Effect of parathyroid-hormone-related protein on human platelet activation

Arantxa ORTEGA*∥[1], Mª Teresa PÉREZ DE PRADA†[1], Petra J. MATEOS-CÁCERES†, Priscila RAMOS MOZO†, Juan J. GONZÁLEZ-ARMENGOL‡, Juan M. GONZÁLEZ DEL CASTILLO‡, Javier MARTÍN SÁNCHEZ‡, Pedro VILLARROEL‡, José L. SANTIAGO§, Ricardo J. BOSCH∥, Carlos MACAYA†, Pedro ESBRIT*[2] and Antonio J. LÓPEZ-FARRÉ†[2]

*Bone and Mineral Metabolism Laboratory, Fundación Jiménez Díaz, 28040 Madrid, Spain, †Cardiovascular Research Unit, Cardiovascular Institute, Hospital Clínico San Carlos, 28040 Madrid, Spain, ‡Emergency Department, Hospital Clínico San Carlos, 28040 Madrid, Spain, §Immunology Department, Hospital Clínico San Carlos, 28040 Madrid, Spain, and ∥Physiology Department, University of Alcalá, Alcalá de Henares, 28801 Madrid, Spain

A B S T R A C T

Evidence suggests that PTHrP [PTH (parathyroid hormone)-related protein] can act as an inflammatory mediator in several pathological settings including cardiovascular disease. The aim of the present study was to determine whether PTHrP might be involved in human platelet activation. We used a turbidimetric method to determine platelet aggregation. The expression of PTH1R (PTH type 1 receptor) in human platelets was analysed by Western blot and flow cytometry analyses. PTHrP-(1–36) (10^{-7} mol/l) by itself failed to modify the activation of platelets. However, it significantly enhanced ADP-induced platelet activation, and also increased the ability of other agonists (thrombin, collagen and arachidonic acid) to induce platelet aggregation. H89 (10^{-6} mol/l) and 25×10^{-6} mol/l Rp-cAMPS (adenosine $3',5'$-cyclic monophosphorothioate Rp-isomer), two protein kinase A inhibitors, and 25×10^{-9} mol/l bisindolylmaleimide I, a protein kinase C inhibitor, partially decreased the enhancing effect of PTHrP-(1–36) on ADP-induced platelet activation. Meanwhile, 10^{-6} mol/l PTHrP-(7–34), a PTH1R antagonist, as well as 10^{-5} mol/l PD098059, a MAPK (mitogen-activated protein kinase) inhibitor, or a farnesyltransferase inhibitor abolished this effect of PTHrP-(1–36). Moreover, 10^{-7} mol/l PTHrP-(1–36) increased (2-fold over control) MAPK activation in human platelets. PTH1R was detected in platelets, and the number of platelets expressing it on their surface in patients during AMI (acute myocardial infarction) was not different from that in a group of patients with similar cardiovascular risk factors without AMI. Western blot analysis showed that total PTH1R protein levels were markedly higher in platelets from control than those from AMI patients. PTH1R was found in plasma, where its levels were increased in AMI patients compared with controls. In conclusion, human platelets express the PTH1R. PTHrP can interact with this receptor to enhance human platelet activation induced by several agonists through a MAPK-dependent mechanism.

INTRODUCTION

Acute coronary syndrome is caused by rupture of the atherosclerotic plaque and subsequent occlusive or subocclusive thrombus formation. Activated platelets then release a variety of vasoactive substances that promote platelet aggregation. Increased platelet activation occurs in cardiovascular diseases related to acute coronary syndrome. However, this process is complex and influenced by a myriad of cellular- and plasma-derived mediators. In fact, the normal response of platelets can be altered by either an increase or decrease in pro-aggregating or anti-aggregating stimuli respectively.

PTHrP [PTH (parathyroid hormone)-related protein], the causative factor of humoral hypercalcaemia of malignancy, is now known to be widespread in normal fetal and adult tissues [1]. Owing to N-terminal sequence homology between PTH and PTHrP, both peptides interact with a common receptor, the G-protein-coupled PTH1R (PTH type 1 receptor), and thus stimulate cAMP formation, and PKC (protein kinase C) and MAPK (mitogen-activated protein kinase) activities in different cell types [2–4]. In contrast with PTH, which acts as a classical endocrine hormone, PTHrP normally exerts its effects locally in an autocrine, paracrine and even intracrine fashion [1,3]. Previous evidence strongly suggests that PTHrP may function as a pro-inflammatory mediator in various pathophysiological settings [5]. Thus PTHrP may contribute to the lethal effects of several pro-inflammatory cytokines during multi-organ failure in endotoxaemia [5–7]. There is also evidence supporting the notion that PTHrP, through its pro-inflammatory properties, could be involved in mechanisms of renal injury [8,9]. Moreover, previous data suggest that PTHrP may also have complex roles in cardiovascular pathology [3]. PTHrP is present in smooth muscle cells and macrophages within human atherosclerotic lesions, where it might act as both a pro-inflammatory and pro-atherogenic factor [10,11].

As mentioned above, platelet activation is a key process related to both plaque instability and the compromised blood flow after coronary ischaemia [12,13]. To our knowledge, there are no current data on a possible effect of PTHrP on platelet activation, but some investigators have reported some conflicting effects of PTH in platelets. One of these studies has shown that acute administration of physiological concentrations of PTH-(1–34) induced an increase in platelet $[Ca^{2+}]_i$ (intracellular $[Ca^{2+}]$) associated with an increased arterial blood pressure in humans [14]. Moreover, a significant correlation between serum PTH and platelet $[Ca^{2+}]_i$ levels has been found in normotensive patients with primary hyperparathyroidism, although other investigators failed to observe such a relationship in human subjects with either essential hypertension or secondary hyperparathyroidism [15,16]. Furthermore, PTH has now been shown to display either pro- or anti-aggregating effects in human platelets [17,18]. However, taken together, these results support the notion that PTH might directly interact with platelets, although no attempts have been made to characterize the putative PTH receptor, e.g. PTH1R or the PTH-specific PTH2R [19], in platelets. Moreover no studies have yet been performed to assess whether PTHrP may also be involved in the regulation of platelet activation. Therefore in the present study we investigated: (i) the effect of PTHrP on platelet aggregation, as well as the putative intracellular mechanism involved in this PTHrP action, and (ii) the presence of PTH1R in human platelets, and possible changes in its expression in platelets from patients during an acute coronary syndrome.

