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Molecular and biochemical evidence for the presence of Type III adenylyl cyclase in human platelets

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The isoform(s) of adenylyl cyclase (AC) present in human platelets has not been identified, and evidence supporting a role for AC in platelet aggregation is equivocal. We recently characterized deaggregation as an active component of the platelet aggregation response that may be an important determinant of the extent and duration of aggregation. G_i-coupled receptors are linked to the inhibition of AC and are targets of antiplatelet drugs. They also affect platelet aggregation by modulating deaggregation, suggesting a role for AC in modulating this response. The purpose of this study was to identify the AC isoform(s) present in human platelets and to identify its physiological modulators. RT-PCR screening of platelet, buffy coat layer cell and bone marrow megakaryocyte cDNA, and Western blot analysis with AC type III (AC-III) antibodies identified AC-III in platelets and in megakaryocytes. Human platelet AC-III was cloned and expressed in HEK293 cells and its characteristics compared to native platelet AC. Both platelet AC and cloned AC-III required Mg^{2+} for activity, were insensitive to Ca^{2+} and were G_s- and G_i-coupled. Zn^{2+} and SQ22536 inhibited platelet AC activity. The affinity of SQ22536 was increased with Mg^{2+}-related stimulation of AC, while that of Zn^{2+} was unchanged, which is consistent with a non-competitive interaction between the two metal ions on AC. The Zn^{2+} chelator TPEN reversed the inhibitory effects of Zn^{2+}. This study identified AC-III as the predominant AC isoform in human platelets, the activity of which may affect the extent and duration of the net aggregation response by modulating deaggregation.

Introduction

The importance of G-protein coupled receptors activated by so-called "weak agonists" in mediating various platelet responses is well documented. Activation of G_q-coupled receptors linked to phospholipase C activation and the release of intracellular Ca^{2+},[1,2] mediate the platelet shape change response and are required but not sufficient to elicit sustained aggregation.[3,4] Similarly, activation of G_i-coupled receptors without G_q-coupled receptor activation is also insufficient to elicit sustained platelet aggregation.[3,5,6] Simultaneous activation of both G_i- and G_q-coupled receptors appears to be necessary to produce sustained aggregation.[7,8] We recently proposed that the activation of G_i-coupled $P2Y_{12}$ and α_{2A} receptors releases a tonic state of inhibited platelet aggregation through the deceleration of an active deaggregation mechanism.[9–11] We demonstrated that blockade of the G_i-coupled $P2Y_{12}$ purinergic receptor by the clinically efficacious anti-platelet agents ticlopidine and clopidogrel[12,13] accelerate deaggregation.[10] Agents that increase AC activity/cAMP content inhibit platelet aggregation[14–17] and can also be classified as potent accelerators of deaggregation.[18] We hypothesized that a tonic state of active deaggregation is related to constitutive AC activity. This suggests a bi-directional role for AC activity in the modulation of platelet aggregation through its effects on deaggregation.

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In order to better understand the potential role of AC in modulating these platelet responses, we sought to identify the isoform(s) present and to characterize the physiological modulation of platelet AC. The different isoforms of AC are differentially modulated by membrane receptors, intermediate metabolites and metal ions including Ca^{2+}, Mg^{2+} and Mn^{2+}. To date, nine transmembrane isoforms of AC have been cloned (see Ref. 19–21). The isoform(s) present in platelets, however, has not been identified and cloning of the cDNA that codes for the AC isoform(s) found in platelets has not been reported. Since platelets are produced by fragmentation of the cytoplasm of bone marrow megakaryocytes, newly released, reticulated platelets contain residual megakaryocyte mRNA that can be reverse-transcribed and PCR-amplified for identification and analysis.[22,23]

We show here that AC-III is the principal isoform of AC expressed in human platelets. Based on pharmacological studies, platelet AC activity is linked to both G_s- and G_i-coupled receptors and is not Ca^{2+}/calmodulin-dependent.[24] Our characterization of platelet AC and that of the AC-III overexpressed in HEK293 cells is consistent with that previously reported for platelet AC and for the AC-III isoform. Zn^{2+} ions inhibited platelet AC and the IC_{50} of Zn^{2+} was unchanged in the presence of Mg^{2+}-related stimulation of AC, demonstrating their non-competitive behavior and implying the importance of these two metal ions in physiological regulation of platelet AC activity.

Materials and methods

Materials

The following chemicals were obtained from the suppliers indicated: epinephrine (EPI), forskolin (FSK), isoproterenol (ISO), pyrithione (PYR), sphingosine 1-phosphate (SphP), 9(tetrahydro-2-furyl) adenine (SQ22536) and *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN) from Sigma (St. Louis, MO); lepirudin (Refludan®) from Hoechst Marion Roussel (Kansas City, MO). Iloprost (ILO) was graciously provided by Schering Aktiengesellschaft (Berlin, Germany). All other chemicals were of the highest grade available.

Cell isolation, RNA extraction and first strand cDNA synthesis

With Institutional Review Board approval and after obtaining informed consent, human platelet rich plasma (PRP) was prepared from freshly collected blood anticoagulated with lepirudin.[11] The platelet pellets were prepared from the upper 2 ml of the PRP by centrifugation at $3000 \times g$ for 5 min at 4°C, quickly washed once with PBS (Gibco BRL, Gaithersburg, MD) under the same conditions and used for total RNA isolation. The buffy coat fraction (about 0.5 ml), a control for leukocyte RNA contamination, was centrifuged and the pellet washed as described above and used for total RNA isolation.

Human megakaryocytes were isolated from excess bone marrow aspirate obtained from a leukemia patient undergoing chemotherapy. The megakaryocyte fraction was isolated by isopycnic separation ($750 \times g$ at 4°C) using an Accudenz gradient (1.5; Accurate Chemical and Scientific Corp., Westbury, NY). Isolated cells were washed twice in PBS as described above, and used for total RNA isolation. Total RNA from all fractions was isolated using TriPure Reagent (Roche Molecular Biochemicals, Indianapolis, IN). Contaminant DNA in total RNA was digested with RQ1 RNase-free DNase (Promega Corp., Madison, WI) for 30 min at 37°C. Reverse transcription was carried out as described previously[25] using oligo dT primers and the SuperScript Preamplification System (Gibco BRL).

