4,4'-Di-isothiocyanatostilbene-2,2'-disulphonic acid ('DIDS') activates protein kinase C and Na⁺/H⁺ exchange in human platelets via α_{2A} -adrenergic receptors

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Most agonists stimulate platelets by inducing Ca²⁺ mobilization, Ca²⁺ influx and protein kinase C (PKC) activation leading to Na⁺/H⁺ exchange, exposure of fibrinogen-binding sites and aggregation. In contrast, previous studies showed that adrenaline induces exposure of fibrinogen-binding sites and aggregation without appreciable changes in cytosolic Ca²⁺ content or PKC activity. In the present study we investigated platelet responses mediated via α_{2A} -adrenergic receptors, using 4,4'-di-isothiocyanatostilbene-2,2'-disulphonic acid (DIDS), which is known to bind to this type of receptor. Addition of DIDS (2–20 μ M) induced (i) a rise in cytosolic pH of 0.23 ± 0.05 pH unit ($n = 5$) as detected by BCECF fluorescence, due to activation of the Na⁺/H⁺ exchanger, (ii) a 3.5–4-fold increase in the phosphorylation of the 47 kDa protein, a major substrate of PKC, (iii) exposure of

81072 ± 7293 ($n = 3$) binding sites for ¹²⁵I-fibrinogen per platelet, and (iv) irreversible aggregation. These responses occurred without changes in cytosolic [Ca²⁺], secretion of dense-granule contents and enhanced phosphoinositide metabolism, and were not affected by inhibition of thromboxane A₂ generation (30 μ M indomethacin). The α_{2A} -adrenergic-receptor antagonists oxy-metazoline (500 μ M) and yohimbine (1 mM) completely abolished DIDS-induced responses. Inhibition of PKC (1 μ M staurosporine) prevented phosphorylation of the 47 kDa protein, the increase in Na⁺/H⁺ exchange and exposure of fibrinogen-binding sites. Thus our present data suggest that activation of PKC is an early event in DIDS-induced platelet activation via the α_{2A} -adrenergic receptor, which precedes any of the other known signal-transducing sequences.

INTRODUCTION

Most agonists stimulate platelets by binding to specific membrane-associated receptors that are linked to phospholipase C (PLC) via a putative pertussis-toxin-sensitive GTP-binding protein. Activation of PLC results in the degradation of PtdIns(4,5)P₂ to Ins(1,4,5)P₃ and 1,2-diacylglycerol. Ins(1,4,5)P₃ induces the release of Ca²⁺ from the dense-tubular system [1], whereas diacylglycerol activates protein kinase C (PKC), a serine/threonine kinase [2]. Subsequently PKC stimulates the Na⁺/H⁺ exchanger, an antiport in the plasma membrane that regulates the cytosolic pH [3], initiates Ca²⁺ sequestration [4], induces the exposure of binding sites for adhesive proteins on the glycoprotein IIb/IIIa complex (GPIIb/IIIa) [5,6], facilitates InsP₃ degradation [7], and affects the interaction of several GTP-binding proteins with their target enzymes [8,9].

Adrenaline is an agonist with unique properties. Initially, it was thought to activate platelets in a synergistic action with trace amounts of other agonists, but recently it has become apparent that adrenaline alone can activate platelets [10]. It induces aggregation, without appreciable activation of PLC [11] and PKC [11,12], and fails to change the cytosolic Ca²⁺ concentration, as detected with Quin-2 or Fura-2 fluorescence [13]. On the other hand, adrenaline activates phospholipase A₂ (PLA₂) via a probably pertussis-toxin-sensitive GTP-binding protein [14,15] and inhibits adenylate cyclase via the inhibitory GTP-binding protein, G_i [9]. Activation of PLA₂ results in rapid liberation of arachi-

donic acid and formation of prostaglandin endoperoxides and thromboxane A₂ [11,16].

In their studies on pH regulation in platelets, Banga et al. [17] observed that an early step in adrenaline-induced signal processing is activation of the Na⁺/H⁺ exchanger. The resulting alkalization preceded activation of PLA₂ and further signal processing, suggesting that the Na⁺/H⁺ exchanger is closely coupled to α_{2A} -adrenergic receptors (α_{2A} -AR) or may even form an intrinsic property of these receptors. This would be in contrast with activation of the Na⁺/H⁺ exchanger in platelets stimulated with α -thrombin, platelet-activating factor ('PAF') or ADP, which appears to be controlled by cytosolic Ca²⁺ and PKC [3,18].