MATERIALS AND METHODS

Reagents

Human PTHrP-(1–36) was kindly supplied by Dr A. F. Stewart and Dr A. García Ocaña (Division of Endocrinology and Metabolism, University of Pittsburgh School of Medicine, Pittsburgh, PA, U.S.A.). Human PTH-(1–34) and human PTHrP-(107–139) were obtained from Sigma, and (Asn^{10}, Leu^{11}, D-Trp^{12}) PTHrP-(7–34) amide [PTHrP-(7–34)] was obtained from Bachem.

H89 (N-{2-[(p-bromocinnamyl)amino]-ethyl}-5-iso-quinolinesulfonamide dihydrochloride), Rp-cAMPS (adenosine 3′,5′-cyclic monophosphorothioate Rp-isomer), BIM (bisindolylmaleimide I), PD098059 (2′-amino-3′-methoxyflavone) and FTase (farnesyltransferase) inhibitor III were obtained from Calbiochem. An affinity-purified mouse monoclonal anti-PTH1R antibody was supplied by Santa Cruz Biotechnology. Rabbit polyclonal antibodies against phosphorylated ERK (extracellular-signal-related kinase) 1/2 (Thr^{202}/Tyr^{204}) were from Cell Signaling Technology. ADP, thrombin, collagen and arachidonic acid were purchased from Chrono-par Reagents.

PRP (platelet-rich plasma) preparation

PRP was obtained from healthy volunteers (31 ± 3 years old) who were not taking aspirin or any other anti-platelet or anti-inflammatory drugs for at least 15 days before blood extraction. Healthy volunteers gave written informed consent for participation in the study. PRP isolation was carried out as previously reported [20]. Briefly, whole blood was obtained in 10 % (v/v) acid–citrate–dextrose, and centrifuged at 800 g for 15 min at 20 °C. PRP was collected, and the platelet number was counted using a Coulter counter. The platelet number was adjusted to 2.5×10^8 cells/ml with platelet-poor plasma obtained from the same subject.

Platelet aggregation

Platelet activation was registered in a lumiaggregometer (Aggrecorder, four channels; Chrono-Log Corporation)

by the change in light transmission. A platelet-poor plasma sample was used as a control for pre-calibration at 100% light transmission. PRP (0.5 ml) was incubated at 37 °C for 10 min with continuous stirring in the aggregometer, and was then stimulated with 10^{-6} mol/l ADP, 5×10^{-4} mol/l arachidonic acid, 0.02 unit/ml thrombin or 2 μg/ml collagen, in the presence or absence of different PTHrP peptides. In some experiments, PRP samples were pre-incubated for 10 min with different inhibitors before PTHrP-(1–36) and ADP were added. For calculations, only turbidimetry values at 8 min were used, a time period corresponding to the maximal or near-maximal value of the first wave of platelet aggregation [20].

Western blot analysis

Platelets were obtained from PRP by centrifugation at 600 g for 10 min at room temperature. Isolated platelets were then homogenized in 8 mol/l urea, 2% (w/v) CHAPS, 40 mmol/l dithiothreitol and 0.01% (w/v) Bromophenol Blue. After centrifugation at 900 g for 15 min at 4 °C, the pellet was discarded. Proteins [40 μg/lane estimated by bicinchoninic acid reagent (Pierce), using BSA as a standard], were separated on SDS/PAGE (10% gels) under reducing conditions. Proteins were then blotted on to nitrocellulose membranes (Amersham Biosciences). Membranes were then blocked with 5% (w/v) BSA in PBS with 0.05% Tween 20, by overnight incubation at 4 °C. Membranes were then incubated with the anti-PTH1R antibody (sc-12722; Santa Cruz Biotechnology) at a 1:1000 dilution, or rabbit polyclonal antibodies against ERK1/2 and phosphorylated ERK1/2 (pERK1/2; Cell Signaling Technology) each at 1:1000 dilution for 1 h at room temperature. An anti-β-actin antibody (Sigma) at 1:5000 dilution was used as a loading control. After extensive washing, the membranes were incubated with a peroxidase-conjugated goat anti-mouse IgG and anti-rabbit IgG respectively for 1 h at room temperature and developed by ECL® (enhanced chemiluminescence; Amersham Biosciences). The bands were quantified by densitometric scanning (ImageQuant; Molecular Dynamics). Pre-stained protein markers were used for molecular mass determinations.

Flow cytometry

For flow cytometry studies, blood samples (0.2 ml) were immediately fixed with 1% paraformaldehyde overnight at 4 °C. The blood cells were then washed twice with PBS and incubated for 30 min at 25 °C with the anti-PTH1R antibody [sc-12722 (FITC conjugated); Santa Cruz Biotechnology]. After extensive washing with PBS, the samples were analysed in a flow cytometer (Beckton Dickinson) as described previously [21]. The number of platelets expressing PTH1R was determined

by analysis of a constant number of platelets (5×10^3) for each sample, using forward and side-light scatter bivariate plots. A non-specific mouse IgG$_1$ isotype antibody (sc-2855; Santa Cruz Biotechnology) was used as a negative control.

Patients

Platelets and plasma were obtained from six consecutive patients [aged 75 ± 4 years of age (range, 69–83 years of age)] during an AMI (acute myocardial infarction), who were admitted to the Emergency Unit of the Hospital Clínico San Carlos, Madrid, Spain. AMI was diagnosed on the basis of the classic criteria of prolonged chest pain accompanied by serial changes on the standard 12-lead ECG, as well as raised plasma CK [creatine kinase; more than twice the upper limit of the normal value measured in the biochemistry laboratory and CK-MB (CK isoenzyme muscle, brain) fraction >10% total CK]. Two patients had electrocardiographic ST elevation, whereas the other four patients had no ST elevation (non-Q-wave infarction). A criterion for enrollment was admission within 24 h after the onset of chest pain. A group of five patients matched by age, sex and cardiovascular risk factors were enrolled as a control group. None of the patients were taking any anti-inflammatory and antithrombotic drugs, except aspirin (250 mg) in the AMI group, administered 20–60 min before blood extraction. Patients with a history of neoplastic or autoimmune disease or infection, or any surgical procedure in the preceding 6 months were not included. Both control and AMI patients gave written informed consent, and the study was approved by the Ethics Committee of Hospital Clínico San Carlos, Madrid, Spain.

