidimetry. The use of electron microscopy allowed us to verify this inhibition (Fig. 4, *a* and *b*).

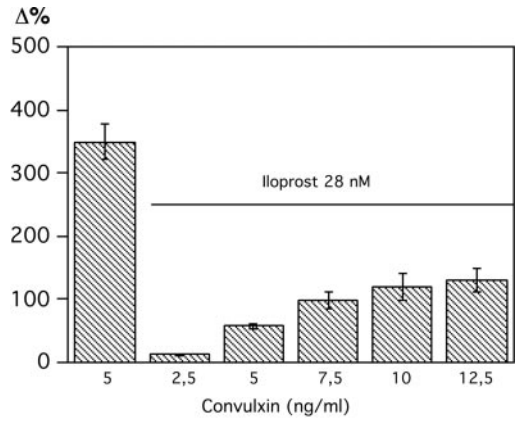

FIG. 5. **The effect of iloprost on increasing concentration of convulxin-induced cytosolic Ca^{2+} mobilization.** Aspirinated platelets were treated with ADP receptor antagonists AR-C66096 (100 nM for 1 min) and MRS-2179 (100 μM), loaded with Fura-2, and stimulated with increasing concentrations of convulxin. The *bar* indicates the samples further treated with iloprost (28 nM for 3 min). Results are presented as mean values ± S.E. of four experiments performed.

Effects of cAMP on Collagen-induced Changes in Intracellular Calcium Concentration and on Myosin Light Chain Phosphorylation—To investigate the role of the increase of cytosolic cAMP on agonist-induced calcium mobilization, Fura-2-loaded platelets were treated with iloprost (28 nM). Cyclic AMP concentrations were enhanced from 10.3 ± 2.1 pmol/10^9cells (basal conditions) to 558.3 ± 59.6 pmol/10^9cells. Higher concentrations of iloprost (3 μM) produced a cAMP concentration of 1164.8 ± 128.6 pmol/10^9 cells. Neither convulxin nor collagen was able to decrease the cAMP levels elicited by iloprost (28 nM) treatment (489.2 ± 72.1 and 495.6 ± 70.3 pmol/10^9 cells respectively).

Stimulation with U46619 (1 μM), which was employed to test the inhibitory action of cAMP on our platelet preparation, evoked a rise in cytosolic Ca^{2+} concentration (729.7 ± 28.9 Δ%) that was completely abolished by iloprost treatment. The stimulation with convulxin (5 ng/ml) caused a rise in intracellular Ca^{2+} mobilization (329.7 ± 28.9 Δ%) that, in agreement with our previous study (13), was strongly inhibited by iloprost treatment (46.2 ± 14.5 Δ%, $p < 0.02$). This inhibition was reduced by increasing convulxin concentration (Fig. 5). Colla-