PCR amplification and cloning

cDNA (1 μl) from all sources was mixed with pairs of oligonucleotide primers (10 μM; Table 1) corresponding to different isoforms of AC, and 45 μl of PCR Super Mix (Gibco BRL) in a 50 μl final volume. PCR was carried out using a Perkin-Elmer Gene Amp PCR System 2400 thermocycler (Perkin-Elmer, Foster City, CA). There were 30 amplification cycles, each including denaturation at 95°C for 10 s, primer annealing at 61°C for 30 s (except for AC-VII, primer annealing at 57°C) and extension at 72°C for 1 min. The reaction mixture was electrophoresed in a 6% polyacrylamide gel and stained with ethidium bromide. Purified PCR-generated fragments were cloned into the multiple cloning site of the pNoTA/T7 vector using the Prime PCR Cloner kit (Eppendorf – 5 Prime, Boulder, CO). Plasmid DNA containing cloned PCR fragments was isolated using the QIAGEN Plasmid Kit (QIAGEN, Valencia, CA) and sequenced from a T7 promoter to confirm AC type authenticity (ABI Prism, ABI50 @ Biotechnology Center, Utah State University, UT). Sequence analysis was carried out using MacVector Software (v.6.5.3, Genetics Computer Group, Inc., Madison, WI)

A complete coding sequence of AC-III[26] was PCR amplified with HF AdvanTaq 2 (High Fidelity) DNA polymerase (Clontech, Palo Alto, CA) from a human platelet cDNA library (gracious gift of Dr. Roth, VAMC, Seattle, WA) with primers (5′-CATGAATTCGAAGCAGACACCAGCCAGTATG-3′ and 5′-TACTCGAGATAGGAGTTGTCCACCACCTG-3′) corresponding to flanking regions of AC-III and containing EcoR I and Xho I sites for subsequent cloning. Amplification of the complete coding sequence was performed using three-step Touchdown PCR. The first two steps included five amplification

Table 1. Oligonucleotide primer sequences used to identify the different isoforms of adenylyl cyclase

AC Isoform	Sequence (5′-...-3′)	Size of product bp/(position)	Origin	NCBI GenBank Accession #	Reference
I	TCCAGAGGCACGACAATGTGAG CTCGTAGTCCCCATTCAAGCAC	473 (49–521)	human	L05500	81
II	CAAAAAAGAGCGGGAGGAGATAG CATGTATGTGCTGCCAATGGTC	325 (669–993)	human	L21993	82
III	TACTCGGTGGAGAAGGAGAAGCAG CGAAAACGCTTGTGGTCGTATTC	554 (2017–2570)	human	AF033861	26
IV	ACTCGTTATCTGGAGTCTTGGGG TTCGTCAGGTGCTCTGAGAAGC	467 (1544–2010)	rat	M80633	54
V	GTGGGGAAACAGAATACTCTGGC GCTGAGGAATTGAAAGTCAGGTTC	575 (169–743)	human	M83533	83
VI	AGGATGAGGTGGACGAGTTTCTGG CATGTTGGCAATGGCAGAGATG	422 (2054–2475)	rat	L01115	84
VII	TGGGTCCATCTTCCTGGAGAAG AGACAGAGCAAGCGATGAAGGC	491 (2295–2785)	bovine	Z49806	85
VIII	CATACCCAGACTAGCGGTCTTTTC CAACACAGCAATGAGCATGGTC	524 (1694-2217)	rat	L26986	86
IX	GAAAACGGACGCCCACTTTG TCCTCCAGGAAGAACACCATCC	375 (2182–2556)	human	AF036927	87
Cytosolic	CTTCCTCTGTGTCTTCGGTTTCC TAGTCGATCTCCCTAACACGACCC	456 (1335–1790)	rat	AF081941	88

cycles each: denaturing at 95°C for 5 s, followed by primer annealing and extension at 68°C for 5 min (step 1) or 66°C for 5 min (step 2). The final step included 31 amplification cycles, which included denaturation at 95°C for 5 s, primer annealing and extension at 64°C for 5 min, and final extension for 10 min at 64°C. A 4149 bp DNA fragment was EcoR I and Xho I digested and ligated in the correct orientation into the pcDNA4/TO/*myc*-His/*lacZ* expression vector (T-Rex system, Invitrogen, San Diego, CA).

Expression of AC-III in HEK (T-Rex) 293 cells

Blasticidin-resistant T-Rex-HEK293 cells expressing the tetracycline repressor protein (T-Rex system) were cultured in DMEM supplemented with 10% fetal calf serum (Gibco BRL) in a humidified 95% air, 5% CO_2 incubator at 37°C. Stably transfected AC-III T-Rex 293 cells were obtained after transfection with 10 μg of the pcACIII/TO/*myc*-His expression vector using cationic liposomes (DC-Chol/DOPE) as described previously.[27] Cell selection was carried out in DMEM containing both blasticidin and zeocin (5 and 200 μg/ml, respectively; Invitrogen). Fresh media containing tetracycline (1 μg/ml) was added to induce AC-III expression 24 h prior to harvesting cells for assay. Blasticidin/zeocin-resistant cells were assayed for AC-III expression and total AC activity.

Immunoblotting

Human PRP (3 ml) was centrifuged at $800 \times g$ for 15 min at 4°C. The pellet was washed in PBS and platelets were lysed in 40 μl of cell culture lysis buffer (Promega). The lysate (30 μg) was electrophoresed on a 7.5% SDS-PAGE gel and transferred to a nitrocellulose membrane (Protran, Schleicher & Schuell, Keene, NH) by electroblotting at 100 V for 1 h at 4°C. Blocked blots were incubated overnight at 4°C with anti-AC-III or anti-AC-V/VI antibodies (1 : 1000, v/v in TBS; both from Santa Cruz Biotechnology, Santa Cruz, CA). The bands were visualized using biotinylated alkaline phosphatase according to manufacturers instructions (Alkaline Phosphatase Conjugate Substrate Kit; Bio-Rad, Hercules, CA). Nonspecific binding was assessed from blots incubated with anti-AC-III antibody that was previously incubated with blocking peptide-antigen (Santa Cruz Biotechnology).

Assay of adenylyl cyclase activity

AC activity was measured in membranes prepared from expired human platelet concentrates (within one day of expiration for clinical use) obtained from the Blood Bank (Mount Sinai Medical Center, New York, NY). Preliminary experiments confirmed that the Michaelis-Menten kinetics of AC activity in outdated platelet membrane preparations were comparable to those determined in platelets from freshly drawn blood. The platelet pellet or HEK293 cells were obtained by centrifugation at $39\,000 \times g$ for 10 min at room temperature and then resuspended in homogenization buffer (one-half the volume of the discarded supernatant) containing: 2 mM EGTA, 1 mM DTT, 1 mM $Mg(CH_3COO)_2$, and 12 mM HEPES-NaOH (pH = 7.4 at room temperature). Pellets were homogenized using a polytron (2×15 s, setting #6; Brinkmann, Westbury, NY) and kept frozen (−20°C) until used. Thawed membranes were

suspended in 500 µl of homogenization buffer, homogenized for 20 s (Omni µH; Omni International, Inc., Warrenton, VA) and diluted to 1.5 ml with the same buffer.