Dot-blot analysis of plasma

For dot-blot analysis, 10 μg of total plasma proteins were loaded on to a nitrocellulose membrane held in a dot-blot filtration manifold apparatus. The amount of protein was determined using bicinchoninic acid reagent. As described above for Western blot analysis, the membranes were blocked with 5% (w/v) BSA in PBS with 0.05% Tween 20, and then incubated with the monoclonal antibody against PTH1R (sc-12722) at a 1:1000 dilution for 1 h at room temperature. Following incubation with a peroxidase-conjugated goat anti-mouse IgG, membranes were developed using ECL®. As a loading control, a parallel blot containing the same amount of samples was performed and stained with Coomassie Blue.

Statistical analysis

Results are expressed as means $\pm$ S.E.M. Statistical significance was assessed by the Mann–Whitney test. $P < 0.05$ was considered statistically significant.
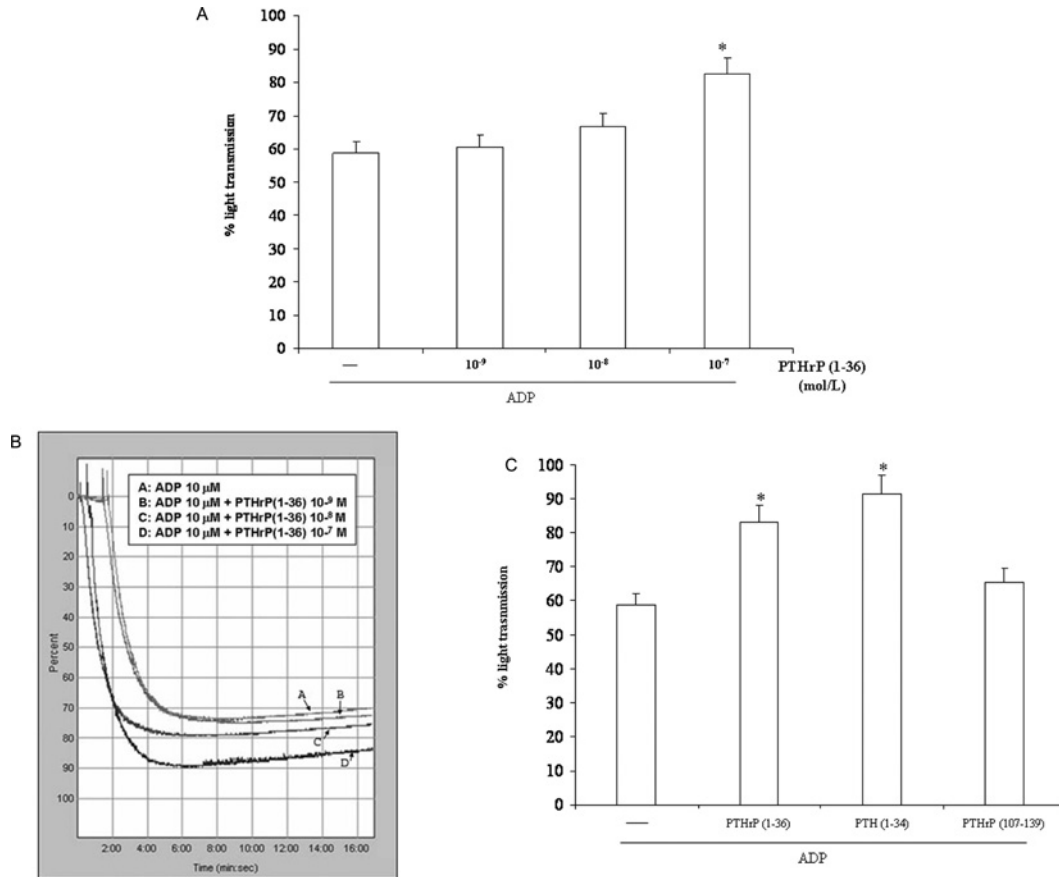
Figure 1   Effect of PTHrP on ADP-induced platelet activation

(A) Effect of increasing concentrations of PTHrP-(1–36) on ADP (10^{-6} mol/l)-induced platelet activation. (B) Representative platelet aggregation trace showing the effect of ADP alone and in the presence of increasing concentrations of PTHrP-(1–36). (C) Effect of ADP + PTH-(1–34) and ADP + PTHrP-(107–139) (each at 10^{-7} mol/l) on ADP-induced platelet activation. For comparison the effect of ADP + PTHrP (10^{-7} mol/l) is also included. Values are means $\pm$ S.E.M. for four independent experiments. *$P < 0.05$ with respect to ADP alone.

RESULTS

Effect of PTHrP-(1–36) on platelet activation

Spontaneous platelet activation (percentage light transmission: < 8 %) was not significantly modified by PRP incubation with increasing concentrations of PTHrP-(1–36) (percentage light transmission: 14 ± 3 %, corresponding to this peptide at 10^{-7} mol/l). However, PTHrP-(1–36) dose-dependently enhanced ADP-induced platelet activation (Figure 1A). Figure 1(B) shows a representative trace of platelet aggregation triggered by ADP in the presence of different PTHrP concentrations. PTHrP potentiated the maximal platelet aggregation response to ADP, but PTHrP did not significantly change the slope of the platelet curve (ADP: 56 ± 4 min; ADP + 10^{-7} mol/l PTHrP: 60 ± 6 min; $P =$ not significant). A similar pro-aggregating effect as with PTHrP was also observed with 10^{-7} mol/l PTH-(1–34) (Figure 1C). In contrast, 10^{-7} mol/l PTHrP-(107–139), which did not

seem to signal through PTH1R [22], failed to modify ADP-induced platelet activation (Figure 1C). Platelet incubation with 10^{-6} mol/l PTHrP-(7–34), a PTH1R antagonist [23], did not by itself modify ADP-induced platelet activation, but blocked the enhancing ability of 10^{-7} mol/l PTHrP-(1–36) on this activation (percentage inhibition: 89 ± 3 %). We next tested whether PTHrP-(1–36) would also interact with the effect of other known platelet agonists. We found that this peptide at 10^{-7} mol/l also increased the ability of thrombin, collagen and arachidonic acid to induce platelet activation (Figure 2).