In the present study, we found that 4,4'-di-isothiocyanatostilbene-2,2'-disulphonic acid (DIDS), which is commonly used to inhibit the Cl⁻/HCO₃⁻ exchanger, strongly activated the α_{2A} -AR, in accordance with an earlier observation by Periyasamy and Somani [19]. This property provided a tool to characterize α_{2A} -AR-mediated signal processing in platelets, especially with respect to the role of Na⁺/H⁺ exchange and its possible control by PKC.

EXPERIMENTAL

Materials

2,7-Biscarboxyethyl-5(6)-carboxyfluorescein tetra-acetoxymethyl ester (BCECF-AM) was obtained from Calbiochem (Frankfurt, Germany). Staurosporine was from Boehringer

Abbreviations used: DIDS, 4,4'-di-isothiocyanatostilbene-2,2'-disulphonic acid; SITS, 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulphonic acid; DTSSP, 3,3'-dithiobis(sulphosuccinimidyl)propionate; AR, adrenergic receptor; H7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine; EIPA, ethylisopropylamiloride; PRP, platelet-rich plasma; PKC, protein kinase C; GPIIb/IIIa, glycoprotein IIb/IIIa complex; PLC, phospholipase C; PLD, phospholipase D; PLA₂, phospholipase A₂; PA, phosphatidic acid; BCECF-AM, 2,7-biscarboxyethyl-5(6)-carboxyfluorescein tetra-acetoxymethyl ester; cAMP, cyclic AMP; cGMP, cyclic GMP; dbcAMP, dibutyl cAMP; PGI₂, prostacyclin; pH_i, intracellular pH; GRGDS: Gly-Arg-Gly-Asp-Ser.

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(Mannheim, Germany). Sepharose 2B was from Pharmacia (Uppsala, Sweden). 4-Acetamido-4'-isothiocyanatostilbene-2,2'-disulphonic acid (SITS) and yohimbine were from Aldrich (Brussels, Belgium). α -Thrombin, nigericin, oxymetazoline, DIDS, indomethacin, (–)-adrenaline, dibutyl cyclic AMP (dbcAMP), H7 [1-(5-isoquinolinylsulphonyl)-2-methylpiperazine], imipramine and 8'-bromo-cGMP were obtained from Sigma (St. Louis, MO, U.S.A.), Na¹²⁵I (sp. radioactivity 629 GBq/mg) and [¹⁴C]hydroxytryptamine creatine sulphate (sp. radioactivity 1.8–2.2 GBq/mmol) were from Amersham International (Amersham, Bucks., U.K.), and [³²P]P_i (sp. radioactivity 314 TBq/mmol) was from New England Nuclear (Boston, MA, U.S.A.). BSA (demineralized) was purchased from Organon Teknika (Turnhout, Belgium), calphostin C from Kamiya Biomedical Co. (Tokyo, Japan), and fibrinogen (grade L) from KABI (Stockholm, Sweden). 3,3'-Dithiobis(sulphosuccinimidyl)propionate (DTSSP) was obtained from Pierce (Rockford, IL, USA), and prostacyclin (PGI₂) from Cayman Chemicals (Ann Arbor, MI, U.S.A.). D-Phenylalanyl-L-prolyl-L-arginylchloromethane was from Bachem (Bubersdorf, Switzerland). Ethylisopropylamiloride (EIPA) was a gift from Dr. W. Siffert, Max-Planck Institut für Biophysik, Frankfurt, Germany, and GRGDS (Gly-Arg-Gly-Asp-Ser) was a gift from A. Bekkers, Department of Biochemistry, University of Utrecht, The Netherlands. All other chemicals were of analytical grade.

Platelet isolation

Freshly drawn venous blood from healthy volunteers (with informed consent) was collected into trisodium citrate (0.1 vol. of 130 mM). The donors claimed not to have taken any medication during the previous 10 days. Platelets were isolated by either gel-filtration or centrifugation (as described below). For the preparation of gel-filtered platelets, citrated blood was centrifuged (200 g, 10 min, 22 °C), and the platelet-rich plasma (PRP) was placed on a Sepharose 2B column equilibrated in Ca²⁺-free Tyrode's solution (137 mM NaCl, 2.68 mM KCl, 0.42 mM NaH₂PO₄, 1.7 mM MgCl₂, 11.9 mM NaHCO₃, pH 7.25) containing 0.1 % glucose and 0.2 % BSA.