Aliquots (50 µl) of the membrane suspensions were added to 200 µl of AC assay medium containing (final concentration): 100 mM NaCl, 10 µM GTP, 2 mM cAMP, 5 mM phosphocreatine, 4 mM theophylline, 2 mM $Mg(CH_3COO)_2$, 0.2 mM ATP, 10 µg of creatine phosphokinase and 75 mM Tris–HCl (pH 7.4). In experiments deriving CRCs for ATP, ATP was added separately to the assay media. Modulators of AC activity were added to the media prior to initiation of the assay. Following pre-incubation for 5 min at 30°C, the assay was initiated by the addition of 25 µl (2–3 µCi) of [^{32}P]α-ATP (specific activity 10–30 Ci/mmol; New England Nuclear, Boston, MA). After 10 min, 150 µl of 50 mM citric acid was added to stop the reaction. AC activity was determined by measuring the conversion of [^{32}P]ATP to [^{32}P]cAMP as described by Salomon.[28] Values for cAMP were corrected for recovery determined from [^{3}H]cAMP (35 000 cpm, New England Nuclear) added to the stopped reaction and counted using a dual label scintillation counting technique. Protein concentration was determined by the method of Bradford[29] using bovine serum albumin as the standard. Enzyme kinetics were estimated by fitting a three-parameter Michaelis-Menten equation to the data.

Measurement of Platelet cAMP Accumulation

PRP was prepared from blood containing 3.8% sodium citrate and 50 U/ml heparin for anticoagulation. PRP containing 10 nM ILO (to prevent aggregation during sedimentation) was centrifuged ($950 \times g$, 7 min, room temperature) and the platelet pellet resuspended in an equal volume of modified Tyrode's buffer (1 mM $CaCl_2$ and 1 mM $MgCl_2$, final concentrations). Platelet counts were measured using a hemocytometer. Washed platelets were pre-incubated (37°C, 3 min) with 10 µM milrinone and, when used, 175 µM SQ22536 or 2 mM $ZnCl_2$ (in the presence of the Zn^{2+} ionophore PYR, 5 µM). Platelets were then incubated for 5 min with ILO (10 nM), FSK (1 µM) or vehicle (saline). Experiments were terminated and platelets lysed by removal of 200 µl samples into 0.2 N HCl. The samples were neutralized with 0.2 M $NaCH_3COO$ and centrifuged for 10 min at $3000 \times g$. The supernatant was transferred to 12×75 mm culture tubes and total cAMP was measured in duplicate by RIA.[30]

Statistics

The goodness of fit of the three-parameter Michaelis-Menten equation by non-linear regression were assessed from cumulative frequency plots of the residuals. Treatment effects were assessed using one way repeated measures analysis of variance (ANOVA). The data were natural log transformed to satisfy the assumptions of the analysis (ANOVA) and all hypothesis tests were conducted at the $P < 0.05$ level of significance. Dunnett's test was used to control the type I experiment-wise error rate for the comparisons of multiple treatment groups against the control group.

Results

Molecular identification of AC-III in platelets

RT-PCR detection of AC isoenzymes The oligonucleotide primers designed for the study detected all 10 known isoforms of AC using cDNA from human duodenum and HEK293 cells, as well as from rat brain, lung and heart. RT-PCR with β-actin primers[31] readily detected residual mRNA in human platelets and was used to screen for the presence of the ten known isoenzymes of AC in cDNA panels from human platelets, buffy coat fraction cells and bone marrow megakaryocytes. A signal for AC-III mRNA was found in platelet (Figure 1) but not buffy coat cDNA. A very faint signal for AC-VI was detected in both preparations. AC-I was also detected in the buffy coat, but not in platelets or megakaryocytes. In megakaryocytes a 554 bp band corresponding to AC-III, and two other bands (325 and 375 bp) corresponding to AC-II and AC-IX, respectively, were detected. Potential contamination of total RNA by genomic DNA was assessed by PCR amplification of human genomic DNA. No bands of the predicted sizes for any of the ten known isoforms of AC were detected by PCR.

The sequence authenticity of the amplified DNA fragments corresponding to the AC isoforms was confirmed by restriction analysis and sequencing (data not presented). To confirm identity with AC-III, the 554 bp DNA fragment from platelets was subcloned and sequenced. Four randomly selected clones demonstrated highest homology with the corresponding region of human AC-III.[26] Subsequently, a complete coding sequence (4142 bp) of AC-III was cloned from a human platelet cDNA library and confirmed by sequencing with multiple primers (data not presented).

AC-III immunoreactivity in human platelets To confirm the expression of AC types in platelets, membranes were probed with antibodies specific for AC-III and AC-V/VI. Western Blot analysis with the AC-III specific antibody detected a polypeptide with a molecular mass of $\sim$140 kDa in human platelet membranes (Figure 2(A)). This corresponds with the molecular mass reported for deglycosylated rat AC-III,[32] which has 95% amino

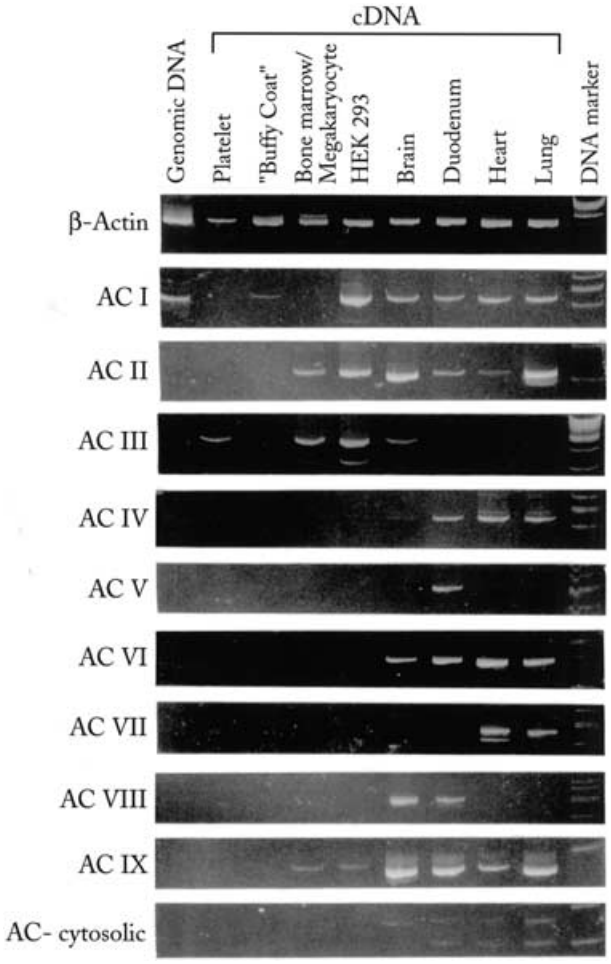

Figure 1. RT-PCR screening of human platelet AC isoforms. The AC isoforms present in human platelets were identified by RT-PCR screening of cDNA derived from human platelet mRNA. The amplified cDNA was separated by PAGE (6%) and visualized with ethidium bromide. A strong signal for AC-III and a faint signal for AC-VI were detected in platelets. Additional human cells and tissues confirmed the ability of the specific oligonucleotide primers (Table 1) to identify all known isoforms of AC. To monitor potential contamination of platelet preparations by genomic DNA and mRNA from the white blood cells, samples of human genomic DNA and cDNA from the buffy coat layer were also included.