PTH1R expression in human platelets

Western blot analysis of human platelet extracts revealed a single band with an apparent molecular mass of 90 kDa [24], consistent with the presence of PTH1R in these cells (Figure 3). This was confirmed further by flow cytometry analysis, showing that PTH1R immunoreactivity was
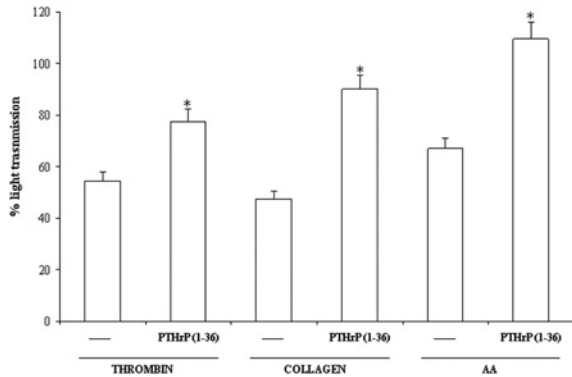
Figure 2 Effect of PTHrP-(1–36) (10^{-7} mol/l) on platelet activation induced by thrombin (0.02 unit/ml), collagen (2 μg/ml) and arachidonic acid (AA; 5×10^{-4} mol/l)

Values are means $\pm$ S.E.M. for three independent experiments. *$P < 0.05$ with respect to the corresponding value in the absence of PTHrP-(1–36).

present on the platelet surface (percentage of platelets expressing the PTH1R: $19.7 \pm 2.2\,\%$).

Intracellular pathways associated with the effect of PTHrP-(1–36) on human platelet activation

Additional studies were performed in order to initially characterize the mechanism involved in platelet activation by PTHrP-(1–36). We found that 10^{-6} mol/l H89 and

Table 1 Effect of PKC (BIM) and PKA (H89 and Rp-cAMPS) inhibitors on the enhancing ability of PTHrP-(1–36) on ADP-induced human platelet activation

Values are means $\pm$ S.E.M. for four different experiments. *$P < 0.05$ with respect to the corresponding value in the presence of ADP. $\dagger P < 0.05$ with respect to ADP + PTHrP-(1–36).

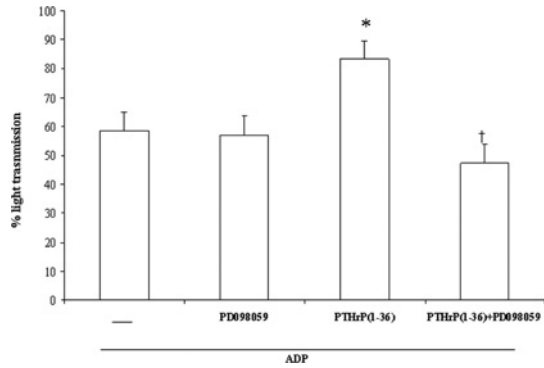| Inhibitor	Platelet aggregation (%)	
	ADP	ADP + PTHrP-(1–36)
–	59 ± 2	83 ± 3*
BIM	53 ± 2	70 ± 2*$\dagger$
H89	48 ± 3	72 ± 3*$\dagger$
Rp-cAMPS	58 ± 2	68 ± 2*$\dagger$

25×10^{-6} mol/l Rp-cAMPS, two PKA inhibitors [25,26], and also 25×10^{-9} mol/l BIM, a PKC inhibitor [27], partially decreased the promoting effect of PTHrP-(1–36) on ADP-induced platelet activation (Table 1). None of these inhibitors significantly modified ADP-induced platelet activation (Table 1).

PD098059 (10^{-5} mol/l), a specific inhibitor of the MAPK upstream of ERK1/2 [28], abolished the effect of PTHrP-(1–36) on ADP-induced platelet activation (Figure 4). In the absence of PTHrP, 10^{-5} mol/l PD098059 failed to significantly modify ADP-induced platelet activation (Figure 4). Moreover, 10^{-7} mol/l PTHrP-(1–36) rapidly ($\leqslant 15$ min) increased (2-fold compared with
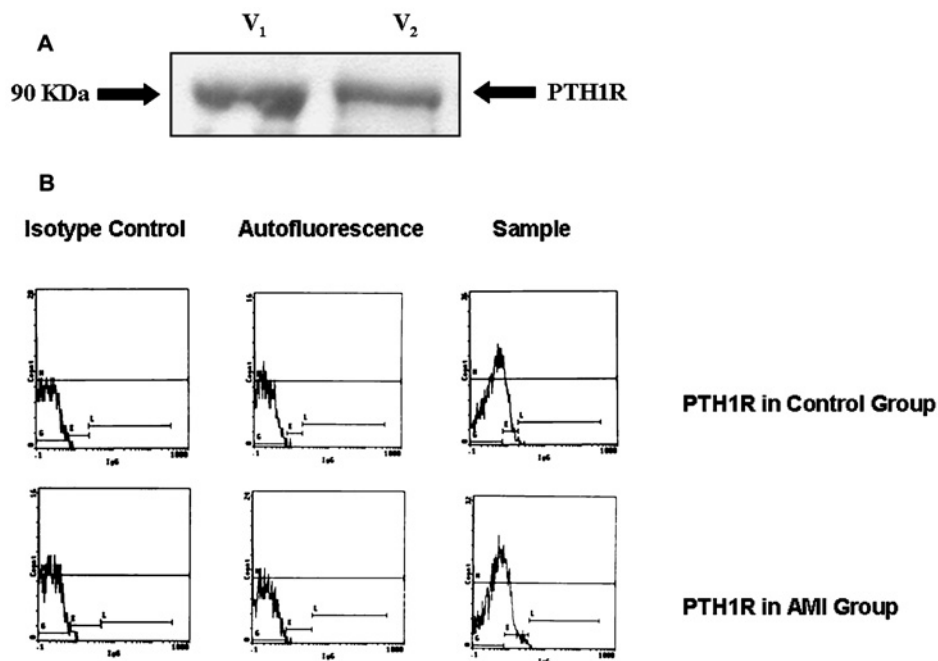