Measurement of intracellular pH (pH_i)

This was done with the fluorescent pH_i-sensitive indicator BCECF-AM. PRP was acidified to pH 6.5 with $\frac{1}{5}$ vol. of ACD (2.5 g of trisodium citrate, 1.5 g of citric acid and 2.0 g of D-glucose in 100 ml of water), and platelets were isolated by centrifugation (700 g, 15 min, 22 °C). Subsequently, the cells were resuspended in 1 ml of Hepes/Tyrode buffer (145 mM NaCl, 5 mM KCl, 0.5 mM Na₂HPO₄, 1 mM MgSO₄, 10 mM Hepes), pH 6.5, containing 0.1 % (w/v) glucose and 0.2 % (w/v) gelatin. The platelets were incubated with BCECF-AM (3 μ M) in the absence or presence of indomethacin (30 μ M) for 30 min at 37 °C and washed on a Sepharose 2B column, equilibrated in Hepes/Tyrode buffer (pH 6.5). Platelets were stored at room temperature until use. Samples (50 μ l) were added to 1450 μ l of prewarmed gelatin-free Hepes/Tyrode buffer (pH 7.2; 37 °C), resulting in a final platelet count of (150–200) $\times 10^3/\mu$ l. Fluorescence was determined on a Hitachi F-3000 spectrofluorimeter, by using wavelengths of 495 and 530 nm for excitation and emission respectively. The suspension was gently stirred (60–100 rev./min). After establishment of a stable baseline, the indicated agonist was added. Calibration of the signal was carried out after diluting the BCECF-loaded platelets in high-K⁺ buffer (120 mM KCl, 30 mM NaCl, 1 mM MgSO₄,

5 mM glucose) in the presence of nigericin (2 μ M), as described by Horne and colleagues [20].

Measurement of PKC activity

Platelets were labelled with 3.7 MBq of carrier-free [³²P]P_i/ml of acidified PRP (pH 6.5), with or without a simultaneous incubation with indomethacin (30 μ M) for 1 h at 37 °C. The platelets were isolated by centrifugation (700 g, 15 min, 22 °C) and were resuspended in Hepes/Tyrode buffer (pH 7.2), resulting in a final platelet count of (150–200) $\times 10^3/\mu$ l. Labelled platelets were stimulated with 20 μ M DIDS or 1 unit/ml α -thrombin at 37 °C, and samples were collected at the times indicated in the Results section, transferred into 0.5 vol. of 3-times-concentrated Laemmli sample buffer and heated for 2 min at 100 °C before electrophoresis. Proteins were separated by electrophoresis through a 11 % polyacrylamide gel, as described by Laemmli [21]. Gels were stained with Coomassie Brilliant Blue, and the distribution of radioactivity was determined by autoradiography of dried gels on Kodak Royal X-Omat film. For determination of the radioactivity of the 47 kDa protein, the specific area was cut out of the dried gels and heated for 2 h at 80 °C in 30 % H₂O₂. The radioactivity was determined by liquid-scintillation counting. In control experiments no difference in phosphorylation patterns could be detected between centrifuged and gel-filtered platelets (G. van Willigen, unpublished work).

Data are expressed as percentage of the maximal phosphorylation induced by 1 unit/ml α -thrombin, to minimize the variation in 47 kDa-protein radioactivity in resting platelets from different donors.

Fibrinogen-binding assay

Gel-filtered platelets [(200–300) $\times 10^3$ platelets/ μ l] were stimulated with α -thrombin (1 unit/ml) (30 nM D-phenylalanyl-L-prolyl-L-arginylchloromethane was added 3 min after stimulation to prevent coagulation), or with DIDS (20 μ M) in the presence of ¹²⁵I-labelled fibrinogen (1 μ M) at 22 °C and 37 °C. Fibrinogen was purified and iodinated as described elsewhere [22]. At the times indicated in the Results section, a sample (in triplicate) of 200 μ l was drawn and layered on 100 μ l of 25 % (w/v) sucrose in Ca²⁺-free Tyrode's solution in micro-sedimentation tubes (Sarstedt, Vienna, Austria) and centrifuged (10000 g, 10 min, 22 °C), in a Beckman Microfuge E. The pellet and supernatant were separated and counted for radioactivity in a γ -radiation counter. The number of molecules of ¹²⁵I-fibrinogen bound per platelet was calculated from the radioactivity in the pellet fraction compared with the total radioactivity in pellet plus supernatant. The data are expressed as means $\pm$ S.D. ($n = 3$) and corrected for non-specific binding, defined as the binding of ¹²⁵I-fibrinogen to unstimulated platelets. Details of these procedures have been described elsewhere [23].