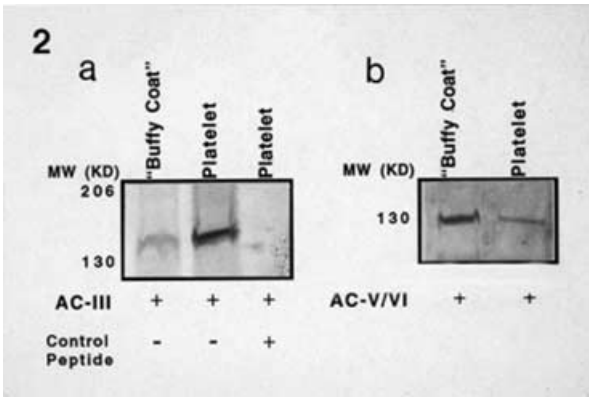

Figure 2. Immunoreactivity of human platelet proteins with antibodies to AC types III and V/VI. Proteins from platelet lysates were separated by SDS-PAGE (7.5%) and transferred to nitrocellulose by electroblotting. The proteins were probed with polyclonal antibodies specific for AC-III or AC-V/VI. Non-specific binding was assessed using antibodies pre-incubated with specific blocking antigens. **A**. A ~ 140 kDa polypeptide immunoreactive to AC-III antibodies was detected. **B.** A ~ 130 kDa polypeptide immunoreactive to AC-V/VI antibodies was also detected.

acid sequence homology with human AC-III.[26] Pre-adsorption of the antibody with blocking peptide-antigen abolished the immunostaining of this band. *N*-glycosidase *F* treatment of platelet proteins did not affect the size of the polypeptide immunoadsorbed by the AC-III antibody (data not presented). Western Blot analysis of platelet and buffy coat cell preparations immunoadsorbed with antibody selective for AC-V/VI detected a polypeptide with a molecular mass of ~130 kDa in both preparations (Figure 2(B)). The signal from the buffy coat cells was greater than that from platelets.

Pharmacology of platelet AC

The pharmacology and physiological modulation of AC in membrane preparations from parental HEK293 cells, stably transfected HEK (ACIII-HEK) cells and platelets were characterized. Parental HEK293 cells exhibited lower AC activity (V_{max}, Table 2) and were included for comparison. Confirming previous reports and consistent with the known structure of AC-III, platelet membrane AC-III required Mg^{2+} for activity[33–35] and was unaffected by Ca^{2+} (0.1–3 μM).[24,36]

G_s-coupling and FSK stimulation of AC-III The effects of FSK and activation of known G_s-coupled receptors on the Michaelis–Menten enzyme kinetics of AC activity were compared in membrane preparations from parental HEK293 cells, stably-transfected ACIII-HEK cells and outdated human platelet concentrates (Figure 3, Table 2). Basal AC activity was higher in ACIII-HEK than in the parental cell membranes, consistent with functional expression of the cloned AC-III. The K_m values and the slope index for ATP of basal AC activity were similar in all three preparations. Preliminary experiments showed that ISO, through the G_s-coupled β-adrenoceptor, did not elicit an increase in AC activity in platelets and ILO, through the G_s-coupled PGI_2 receptor, had no effect on AC activity in HEK cells, therefore, we activated different G_s-coupled receptors in the platelet and HEK cell preparations. ILO increased the V_{max} value in platelets over basal, confirming previous reports.[16,17] Similar to platelets, AC activity was increased by ISO (increase in the V_{max} values over basal) in both parental and ACIII-HEK cell preparations. The increase in the V_{max} value in ACIII-HEK membranes was more than double that of the parental HEK membranes, confirming the G_s-coupling of the cloned AC-III. The direct AC activator FSK also

Table 2. Comparison of kinetic parameters of basal and stimulated (FSK and ILO) AC activity from platelet, HEK cell and stably transfected AC-III-HEK cell membrane preparations. Membranes were prepared from human platelets and HEK cells and AC activity was measured as described in Methods. Membranes were incubated for 10 min with vehicle (basal), FSK (10 μM) or ILO (100 nM) or ISO (1.0 μM), and with varying concentrations of ATP. Kinetic parameters of AC activity (V_{max} (pmol/min × mg); K_m (μM)) were determined by fitting a three-parameter Michaelis–Menten equation to the data. Results are presented as mean values (± S.D.; N = 3).

	PLATELET			HEK			ACIII-HEK		
Treatment	V_{max}	K_m (μM)	Slope	V_{max}	K_m (μM)	Slope	V_{max}	K_m (μM)	Slope
Basal	95 ± 5	44 ± 16	1.04 ± 0.13	21 ± 9	60 ± 36	0.96 ± 0.14	41 ± 2	72 ± 68	1.14 ± 0.52
FSK	402 ± 352	228 ± 40	0.98 ± 0.12	414 ± 263	145 ± 31	1.13 ± 0.12	673 ± 400	194 ± 213	1.21 ± 0.33
ISO	*	*	*	35 ± 14	59 ± 17	0.94 ± 0.11	80 ± 49	46 ± 25	1.32 ± 0.32
ILO	416 ± 176	159 ± 16	0.83 ± 0.03	*	*	*	*	*	*

* Not assessed (see Results).

increased AC activity in all three preparations (V_{max}, Table 2). In platelet membranes, FSK induced a 4-fold increase in AC activity over basal. FSK induced a greater increase in the V_{max} value for ATP in ACIII-HEK than in parental cell membrane preparations. K_m values for ATP were increased by FSK-stimulation in all three preparations, though the increase was greater in platelet than in HEK or ACIII-HEK cell preparations.

G_i-coupling of AC-III G_i-coupled receptor activation inhibits stimulated AC activity in human platelets.[14,37–39] We confirmed the ability to inhibit platelet AC activity through the G_i-coupled α_2-adrenoceptor[14,40] (29% inhibition of ILO-stimulated platelet AC activity by 10 μM EPI). The sphingolipid SphP interacts with a high affinity G_i-coupled receptor in the plasma membrane of HEK cells.[41] SphP reduced the V_{max} values for ATP of FSK-stimulated AC activity by 19% and 15% in membrane preparations from HEK cells and ACIII-HEK cells, respectively. A slight inhibition of basal AC activity was also detected in these preparations. This confirmed the G_i-receptor coupling of the cloned AC-III.

Inhibition of stimulated platelet AC activity by Zn^{2+} and the P-site inhibitor SQ22536 The structure of the AC-III also suggested that it might be affected by Zn^{2+}.[35] The inhibition by Zn^{2+} and the P-site inhibitor SQ22536, therefore were compared on G_s-receptor (PGI_2 by ILO) and directly (FSK) stimulated AC activity. ILO and FSK increased AC activity in a saturable manner with EC_{50} values of 7 nM and 8 μM, respectively, with slope indices not different from unity. Mg^{2+} was required for stimulation by either ILO or FSK. Increasing Mg^{2+} concentrations up to 6 mM produced a concentration-dependent, but apparently non-saturable increase in ILO- (Table 3) and FSK- (not shown) stimulated AC activity.