Figure 3 PTH1R expression in human platelets

(A) Representative autoradiogram showing PTH1R protein expression in human platelet extracts from two different normal subjects (V_1 and V_2) as measured by Western blotting (see text for details). (B) Flow cytometry analysis to detect PTH1R expression on the platelet surface obtained from patients during AMI and patients with similar cardiovascular risk factors without AMI. The upper panels of (B) show the autofluorescence of platelets used as a negative control which was substracted from the positive value of the samples. In addition, a non-specific mouse IgG$_1$-isotype antibody was also used as a negative control (B, left-hand side columns).

Figure 4   Abrogating effect of the MAPK inhibitor PD098059 (10^{-5} mol/l) on the pro-aggregating ability of PTHrP-(1–36) (10^{-7} mol/l) in ADP-stimulated human platelets

Values are means $\pm$ S.E.M. for three independent experiments. *$P < 0.05$, with respect to ADP alone. †$P < 0.05$, with respect to ADP + PTHrP-(1–36).
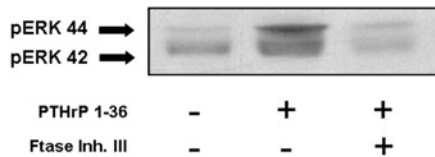
Figure 5   ERK1/2 activation by PTHrP-(1–36) (10^{-7} mol/l) and its blockade by the FTase inhibitor III (10^{-8} mol/l) by Western blotting

A representative autoradiogram of two experiments is shown. Protein loading was similar in each well, as assessed by Ponceau S staining (results not shown).

Table 2   Clinical features of AMI patients and patients with similar cardiovascular risk factors

ARAII, angiotensin II receptor antagonist.

	Control group ($n = 5$)	AMI group ($n = 6$)
Age (years)	61.8 ± 2.26	69 ± 5.1
Male/female ratio (n)	3/2	3/3
Hypertension (n)	1 (20 %)	3 (50 %)
Hypercholesterolaemia (n)	5 (100 %)	5 (83.3 %)
Tobacco smoking (n)	1 (20 %)	2 (33.3 %)
Diabetes mellitus (n)	1 (20 %)	2 (33.3 %)
Medication (n)		
ARAII	0 (0 %)	1(16,7 %)
Statins	1 (20 %)	2 (33.3 %)
β-Blockers	1 (20 %)	1 (16.7 %)
Aspirin	0 (0 %)	6 (100 %)

Figure 6   Total content of PTH1R in platelets from patients during AMI and patients in the absence of AMI (control group)

A representative Western blot is shown. Densitometric values are means $\pm$ S.E.M. *$P < 0.05$ with respect to the control group.

control) ERK1/2 phosphorylation in human platelets (Figure 5). No changes in total ERK1/2 expression in platelets was observed under the different experimental conditions (results not shown).

ERK activation by G-protein-coupled receptors may occur via Ras [29]. In the present study we found that FTase inhibitor III (at 10^{-8} mol/l), which inhibits protein prenylation and thus Ras activation [30,31], abrogated the effect of PTHrP-(1–36) on ERK1/2 phosphorylation in human platelets (Figure 5), as well as on ADP-induced platelet aggregation. Thus this aggregation in the presence of this PTHrP peptide (at 10^{-7} mol/l) plus the FTase inhibitor III was 62 ± 4 % (percentage light transmission), which was not significantly different than the corresponding value with ADP alone (percentage light transmission: 60 ± 3 %).
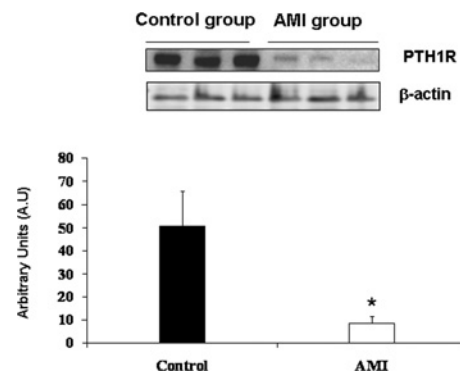
PTH1R expression in platelets from patients during an AMI

Table 2 shows the clinical features of both AMI patients and patients with similar cardiovascular risk factors without AMI (control group). The percentage of platelets from patients during AMI expressing PTH1R on their surface was 17.2 ± 2.8 %, which was not significantly different from that observed in platelets from the control

group (19.4 ± 2.6 %; $P =$ not significant). Figure 3(B) shows a representative flow cytometry analysis of PTH1R in platelets from an AMI patient. However, Western blot analysis demonstrated that the platelet content of PTH1R was significantly higher in platelets from the control group compared with that observed in the platelets from AMI patients (Figure 6). Moreover, dot-blot analysis demonstrated that plasma obtained from patients during AMI contained higher amounts of circulating PTH1R levels than those in plasma from patients in the control group (Figure 7).

DISCUSSION

PTHrP was initially isolated from hypercalcaemia-associated tumours. However, in the absence of malignancy, PTHrP acts as a local growth factor or cytokine in a broad variety of tissues [1,3]. A growing body of evidence suggests that PTHrP might be an important mediator
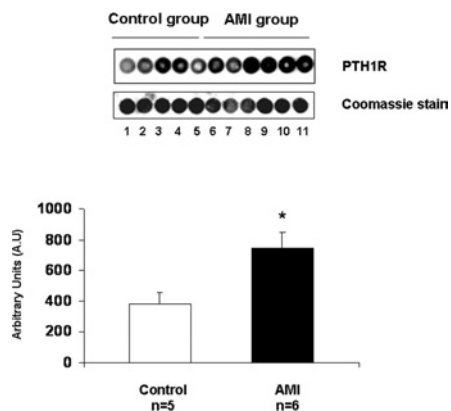
Figure 7   Presence of PTH1R protein in plasma from patients with AMI and patients in the absence of AMI (control group)

A representative dot blot is shown. Protein loading was similar in each well as assessed by Coomassie Blue staining. Corresponding densitometric values are means $\pm$ S.E.M. *$P < 0.05$ with respect to the control group.

of the inflammatory host response. In this setting, PTHrP would participate in a network of redundant pathways involving various cytokines and growth factors, e.g. interleukin-6 and angiotensin II, to promote inflammation and fibrosis (reviewed in [6]).