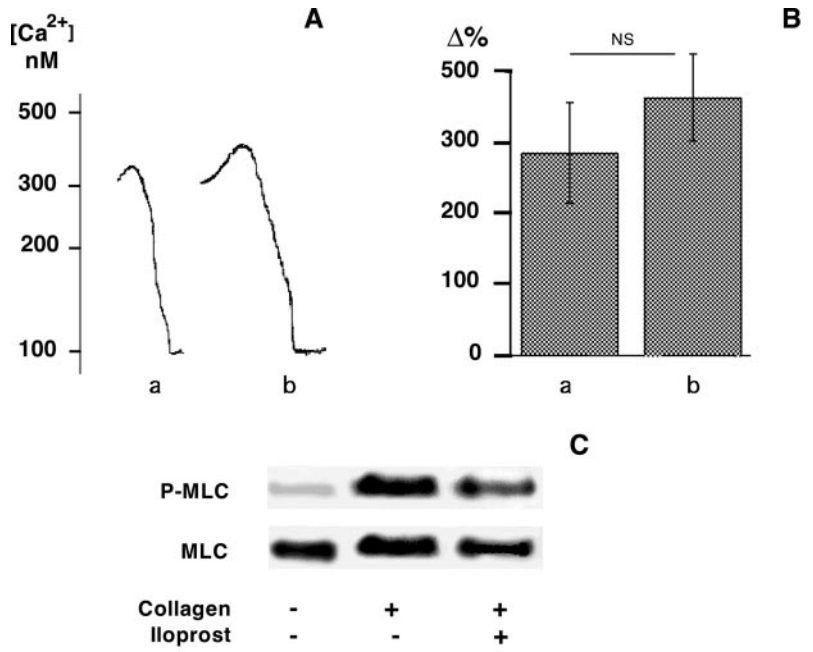

FIG. 6. **The effect of iloprost on collagen-induced increase in cytosolic Ca^{2+} and on levels of myosin light chain phosphorylation.** *A* and *B*, representative ($n = 5$) traces (*A*) and percentage of change (Δ%) (*B*) of Ca^{2+} mobilization in collagen-stimulated platelets in the absence (*a*) and in the presence (*b*) of iloprost (28 nM for 3 min). *C*, alkaline-urea-PAGE of pellets from platelets treated with either vehicle (Me_2SO) or iloprost, stimulated with 25 μg/ml collagen. Phospho-MLC (*P-MLC*, *upper panel*) and control MLC (*lower panel*) antibodies were used. These results are representative of three experiments performed.

gen (25 μg/ml) evoked a much lower rise in cytosolic Ca^{2+} concentration (184.5 ± 46.2 Δ%), which was slightly, although not significantly, enhanced by iloprost treatment (236.2 ± 39.7 Δ%) (Fig. 6*A*), as also found by Smith *et al.* (9, 17). Conversely, the extent of collagen-induced MLC phosphorylation was reduced by the enhanced intracellular levels of cAMP (Fig. 6*B*).

Effects of Ca^{2+}/Calmodulin Pathway Inhibition on Collagen-induced Changes in Intracellular Calcium Concentration and on Myosin Light Chain Phosphorylation—To verify that collagen-induced platelet MLC phosphorylation follows the Ca^{2+}-dependent pathway, platelets were treated with the Ca^{2+}/calmodulin inhibitors W-7 (60 μM) and CPZ (60 μM), a naphthalenesulfonamide derivative. Our data demonstrated that treatment with W-7 or CPZ dramatically inhibited MLC phosphorylation in response to collagen, supporting a role for the Ca^{2+}/calmodulin pathway in this process (Fig. 7).

Effects of PKA Inhibition on Collagen-induced Changes in Intracellular Calcium Concentration and on Myosin Light Chain Phosphorylation—The effects of cAMP-dependent protein kinase inhibition were determined. For this purpose, the PKA active site inhibitors KT-5720 (3 μM) and myristoylated PKI peptide-14,22 (5 μM) were used. Our results demonstrate that the PKA inhibitors did not significantly modify the collagen-induced Ca^{2+} response when compared with control (Fig. 8*A*). When the PKA inhibitors were tested on the responses elicited by U46619 and convulxin, iloprost-inhibited Ca^{2+} mobilization was almost completely reversed by pretreatment with KT-5720 (Fig. 9). This result is in agreement with previous reports demonstrating that it is possible to restore cAMP-inhibited platelet aggregation induced by a thromboxane analogue using a PKA antagonist (26). As expected, the inhibition of collagen-induced MLC phosphorylation achieved with iloprost was reversed by preincubation with the PKA inhibitors (Fig. 8*B*).

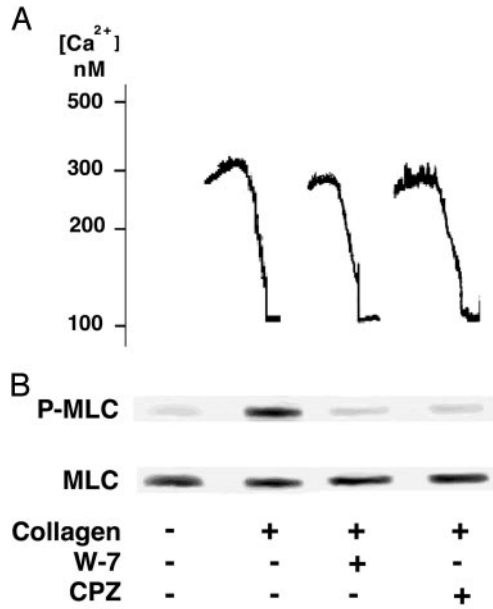

FIG. 7. **Role of Ca^{2+}/calmodulin in collagen-induced Ca^{2+} mobilization and myosin light chain phosphorylation.** *A*, representative ($n = 4$) traces of Ca^{2+} mobilization in collagen-stimulated (25 μg/ml) platelets in the absence and in the presence of Ca^{2+}/calmodulin inhibitors W-7 (60 μM) or CPZ (60 μM). *B*, alkaline-urea-PAGE of pellets from platelets treated with vehicle (Me_2SO), W-7, or CPZ, stimulated with 25 μg/ml collagen. Phospho-MLC (*P-MLC*, *upper panel*) and control MLC (*lower panel*) antibodies were used. These results are representative of four experiments performed.