Zn^{2+} inhibited the Mg^{2+}-related potentiation of AC activity stimulated by either ILO (Figure 4(A), Table 3) or FSK (data not presented) in a concentration-dependent manner. Characteristic of non-competitive behavior, the estimated IC_{50} value for Zn^{2+} (145 ± 20 μM) was independent of Mg^{2+}, ILO or FSK concentrations. SQ22536 also inhibited the Mg^{2+}-related potentiation of ILO- (Figure 4(B)) and FSK- (data not presented) stimulated AC activity in a concentration-dependent and saturable manner. In contrast to the effects of Zn^{2+}, the estimated IC_{50} value for SQ22536 decreased 6 times with increased Mg^{2+}-related stimulation of AC (from 82.2 ± 5.1 μM at 1.5 mM Mg^{2+} to 13.1 ± 6.9 μM at 6 mM Mg^{2+}) (Figure 4(B) and (C), Table 3). This increase of the affinity of SQ22536 for AC-III is consistent with results reported for other AC types in which increased potency of P-site inhibitors was observed with increased enzyme activity.[42,43] Inhibition of AC activity by SQ22536 was saturable at the level of 90% inhibition in contrast to the apparent non-competitive behavior between Zn^{2+} and Mg^{2+}. Zn^{2+}, at concentrations above 0.6 mM, completely inhibited ILO- (or FSK-) Mg^{2+} stimulated AC activity. As shown in Figure 5, the inhibitory modulation of platelet AC by Zn^{2+} could be reversed by the Zn^{2+} chelator-TPEN (1 mM), when added after Zn^{2+} addition (400 μM). When added before, it completely prevented the inhibitory effect of Zn^{2+} on platelet AC activity. TPEN did not affect the activation of AC by Mg^{2+} (1 mM) or ILO (10 nM).

The physiological relevance of these observations on platelet membrane AC was assessed by determining the effects of inhibitors and activators of AC on cAMP accumulation (in the presence of the type III phosphodiesterase inhibitor milrinone[44]) in intact washed platelets (Figure 6). Confirming previously reported results,[14,16,45] ILO and FSK induced a more than 3- and 5-fold (respectively) increase in total cAMP accumulation. These increases in cAMP accumulation were inhibited (2-fold) by SQ22536. The same degree of inhibition by Zn^{2+} was noted in FSK- but not in ILO-stimulated cAMP accumulation.

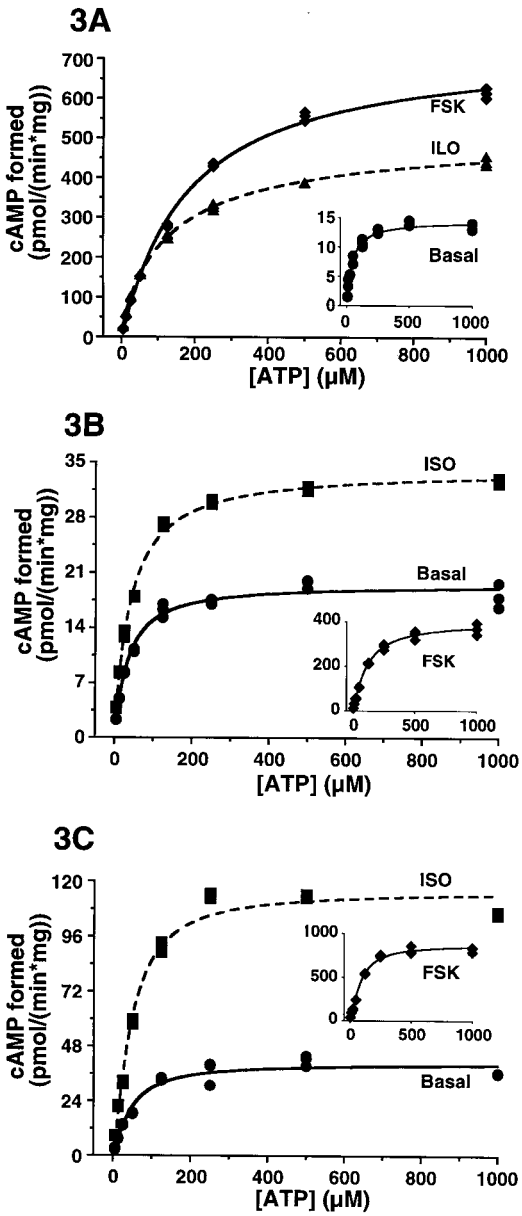

Figure 3. Comparison of the AC enzyme kinetics from three cell preparations. AC activity in membrane preparations was determined from the conversion of [^{32}P]αATP to [^{32}P]cAMP and the kinetic parameters calculated as described in Methods. Membranes were incubated for 10 min with vehicle (basal, ●), 10 μM FSK (◆), 100 nM ILO (▲) or 1.0 μM ISO (■). Representative data from a single experiment are shown ($N = 3$). AC activity was determined in triplicate at each ATP concentration. The V_{max} (pmol/(min × mg)), K_m (μM) and slope index for ATP, respectively were (Mean ± SE):**A.** Platelets: Basal, 15 ± 0.47, 43 ± 4.5, 0.99 ± 0.01; +FSK, 735 ± 19, 183 ± 12, 1.04 ± 0.004; +ILO, 605 ± 10, 142 ± 7.5, 0.84 ± 0.002; **B.** HEK cells: Basal, 20 ± 0.49, 34 ± 2.9, 1.09 ± 0.08; +FSK, 396 ± 11, 110 ± 7.6, 1.2 ± 0.06; +ISO, 34 ± 0.42, 40 ± 1.7, 1.02 ± 0.04; **C.** stably transfected ACIII-HEK cells: Basal, 40 ± 1.6, 46 ± 5.5, 1.3 ± 0.16; +FSK, 865 ± 20, 86 ± 5.5, 1.4 ± 94; +ISO, 116 ± 2.8, 46 ± 3.5, 1.4 ± 0.11.

Table 3. Zn^{2+} and SQ22536 inhibition of ILO-stimulated AC activity from human platelet membrane preparations. Membranes were prepared from human platelets and AC activity was measured as described in Methods. Membranes were incubated for 10 min with vehicle (basal), ILO (10 nM) and with the indicated concentrations of Mg^{2+} using 200 μM ATP as the substrate. AC activities (V (pmol/min × mg)) are shown in the absence of Zn^{2+} or SQ22536 for each of the indicated Mg^{2+} concentrations. The effect of increasing Zn^{2+} (0.05–1.0 mM) or SQ22536 (0.3–1000 μM) were assessed for each of the indicated Mg^{2+} concentrations. The IC_{50} concentration of the effect of Zn^{2+} or SQ22536 was determined by fitting a three-parameter Michaelis–Menten equation to the data (see Figure 4). Results are presented as mean values (± S.D.; N = 6 for Zn^{2+} and N = 5 for SQ22536).