Inflammation is a key process associated with the instability of atherosclerotic lesions [32]. Following atheroma rupture, the formation of an occlusive thrombus is the major event related to myocardial infarction and unstable angina. This is accompanied by an inflammatory response which is responsible for most acute coronary syndromes. In fact, increased plasma levels of inflammatory markers have been detected in this scenario, and the degree of inflammation has been associated with the outcome of these patients [12,13,33,34].

Studies in human atherosclerotic lesions following atherectomy have shown an intense PTHrP staining in both macrophages and smooth muscle cells which seems to correlate with the severity of the lesion [10,11,35]. Moreover, we have demonstrated previously that PTHrP and monocyte chemoattractant factor-1 co-localize in these cells within the atherosclerotic plaque [11]. These findings suggest that PTHrP may act as a pro-inflammatory factor contributing to plaque breakdown and thrombus formation. The factors responsible for PTHrP overexpression in atherosclerosis are unknown. However, vasoactive factors such as thrombin and noradrenaline (norepinephrine), two agents up-regulated in this condition and well-known platelet activators, could induce PTHrP in the plaque neointima [36,37].

In the present study, we examined whether PTHrP might directly interact with platelet activation. PTHrP-(1–36) was found to significantly increase the effect of various known inducers of platelet aggregation in human platelets. Our present findings also indicate that this

effect is likely to occur through the PTH1R, on the basis of the following evidence. First, this receptor was detected, for the first time to our knowledge, in human platelets using Western blotting and flow cytometry. Secondly, this effect of PTHrP-(1–36) was similar to that of PTH-(1–34), which binds to the PTH1R with the same affinity [3]. Thirdly, the effect was abolished by PTHrP-(7–34), a PTH1R antagonist [23]. Moreover, our results suggest that the N-terminal region of PTHrP is essential in enhancing platelet activation, since PTHrP-(107–139), which does not interact with the PTH1R [22], was inefficient in enhancing platelet activation.

The finding that PTHrP-(1–36) not only enhanced the effect of ADP, but also that of other inducers of platelet activation suggests that this peptide might interact with an ADP-postreceptor mechanism in human platelets. PTH1R activation usually stimulates both cAMP/PKA and $[Ca^{2+}]_i$/PKC pathways through $G_{\alpha s}$ and $G_{\alpha q}$ respectively, in many target cells [3,38]. In the present study, the promoting effect of PTHrP-(1–36) on platelet activation was partially inhibited by either PKA or PKC inhibitors. This suggests that both signal transduction pathways can act independently as mediators of this effect of PTHrP-(1–36) in human platelets. Interaction with the PTH1R may also activate MAPK signalling in a Ras-dependent or -independent manner in different cell types [2,4,39]. Our present findings suggest the existence of a cross-talk between PTHrP-(1–36) activation of PKA and PKC and MAPK pathways in human platelets, since the enhancing effect of this peptide on ADP-inducing platelet activation was abrogated by a MAPK inhibitor. It is noteworthy that, in the absence of PTHrP-(1–36), this MAPK inhibitor did not affect ADP-induced platelet activation, in agreement with previous findings using platelet activators [40]. In accordance with the involvement of MAPK pathways in the PTHrP-dependent platelet activation, PTHrP-(1–36) rapidly activated ERK1/2 phosphorylation in human platelets. The present results also support the notion that Ras-dependent ERK activation is a key distal signalling pathway integrating the control of platelet activation by PTHrP-(1–36). It is of interest that this PTHrP domain has been shown to activate several G-protein-coupled pathways apparently converging in ERK activation to modulate osteoblast cell differentiation [2]. The true role of ERK activation in platelet activation is currently controversial [40,41]. However, this activation might induce Ca^{2+} influx [41], and thus be responsible, at least in part, for the pro-aggregating effect of PTHrP in human platelets. This hypothesis awaits further investigation.

As mentioned above, PTHrP is abundant in human atherosclerotic plaques [10,11]. Previous data [10,11] and the present data support the hypothesis that, after plaque rupture, human platelets would be exposed to PTHrP and thus might promote both the inflammatory reaction and platelet activation. The presence of PTH1R

in platelets, as demonstrated in the present study, might provide a new significance to the previously reported increase in serum PTH early after AMI [42]. It is unknown whether the previously identified PTH-specific receptor, PTH2R [19], could also be present in platelets. However, the present data suggest that in this pathological setting both PTH and PTHrP might act alone or in concert through the PTH1R to promote platelet aggregation. In the present study, we failed to find a significant alteration in the number of platelets expressing PTH1R on their surface in AMI patients compared with that in another group of cardiovascular patients without AMI, matched for age, gender and cardiovascular risk factors (used as controls). However, despite the fact that AMI patients were administered anti-platelet therapy (aspirin) before platelet isolation, the total amount of PTH1R in platelets from these patients were markedly reduced compared with those from controls. Therefore taken together these findings suggest that PTH1R may also be contained in the platelets, probably stored in the platelet granules, and when platelets are activated, as occurs during AMI, they may be rapidly released into the circulation. In this regard, platelets contain other receptors, such as CD40 ligand and P-selectin, which localize to the platelet surface upon activation, and are rapidly shed into the circulation [43–45]. Therefore in the present study we assessed whether such a mechanism might also apply to the PTH1R, which could thus be detected in plasma in AMI patients. The present results reveal, for the first time to our knowledge, the presence of this receptor in plasma. In addition, its levels in plasma were significantly increased during AMI. However, the source of the circulating PTH1R is presently unknown, since it might originate not only from platelets but also from other cells of the ruptured plaque environment. In addition, it did not disprove that platelets during AMI may interact with PTHrP in ruptured atherosclerotic plaques, which may favour the activation of the expressed PTH1R on the platelet surface enhancing platelet activation during the acute coronary syndrome. In any case, our finding is intriguing and raises the question of the putative functional role of the PTH1R in AMI as a marker of thrombosis or inflammation. In future studies it would be interesting to explore the possible relationship of the increase in PTH1R plasma levels with that of other soluble platelet receptors, namely CD40 ligand and P-selectin [43,46,47], in cardiovascular disease.