DISCUSSION

In the present study, the use of transmission electron microscopy allowed us to shed light on the controversial matter of the capability of collagen to induce platelet shape change without the necessary presence of positive amplificatory pathways represented by released ADP and TxA_2. Our results clearly demonstrated that collagen induces morphological changes accompanied by secretory granule centralization of the few adherent platelets, as evidenced by the electron microscopy images, a process that turbidimetry failed to detect. This might explain the results obtained by Jarvis *et al.* (8), who, monitoring platelet shape change in response to collagen by means of an aggregometer, found that this early phenomenon of platelet activation was substantially blocked by preincubation with TxA_2 and ADP inhibitors. The reason for these apparently contrasting results might reside in the fact that fibrillar collagen is an insoluble macromolecule, and therefore, only a small number of platelets come into direct contact with it (27), whereas in the presence of soluble second mediators released from collagen-activated platelets, a larger number of cells be-

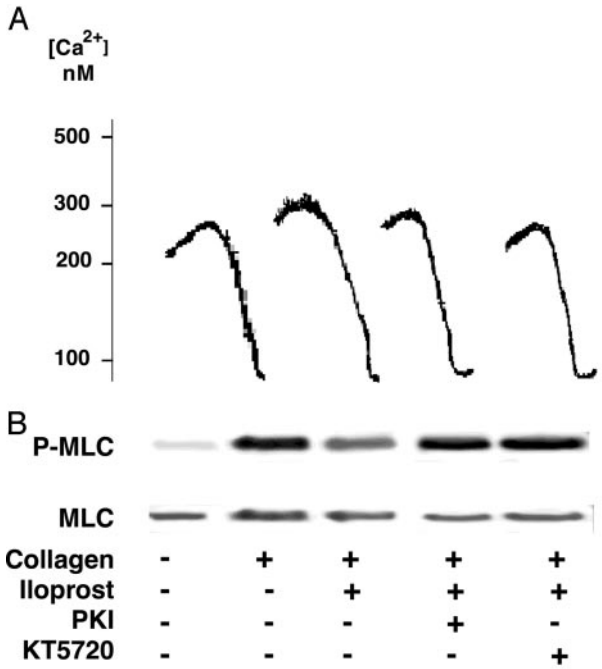

FIG. 8. **Role of PKA in collagen-induced Ca^{2+} mobilization and myosin light chain phosphorylation.** *A*, representative (n = 4) traces of Ca^{2+} mobilization in collagen-stimulated (25 μg/ml) platelets in the absence and in the presence of PKA active-site inhibitors, KT-5720 (3 μM), and myristoylated PKI peptide-14,22 (5 μM). *B*, alkaline-urea-PAGE of pellets from platelets treated with vehicle (Me_2SO), PKI-14,22, or KT-5720, prior to iloprost (28 nM for 3 min) addition, stimulated with 25 μg/ml collagen. Phospho-MLC (*P-MLC*, *upper panel*) and control MLC (*lower panel*) antibodies were used. These results are representative of four experiments performed.

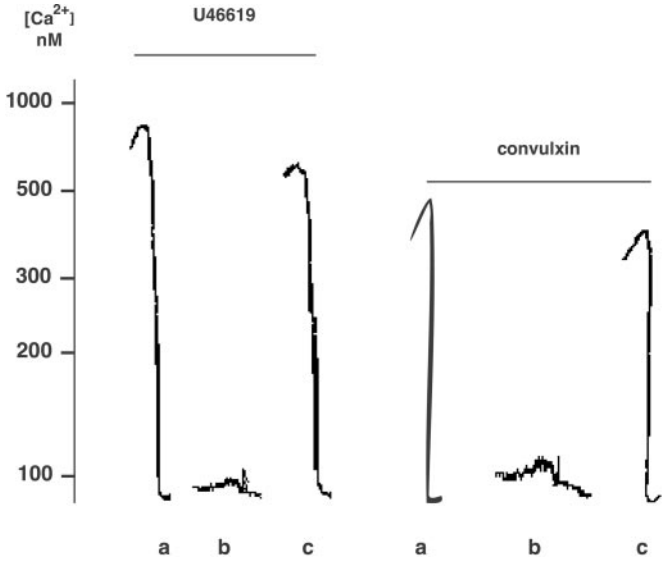

FIG. 9. **Role of PKA in U46619- and convulxin-induced Ca^{2+} mobilization.** Representative (n = 4) traces of Ca^{2+} mobilization in iloprost-treated (28 nM for 3 min) platelets stimulated with U46619 (1 μM) or convulxin (5 ng/ml) in the absence (*b*) and in the presence (*c*) of PKA active-site inhibitor, KT-5720 (3 μM). *a* represents control, untreated, stimulated platelets.