$[Mg^{2+}]$ (mM)	V (pmol/min*mg)	$[Zn^{2+}]$ IC_{50} (μM)	V (pmol/min*mg)	[SQ22536]IC_{50} (μM)
1/1.5	31.7 ± 9.1	164.4 ± 9.6	36.7 ± 4.1	82.2 ± 5.1
2/2.5	57.7 ± 27.2	146.6 ± 7.3	74.3 ± 23.3	38.7 ± 18.4
3/3.5	70.1 ± 23.5	155.8 ± 5.7	84.1 ± 16.7	38.8 ± 0.1
4/5	85.7 ± 11.2	148.1 ± 4.7	105.7 ± 20.1	23.3 ± 0.01
6	135.2 ± 61.7	112 ± 10.8	146.8 ± 53.5	13.1 ± 6.9

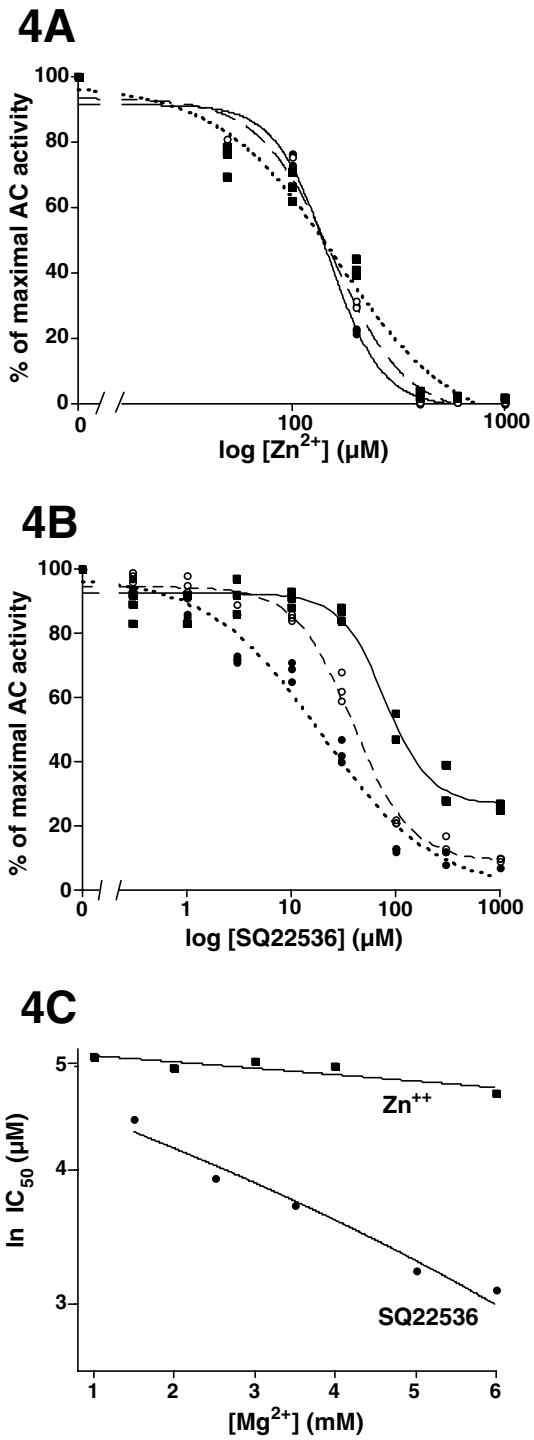

Figure 4. Inhibition of ILO-stimulated platelet AC activity by Zn^{2+} and SQ22536. The inhibitory effects of Zn^{2+} and SQ22536 on ILO-stimulated platelet membrane AC activity were characterized in the presence of varying Mg^{2+} concentrations. Platelet membranes were prepared as described in Methods and the kinetic parameters calculated by fitting a three-parameter logistic equation to the data. AC activity was stimulated (10 min, 30°C) with 10 nM ILO using 200 μM ATP as the substrate. AC activity was determined in triplicate at each Zn^{2+} and SQ22536 concentration and the activity was normalized to the maximal activity obtained at each Mg^{2+} concentration for comparison. **A.** The inhibitory effects of increasing Zn^{2+} concentrations (0.05–1.0 mM) were assessed in the presence of 1.0, 2.0, 3.0, 4.0 and 6.0 mM Mg^{2+}. Data presented are from a single representative subject ($N = 6$) and only the data for 1.0 (■), 3.0 (○) and 6.0 (●) mM Mg^{2+} are presented for clarity. The IC_{50} concentrations of Zn^{2+} were similar across Mg^{2+} concentrations. The calculated V_{max} (Mean ± SE, pM cAMP formed/(mg × min)) values for the presented data were 22.1 ± 1.1, 78.3 ± 2.9, 98.2 ± 2.5 for 1.0, 3.0 and 6.0 mM Mg^{2+}, respectively. **B.** The inhibitory effects of increasing SQ22536 concentrations (0.3–1000 μM) were assessed in the presence of 1.5, 2.5, 3.5, 5.0 and 6.0 mM Mg^{2+}. Data presented are from a single representative subject ($N = 5$) and only the data for 1.5, 3.5 and 6.0 mM Mg^{2+} are presented for clarity. The IC_{50} concentrations of SQ22536 decreased with increasing Mg^{2+} concentrations. The calculated V_{max} (Mean ± SE, (pmol cAMP formed/(mg × min)) values for the presented data were 40.1 ± 3.1, 96.8 ± 6.8 and 122.0 ± 2.3 for 1.5 (■), 3.5 (○) and 6.0 (●) mM Mg^{2+}, respectively. **C.** The effects of Zn^{2+} and SQ22536 were compared by plotting the ln of the IC_{50} concentrations of each inhibitor as a function of Mg^{2+} concentration. While there was no apparent change in the IC_{50} concentration of Zn^{2+} across Mg^{2+} concentrations, the IC_{50} concentration of SQ22536 decreased with increasing Mg^{2+} concentration. Data presented are the mean IC_{50} values (Zn^{2+}, $N = 6$ and SQ22536, $N = 5$) at each Mg^{2+} concentration.