In conclusion, our findings demonstrate, for the first time to our knowledge, that human platelets express PTH1R. In addition, this receptor seems to mediate a pro-aggregating effect of both PTH and PTHrP through a mechanism involving ERK activation. Our findings provide new insight into the mechanisms of platelet activation, which may lead to the development of new pharmacological strategies for patients with acute coronary syndrome.

ACKNOWLEDGMENTS

REFERENCES

1   Philbrick, W. M., Wysolmerski, J. J., Galbraith, S. et al. (1996) Defining the roles of parathyroid hormone-related protein in normal physiology. Physiol. Rev. **76**, 127–173

2   Carpio, L., Gladu, J., Goltzman, D. and Rabbani, S. A. (2001) Induction of osteoblast differentiation indexes by PTHrP in MG-63 cells involves multiple signaling pathways. Am. J. Physiol. Endocrinol. Metab. **281**, E489–E499

3   Clemens, T. L., Cormier, S., Eichinger, A. et al. (2001) Parathyroid hormone-related protein and its receptors: nuclear functions and roles in the renal and cardiovascular systems, the placental trophoblasts and the pancreatic islets. Br. J. Phamacol. **134**, 1113–1136

4   Fortino, V., Torricelli, C., Gardi, C., Valacchi, G., Rossi Paccani, S. and Maioli, E. (2002) ERKs are the point of divergence of PKA and PKC activation by PTHrP in human skin fibroblasts. Cell. Mol. Life Sci. **59**, 2165–2171

5   Funk, J. L. (2001) A role for parathyroid hormone-related protein in the pathogenesis of inflammatory/autoimmune diseases. Int. Immunopharmacol. **1**, 1101–1121

6   Funk, J. L., Krul, E. J., Moser, A. H. et al. (1993) Endotoxin increases parathyroid hormone-related protein mRNA levels in mouse spleen. Mediation by tumor necrosis factor. J. Clin. Invest. **92**, 2546–2552

7   Funk, J. L., Moser, A. H., Grunfeld, C. and Feingold, K. R. (1997) Parathyroid hormone-related protein is induced in the adult liver during endotoxemia and stimulates the hepatic acute phase response. Endocrinology **138**, 2665–2673

8   Ortega, A., Rámila, D., Ardura, J. A. et al. (2006) Role of parathyroid hormone-related protein in tubulointerstitial apoptosis and fibrosis after folic acid-induced nephrotoxicity. J. Am. Soc. Nephrol. **17**, 1594–1603

9   Ortega, A., Rámila, D., Izquierdo, A. et al. (2005) Role of the renin–angiotensin system on the parathyroid hormone-related protein overexpression induced by nephrotoxic acute renal failure in the rat. J. Am. Soc. Nephrol. **16**, 939–949

10   Ishikawa, M., Akishita, M., Kozaki, K. et al. (2000) Expression of parathyroid hormone-related protein in human and experimental atherosclerotic lesions: functional role in arterial intimal thickening. Atherosclerosis **152**, 97–105

11   Martín-Ventura, J. L., Ortego, M., Esbrit, P., Hernández-Presa, M. A., Ortega, L. and Egido, J. (2003) Possible role of parathyroid hormone-related protein as a proinflammatory cytokine in atherosclerosis. Stroke **34**, 1783–1789

12   Gawaz, M. (2004) Role of platelet in coronary thrombosis and reperfusion of ischemic myocardium. Cardiovasc. Res. **61**, 498–511

13 Hughes, A., Daunt, S., Vass, G. and Wickes, J. (1982) *In vivo* platelet activation following myocardial infarction and acute coronary ischaemia. Thromb. Haemostasis **48**, 133–135

14 Fliser, D., Franek, E. and Fode, P. et al (1997) Subacute infusion of physiological doses of parathyroid hormone raises blood pressure in humans. Nephrol. Dial. Transplant. **12**, 933–938

15 Fardella, C. and Rodríguez-Portales, J. A. (1995) Intracellular calcium and blood pressure: comparison between primary hyperparathyroidism and essential hypertension. J. Endocrinol. Invest. **18**, 827–832

16 Poch, E., Fernández-Llama, P., Botey, A. et al. (1995) Parathyroid hormone and platelet cytosolic calcium concentration in essential hypertension. Nephrol. Dial. Transplant. **10**, 366–371

17 Benigni, A., Livio, M., Dodesini, P. et al. (1985) Inhibition of human platelet aggregation by parathyroid hormone: is cyclic AMP implicated? Am. J. Nephrol. **5**, 243–247

18 Kirichuk, V. F. and Aref'ev, F. G. (2001) Role of parathyroid hormone in regulating the functional activity of platelets. Tsitologiia **43**, 583–589

19 Usdin, T. B., Gruber, C. and Bonner, T. I. (1995) Identification and functional expression of a receptor selectively recognizing parathyroid hormone, the PTH2 receptor. J. Biol. Chem. **270**, 15455–15458

20 López-Farré, A., Caramelo, C., Esteban, A. et al. (1995) Effects of aspirin on platelet-neutrophil interactions. Role of nitric oxide and endothelin-1. Circulation **91**, 2080–2088

21 De Miguel, L. S., Jiménez, A., Monton, M. et al. (2000) A 4-trifluoromethyl derivative of salicylate, triflusal, stimulates nitric oxide production by human neutrophils: role in platelet function. Eur. J. Clin. Invest. **30**, 811–817