come more promptly activated. Our result is consistent with previous findings (9), indicating 30.0% platelet adhesion to collagen fibrils in the presence of ADP and TxA_2 inhibitors. It also provides an explanation for the evidence that convulxin and collagen-related peptide, soluble agonists that activate platelets through the same collagen receptor complex, GPVI/Fc receptor γ-chain, induce a shape change of greater magnitude and slope, even in the presence of inhibitors of cyclooxygenase and antagonists of ADP receptors $P2Y_1$ and $P2Y_{12}$ (8, 17, 27, 29). These findings were substantiated in the present study by transmission electron microscopy analyses of convulxin-stimulated platelets depicting evident signs of morphological change in a higher number of cells.

It is worth noting that granule centralization has to be considered the earliest evidence of platelet shape change, preceding the extrusion of filopodia, and transmission electron microscopy can be considered the ideal tool for its assessment. The demonstration that collagen stimulation phosphorylates MLC further corroborates our results, indicating that collagen induces MLCK activation, a biochemical event that leads to platelet shape change (7).

Collagen-induced shape change is insensitive to cyclic AMP-mediated inhibition. Indeed, we did not observe, by electron microscopy, any modification of iloprost-treated platelet morphology when compared with control. This finding is not surprising since previous studies demonstrated that elevation of cyclic AMP did not inhibit either platelet adhesion to collagen or the associated Ca^{2+} mobilization (9). Accordingly, in our study, we also found that iloprost not only failed to inhibit collagen-induced Ca^{2+} mobilization but even enhanced it. This particular behavior of the response to collagen is not dependent upon the capability of collagen to lower elevated levels of cAMP (the present study and Ref. 9).

The fact that collagen-elicited platelet shape change is not subject to cAMP inhibition has been further confirmed by the studies on the phosphorylation of MLC, which involves Ca^{2+}/calmodulin-dependent MLCK (7) and is the preferential pathway leading to platelet shape change following collagen stimulation (9). When analyzing how cAMP influences collagen-mediated MLC phosphorylation, we found the amount of phosphorylated proteins to be only slightly reduced by treatment with iloprost in the presence of inhibitors of cyclooxygenase and antagonists of ADP receptors $P2Y_1$ and $P2Y_{12}$. The fact that in response to collagen, MLC phosphorylation is reduced, albeit weakly, by high cytosolic levels of cAMP, whereas Ca^{2+} mobilization is enhanced, requires careful analysis.

Indeed, that MLC phosphorylation in response to collagen is strongly related to the Ca^{2+}/calmodulin-dependent pathway has been proved by antagonizing the Ca^{2+}-dependent pathway with the Ca^{2+}/calmodulin inhibitors W-7 and chlorpromazine. In fact, collagen-evoked MLC phosphorylation was dramatically inhibited by treatment with inhibitors of the Ca^{2+}/calmodulin interaction.

The discrepancy between Ca^{2+} mobilization and MLC phosphorylation observed in the presence of cAMP-enhancing compounds might thus be explained by considering that PKA phosphorylation directly inhibits MLCK activity and the subsequent MLC phosphorylation, as already demonstrated by others using different cellular approaches (30). Consistent with this hypothesis, our data show that also in platelets, PKA phosphorylation inhibits MLCK. In fact, treatment with the PKA inhibitors KT-5720 and myristoylated PKI peptide-14,22 before incubation with iloprost, while having no effects on the Ca^{2+} response to collagen, reversed collagen-induced MLC phosphorylation to pretreatment levels.

Taken together, our findings indicate that collagen is able *per se* to cause platelet shape change through activation of Ca^{2+}/calmodulin-dependent MLCK, without the necessary involvement of the amplificatory pathway represented by released ADP and TxA_2. Enhanced cytosolic levels of cAMP exert an inhibitory action directly on MLCK, that, however, is too weak to inhibit collagen-induced platelet shape change.

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