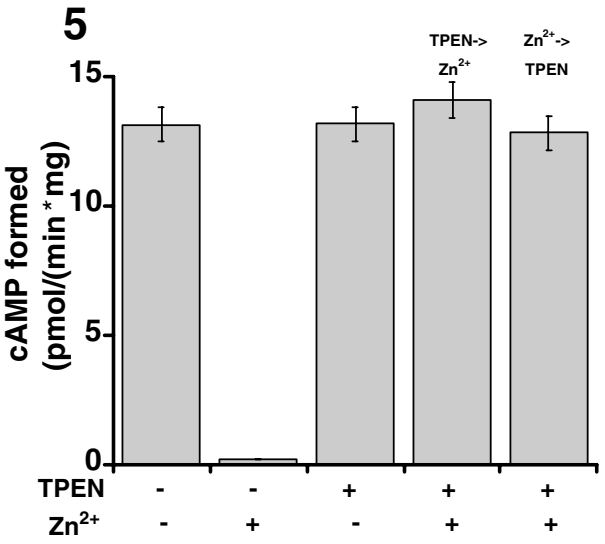

Figure 5. Treatment with the Zn^{2+} chelator TPEN prevented and reversed Zn^{2+}-mediated inhibition of ILO/Mg^{2+}-stimulated platelet AC activity in membrane preparations. The inhibitory effect of Zn^{2+} (400 μM) on ILO-stimulated platelet membrane AC activity in the presence of Mg^{2+} was eliminated by the addition of TPEN (1 mM) either before or following Zn^{2+} addition. Platelet membranes were prepared as described in Methods. AC activity was stimulated (10 min, 30°C) with 10 nM ILO in the presence of Mg^{2+} (1 mM) and Zn^{2+} (400 μM) using 200 μM ATP (EC_{50} concentration) as the substrate. AC activity was determined in triplicate for each treatment. Data presented are from a single representative subject ($N = 3$).

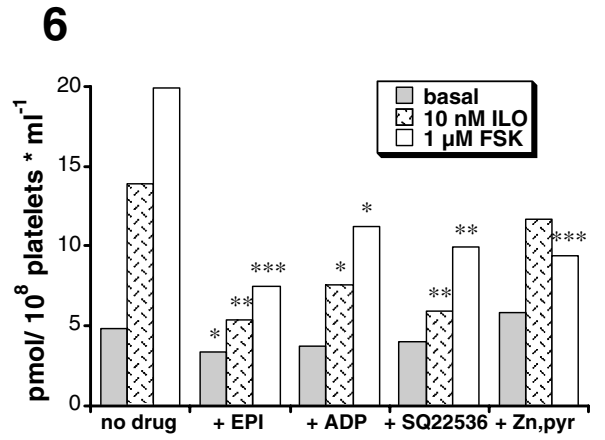

Figure 6. Effects of AC modulating agents on cAMP accumulation in washed platelets. Platelet rich plasma was prepared from whole blood anticoagulated with sodium citrate (3.8%) and heparin (50 U/ml), and the platelets subsequently washed as described in Methods. Washed platelets were preincubated for 3 min with milrinone (10 μM) and either EPI (10 μM), ADP (10 μM), SQ22536 (175 μM), Zn^{2+} (2 mM with the Zn^{2+}-ionophore PYR) or vehicle as indicated. AC activity was stimulated by the addition of FSK (1 μM) or ILO (10 nM) or vehicle only to determine basal activity. The concentration of cAMP (pmol/10^8 platelets) was determined by RIA (Methods) after a 5 min incubation. Treatment effects were assessed using one way repeated measures analysis of variance (ANOVA). The data were natural log transformed to satisfy the assumptions of the analysis (ANOVA) and all hypothesis tests were conducted at the $P<0.05$ level of significance. Dunnett's test was used to control the type I experiment-wise error rate for the comparisons of multiple treatment groups against the control group. Data represent the mean values ± SD ($N = 5$). * = $P<0.05$; ** = $P<0.01$; *** = $P<0.001$.

Discussion

The purpose of this study was to identify the isoform(s) of AC expressed in human platelets and to characterize the effects of several of its physiological modulators. PCR cloning identified AC-III as the principal AC isoform, and its expression in human platelets was verified using specific antibodies. The complete coding sequence of platelet AC-III was subcloned and expressed in HEK293 cells to compare its kinetic characteristics and to verify the functionality of this isoenzyme from platelets. In addition, we characterized the effects of three modulators, Mg^{2+}, Zn^{2+} and a P-site inhibitor SQ22536 on platelet AC activity.

The data on the molecular characterization of platelet AC indicate that AC-III is the principal isoenzyme expressed in human platelets (Figure 1; Figure 2, Panel A). The presence of AC-III in megakaryocytes and its absence in the cDNA prepared from the buffy coat preparation suggests that the AC-III detected in platelets is not due to white blood cell contamination of the platelet preparations. A faint signal for AC-VI was also detected in cDNA prepared from platelets and buffy coat cells. The absence of a signal for AC-VI in megakaryocytes, however, suggests that the source of signal for AC-VI in cDNA from platelets is likely due to the presence of white blood cells in these preparations. Additionally, a faint signal was also detected in platelet membranes by a type AC-V/VI antibody compared to the abundant signal detected in cell membranes from buffy coat preparations (Figure 2, Panel B). The presence of a low concentration of AC-VI in platelets, however, cannot be excluded. Because AC-VI, like AC-V, is a Ca^{2+}-inhibitable AC isoform,[21] and AC activity in platelet membrane preparations were unaffected by Ca^{2+} (0.1–3 mM), it is unlikely that AC-VI is a significant contributor to the AC activity of platelets.

The oligonucleotide primers designed for this study detected all 10 known isoenzymes of AC in various preparations (Figure 1). AC-III expression was also detected in parental HEK293 cells and rat brain cortex, consistent with the previously reported wide distribution of AC-III.[46] Positive signals for the other AC isoforms were observed in other tissues and HEK293 cells (Figure 1). AC-VII was previously identified in human erythroleukemia (HEL) cells which are used as a model for platelet signalling events.[47] This, along with the biochemical characteristics of AC-VII,[48,49] suggested that this isoenzyme might be expressed in human platelets. However, we were unable to detect AC-VII in human platelet cDNA. It is possible that the failure to detect AC-VII was due to the fact that the primers used in this study were designed from the sequence of bovine AC-VII. The amplified region, however, is homologous to the corresponding region within the sequence of human

AC-VII (GenBank, Accession D25538). Positive signals for AC-VII were demonstrated in cDNA from rat heart and lung. The AC-VII is a member of the group of the Ca^{2+}/calmodulin-insensitive ACs, which also include types AC-II and -IV.[50] The G_i-coupled receptor modulation of these AC isoforms has been demonstrated to be different from the other groups of ACs. The $G_{\beta\gamma}$ subunits released upon activation of G_{i2} protein have been shown to stimulate AC-II[51, 52] and pretreatment with antagonist of the $G_{\beta\gamma}$ subunits blocked this G_i-mediated activation of AC-II.[51] Similar effects of $G_{\beta\gamma}$ subunits have been observed on AC-VII[53] and AC-IV.[54] As previously reported[14,38,55,56] and as shown here (Results) in membrane preparations from human platelets, the activation of G_i-coupled receptors by EPI and ADP inhibits AC activity. Because AC-VII activity is stimulated by the activation of the G_i proteins while AC activity is inhibited by the activation of G_i-coupled receptor in platelets, AC-VII is unlikely to be a major AC isoform in platelets.