Platelet aggregation, single platelet disappearance and ATP secretion

Gel-filtered platelets, treated with or without indomethacin (30 μ M) for 30 min at 22 °C, were prepared as described above. The platelet count was adjusted with Ca²⁺-free Tyrode's solution to 200 $\times 10^3$ cells/ μ l. After addition of fibrinogen (1 μ M) the agonists were added and aggregation was measured by two techniques; first, by measuring the disappearance of single platelets [24], and second by measuring the change in light transmission in the aggregometer (designated as optical aggregation). For the first-mentioned procedure, samples were

collected at 2 min after addition of the agonist and immediately fixed with 9 vol. of ice-cold 0.9% NaCl/0.5% (v/v) glutaraldehyde. The platelets were counted in a Platelet Analyzer 810 (Baker Instruments, Allentown, PA, U.S.A.) with apertures set at 3.2 and 16 μm^3 . Optical aggregation was measured in a Chronolog lumiaggregometer at 37 °C and a stirring speed of 900 rev./min. Simultaneously, the secretion of ATP was measured by using the Chronology firefly reagent.

Secretion of hydroxy[^{14}C]tryptamine

Platelets were loaded with hydroxy[^{14}C]tryptamine (1 μM) in the presence of indomethacin (30 μM) for 30 min at 37 °C in acidified PRP. Subsequently, the platelets were washed on a Sepharose 2B column, equilibrated in Ca^{2+} -free Tyrode's solution. The platelet count was adjusted to 200×10^3 cells/ μl . Secretion was analysed by measuring the liberation of 5-hydroxy[^{14}C]tryptamine from the dense granules. The experiments were performed under stirring (900 rev./min) at 37 °C in the presence of 2.5 μM imipramine, to prevent uptake of secreted 5-hydroxytryptamine into the platelets. Samples were collected 5 min after addition of the agonists in 0.15 vol. of 1.035 M formaldehyde in 0.9% NaCl (0 °C) to stop secretion. After centrifugation (10000 g for 1 min at 4 °C), the supernatants were collected and used for analysis of 5-hydroxy[^{14}C]tryptamine and counted for radioactivity by standard procedures. The data are expressed as percentage of maximal secretion (α -thrombin, 5 units/ml, 5 min at 37 °C).

Measurement of $\text{PtdIns}(4,5)\text{P}_2$ and phosphatidic acid (PA)

Changes in PtdInsP_2 and PA were measured by a modified procedure described by Jolles et al. [25] for rat brain cells. In short, platelets were labelled with [^{32}P]P_i and isolated by centrifugation as described above under 'Measurement of PKC activity'. Platelets were stimulated with the various agonists, and the reactions were terminated at different time points by adding 2 ml of chloroform/methanol/13 M HCl (100:50:1, by vol.; 0 °C). The lipids were extracted as described by Bligh and Dyer [26]. Phospholipids were separated by high-performance t.l.c., and the radioactivity was determined by autoradiography of the plate on Kodak Royal X-Omat film. The spots were scraped off and counted for ^{32}P by liquid-scintillation counting. The ^{32}P content is expressed as a percentage of that of unstimulated platelets.

Presentation of data

Data are expressed as means $\pm$ S.D. unless indicated otherwise. Statistical significances were determined by Student's t test, and were considered significant at $P < 0.05$. All experiments were performed with at least three different platelet preparations (in duplicate or triplicate per donor).

RESULTS

DIDS-Induced changes in pH_i

Addition of DIDS (20 μM) to BCECF-loaded platelets raised the pH_i from 7.12 ± 0.04 to 7.32 ± 0.06 ($n = 5$; Figure 1a). This alkalinization was preceded by an acidification of 0.03 ± 0.01 pH unit within 20 s after addition of DIDS. The alkalinization was comparable with the changes in BCECF fluorescence induced by 1 unit/ml α -thrombin (Figure 1b). The DIDS-induced rise in pH_i was completely the result of enhanced Na^+/H^+ exchange, since pretreatment with EIPA (25 μM), an inhibitor of this antiporter [3], completely abolished the alkalinization and instead led to a

fall in pH_i of 0.09 ± 0.02 pH unit in 60 s (Figure 1c). The α_{2A} -AR antagonist oxymetazoline (500 μM ; [27,28]) completely blocked DIDS-induced changes in pH_i (Figure 1d). This effect was dose-dependent, showing a half-maximal effect at about 130 μM oxymetazoline. A second α_{2A} -antagonist, yohimbine, also inhibited the DIDS-induced changes in pH_i , with a half-maximal effect at about 500 μM (Figure 1, insert).