22 Valín, A., Guillén, C. and Esbrit, P. (2001) C-terminal parathyroid hormone-related protein (PTHrP) (107–139) stimulates intracellular Ca^{2+} through a receptor different from the type 1 PTH/PTHrP receptor in osteoblastic osteosarcoma UMR 106 cells. Endocrinology **142**, 2752–2759

23 Nutt, R. F., Caulfield, M. P., Levy, J. J., Gibbons, S. W., Rosenblatt, M. and McKee, R. L. (1990) Removal of partial agonism from parathyroid hormone (PTH)-related protein-(7–34)NH$_2$ by substitution of PTH amino acids at positions 10 and 11. Endocrinology **127**, 491–493

24 Izquierdo, A., López-Luna, P., Ortega, A. et al. (2006) The parathyroid hormone-related protein system and diabetic nephropathy outcome in mice with steptozotocin-induced diabetes. Kidney Int. **69**, 2171–2177

25 Chijiwa, T., Mishima, A., Hagiwara, M. et al. (1990) Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, N-{2-[(p-bromocinnamyl)-amino]ethyl}-5-isoquinolinesulfonamide dihydrochloride (H89), of PC12D pheocromocytoma cells. J. Biol. Chem. **265**, 5267–5272

26 Wang, L-Y., Salter, M. W. and MacDonald, J. F. (1992) Regulation of kainate receptors by cAMP-dependent protein kinase and phosphatase. Science **253**, 1132–1135

27 Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T. and Saltiel, A. R. (1995) PD098059 is a specific inhibitor of the activation of mitogen-activated kinase kinase *in vitro* and *in vivo*. J. Biol. Chem. **270**, 27489–27494

28 Toullec, D., Pianetti, P., Coste, H. et al. (1991) The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase. J. Biol. Chem **266**, 15771–15781

29 Luttrell, L. M., Daaka, Y. and Lefkowitz, R. J. (1999) Regulation of tyrosine kinase cascades by G-protein-coupled receptors. Curr. Opin. Cell Biol. **11**, 177–183

30 Nagasu, T., Yoshimatsu, K., Rowell, C., Lewis, M. D. and García, A. M. (1995) Inhibition of human tumor xenograft growth by treatment with the farnesyl transferase inhibitor B956. Cancer Res. **55**, 5310–5314

31 Qian, Y., Blaskovich, M. A., Saleem, M. et al. (1994) Design and structural requirements of potent peptidomimetic inhibitors of p21ras farnesyltransferase. J. Biol. Chem. **269**, 12410–12413

32 Ross, R. (1999) Atherosclerosis: an inflammatory disease. N. Engl. J. Med. **340**, 115–126

33 Eikelboom, J. W. and Anand, S. (2003) Antiplatelet agents in acute coronary syndromes. Semin. Vasc. Med. **3**, 403–414

34 Mateos-Cáceres, P. J., García-Méndez, A., López-Farré, A. et al. (2004) Proteomic analysis of plasma from patients during an acute coronary syndrome. J. Am. Coll. Cardiol. **44**, 1578–1583

35 Nakayama, T., Ohtsuru, A., Enomoto, H. et al. (1994) Coronary atherosclerotic smooth muscle cells overexpress human parathyroid hormone-related peptides. Biochem. Biophys. Res. Commun. **200**, 1028–1035

36 Hongo, T., Kupfer, J., Enomoto, H. et al. (1991) Abundant expression of parathyroid hormone-related protein in primary rat aortic smooth muscle cells accompanies serum-induced proliferation. J. Clin. Invest. **88**, 1841–1847

37 Pirola, C. J., Wang, H. M., Kamyar, A. et al. (1993) Angiotensin II regulates parathyroid hormone-related protein expression in cultured rat aortic smooth muscle cells through transcriptional and post-transcriptional mechanisms. J. Biol. Chem. **268**, 1987–1994

38 Mannstadt, M., Jüppner, H. and Gardella, T. J. (1999) Receptors for PTH and PTHrP: their biological importance and functional properties. Am. J. Physiol. **277**, F665–F675

39 Swarthout, J. T., Doggett, T. A., Lemker, J. L. and Partridge, N. C. (2001) Stimulation of extracellular signal-regulated kinases and proliferation in rat osteoblastic cells by parathyroid hormone is protein kinase C-dependent. J. Biol. Chem. **276**, 7586–7592

40 Borsch-Haubold, A. G., Kramer, R. M. and Watson, S. P. (1996) Inhibition of mitogen-activated protein kinase kinase does not impair primary activation of human platelets. J. Biol. Chem. **318**, 207–212

41 Rosado, J. and Stewart, S. O. (2001) Role of the ERK pathway in the activation of store-mediated calcium entry in human platelets. J. Biol. Chem. **276**, 15659–15665

42 Joborn, H., Hjemdahl, P., Larsson, P. T. et al. (1990) Platelet and plasma catecholamines in relation to plasma minerals and parathyroid hormone following acute myocardial infarction. Chest **97**, 1098–1105

43 Chiu, C. A., Wu, C. J., Yang, C. H. et al. (2005) Levels and value of soluble P-selectin following acute myocardial infarction: evaluating the link between soluble P-selectin levels and recruitment of circulating white blood cells and the marker for the rapid diagnosis of chest pain. Chang Gung Med. J. **28**, 699–707

44 Henn, V., Steinbach, S., Buchner, K., Presek, P. and Kroczek, R. A. (2001) The inflammatory action of CD40 ligand (CD154) expressed on activated human platelets is temporally limited by coexpressed CD40. Blood **98**, 1047–1057

45 Nannizzi-Alaimo, L., Alves, V. E. and Philips, D. R. (2003) Inhibitory effects of glycoprotein IIb/IIIa antagonists and aspirin on the release of soluble CD40 ligand during platelet stimulation. Circulation **107**, 1123–1128

46 Garlichs, C. D., Eskafi, S., Raaz, D. et al. (2001) Patients with acute coronary syndromes express enhanced CD40 ligand/CD154 on platelets. Heart **86**, 649–655

47 Solheim, S., Pettersen, A. A., Arnesen, H. and Seljeflot, I. (2006) No difference in the effects of clopidogrel and aspirin on inflammatory markers in patients with coronary heart disease. Thromb. Haemostasis **96**, 660–664