To further substantiate the identity of AC-III as the predominant isoform of AC in platelets, the physiological regulation of AC in transfected ACIII-HEK cells was compared to that of platelets. As noted above, AC-III was detected in parental HEK cells, however, the V_{max} of AC assayed in the ACIII-HEK cells was greater than that of the parental cells suggesting that the AC-III was functionally expressed (Table 2). Consistent with the behavior of the native AC activity present in platelet membranes, the AC activity in the ACIII-HEK cell membranes was coupled to both excitatory G_s- (Figure 3, Table 2), and inhibitory G_i-coupled receptors. Analysis of the Michaelis–Menten kinetics revealed similar values of the K_m for ATP and slope indices for platelet and ACIII-HEK membrane preparations (Table 2). Agents known to directly activate or inhibit AC activity had similar results in both platelet and ACIII-HEK membrane preparations (Table 2).

In addition to the known receptor-linked signalling pathways and other biologically active compounds that directly alter AC activity, metal ions including Ca^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} exhibit isoform-dependent differential modulation of AC activity.[19,35] Consistent with the known characteristics of AC-III, physiologically relevant Ca^{2+} concentrations had no effect on AC activity in platelet membrane preparations. Two Mg^{2+} ions were shown to be directly involved in AC catalytic activity.[35] One binds the metal binding site in the C_{1a} domain of the enzyme that is presumably involved in the co-ordination of the 3′ hydroxyl group of the ribose ring of ATP in the catalytic core.[57] The second ion binds as a complex with ATP.[33] This study demonstrated a significant increase in platelet membrane AC activity with increasing Mg^{2+} concentrations up to 6 mM (Table 3), and which was saturated at 10 mM (data not shown). Increased basal enzymatic activities of AC-II and AC-VI isoforms were also associated with increasing Mg^{2+} concentrations and differed by an order of magnitude for the same Mg^{2+} concentration, AC-II had greater activity than AC-VI.[58] These effects cannot be explained solely on the basis of intrinsic differences in the catalytic capabilities of these isoforms and suggest an additional modulatory effect of Mg^{2+} that may be isoform-dependent. Evidence for Mg^{2+}-related stimulation of AC in platelets has been reported[59,60] and the physiological basis for this modulation appears to exist in platelets. For instance, the intracellular free Mg^{2+} concentration in intact resting platelets (0.3–0.7 mM[61,62]) can be increased up to 1.3 mM in response to thrombin or collagen stimulation, presumably from intracellular mobilization.[61]

The inhibitory effects of Zn^{2+} on platelet AC activity are also consistent with the known structure of the catalytic domains of the enzyme and should presumably compete for the catalytic Mg^{2+} binding sites.[35] Although Zn^{2+} inhibited ILO- and FSK-stimulated activity and their potentiation by Mg^{2+}, it appeared to be a non-competitive AC inhibitor with respect to Mg^{2+} binding. At the concentrations of Mg^{2+} used (1–6 mM) in these experiments, the inhibitory effect of Zn^{2+} was not surmountable. In contrast, the Zn^{2+}-mediated inhibition was reversed by TPEN, which did not affect the ILO/Mg^{2+}-related stimulation of AC activity (Figure 5). This suggests the possibility of the presence of different Mg^{2+} binding sites on AC. The reported IC_{50} concentration for Zn^{2+} for the catalytic C_{1A}/C_{2A} heterodimer of AC-V/II was 15 μM,[35] which is significantly lower than that found for platelet AC in this study, suggesting that the modulatory effect of Zn^{2+} may also be AC isoform-dependent.

Zn^{2+} binding inhibits AC activity and is also associated with the potentiation of platelet aggregation.[63,64] Conversely, Mg^{2+} is required for AC catalytic activity[33,35] and is associated with inhibition of platelet aggregation.[59,60] Substantial intracellular stores of both Zn^{2+}[65] and Mg^{2+}[66] are present in platelets. The balance between these intracellular ions may represent a significant regulatory mechanism for platelet AC-III activity as well as the modulation of the net aggregation response.

Although the relationship between activation of G_i-coupled receptors linked to the inhibition of stimulated AC and sustained platelet aggregation is well established,[7,8,67] arguments discounting the bi-directional modulation of platelet aggregation by AC were based on the failure of the P-site inhibitor SQ22536 to inhibit basal AC activity.[45,68–71] In this study, SQ22536 inhibited ILO- and FSK-stimulated AC activity and their potentiation by Mg^{2+} in membrane preparations. Confirming previous reports,[72,73] the IC_{50} of SQ22536, unlike that of Zn^{2+}, decreased with increased stimulation of enzyme activity. Zn^{2+} was able to completely inhibit AC activity, SQ22536

could not. This behavior is consistent with the dead-end P-site inhibitor model,[72] which suggests that a higher concentration of P-site inhibitor is required if the enzyme–product complex is present at low steady-state concentrations.[74] This would also explain the difficulty of measuring an effect of SQ22536 on the low basal AC activity and its ability to potentiate platelet net aggregation in platelets in which AC activity was stimulated but not in unstimulated controls.[68]

Alternatively, a different G_i-coupled signalling pathway may be involved in the modulation of platelet aggregation. Sustained platelet aggregation is associated with activation of and the translocation of phosphoinositide-3-OH kinase (PI3K) to the cytoskeleton and its association with actin filaments.[75] The $G_{\beta\gamma}$ subunits released by G_i-coupled receptor activation stimulate $PI3K_\gamma$-activity, an isoform found in platelets.[76–78] Data from preliminary experiments indicate that an inhibitor of PI3K (wortmannin) accelerates deaggregation elicited by U46619 (in preparation), implicating PI3K activation as an additional signalling pathway that may contribute to maintaining the balance between aggregation and deaggregation in what appears to be a reciprocal manner to that of AC involved signalling. Interestingly, cAMP was recently found to inhibit the lipid kinase activity of PI3K and decrease the levels of phosphatidylinositol 3,4,5-triphosphate in COS cells transfected with the catalytic subunit of PI3K,[79] suggesting an interaction between these two signalling pathways.

In summary, we demonstrate that AC-III is the principal isoform of AC present in platelets. We also characterized the modulation of platelet AC activity by two metal ions as well as a P-site inhibitor. These results provide experimental support for the hypothesis that the native platelet net aggregation response mediated by proaggregatory stimuli is an inhibited response due to the presence of constitutive AC activity and the deaggregation it maintains. Based on our previous communications[9–11,80] and on the data presented here we propose that the default state of circulating platelets is constitutive deaggregation, which requires deceleration to enhance and sustain aggregation. We further propose that the requirement for the stability of platelet aggregation may be related to the deceleration of this AC-III/cAMP-related active deaggregation mechanism.

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