DIDS is known as an inhibitor of the $\text{Cl}^-/\text{HCO}_3^-$ exchanger, which in many cell types contributes to pH_i control [29,30]. In addition, DIDS induces cross-linking of membrane proteins in human platelets [31]. Therefore, control experiments were performed to ascertain that DIDS-induced Na^+/H^+ exchange was exclusively due to activation of the α_{2A} -AR. First, the experiments were repeated in the presence of 30 mM HCO_3^- to enable the operation of the $\text{Cl}^-/\text{HCO}_3^-$ exchanger, but this treatment failed to affect the changes in pH_i induced by DIDS or α -thrombin (results not shown). Second, platelets were treated with SITS (20 μM), like DIDS an inhibitor of $\text{Cl}^-/\text{HCO}_3^-$ exchange and possessing a structure similar to DIDS, apart from lacking one of the two isothiocyanato groups [31]. SITS failed to affect the pH_i at even a 10-fold higher concentration than DIDS (Figure 1e). The property of DIDS to cross-link platelet membrane glycoproteins was mimicked by addition of DTSSP (20 μM), which also cross-links platelet membrane surface proteins [32], but again no change in pH_i was observed (Figure 1f). In Table 1 the pH_i responses upon addition of DIDS and α -thrombin as well as the different control studies are summarized. Taken together, these findings indicate that DIDS modulates Na^+/H^+ exchange in platelets via a mechanism that is apparently not affected by inhibition of $\text{Cl}^-/\text{HCO}_3^-$ exchange or cross-linking of membrane proteins. Noteworthy is the observation that the sensitivity of the platelets from different donors to DIDS appeared to fall in two groups, with most donors showing maximal responses at 20 μM DIDS and a minor group already

Figure 1 DIDS-induced changes in pH_i

Gel-filtered BCECF-loaded platelets were stimulated at 37 °C with DIDS (20 μM ; a), α -thrombin (1 unit/ml; b) or DIDS (20 μM) after preincubation with (c) EIPA (25 μM) or (d) oxymetazoline (500 μM), (e) SITS (20 μM) or (f) DTSSP (20 μM). The arrows indicate the addition of the agonists. All tracings were obtained within one experiment, but are representative of five similar experiments. Insert: BCECF-loaded platelets were stimulated at 37 °C with DIDS (20 μM) after preincubation (2 min, 37 °C) with different concentrations of oxymetazoline (●) or yohimbine (▼). The data are expressed as a percentage of the DIDS-induced increase in pH_i (means $\pm$ S.D.; $n = 4$).

Table 1 DIDS-induced alterations in pH_i

Gel-filtered BCECF-loaded platelets were stimulated with DIDS (20 μ M), α -thrombin (1 unit/ml), SITS (20 μ M) or DTSSP (20 μ M). Alternatively, gel-filtered BCECF-loaded platelets were stimulated with DIDS (20 μ M) after a preincubation with (final concns.) oxymetazoline (500 μ M), yohimbine (1 mM), indomethacin (30 μ M), 8'-bromo-cGMP (1.5 mM), dbcAMP (2.5 mM), PGI₂ (10 ng/ml), staurosporine (1 μ M), H7 (50 μ M), calphostin C (1 μ M), EIPA (25 μ M), fibrinogen (200 μ M) or fibrinogen (200 μ M) in the presence of GRGDS (4.9 μ g/ml). All preincubations were performed for 2 min at 37 °C, except for calphostin C and indomethacin, which were preincubated for 5 or 30 min at 37 °C respectively. The preincubations did not affect the resting pH_i . After addition of the agonist, changes in BCECF fluorescence were measured and changes in pH_i calculated. Data are the changes in pH_i compared with resting values and are expressed in pH units. Data are means $\pm$ S.D. from five different platelet suspensions: *significantly different ($P < 0.05$) from control (20 μ M DIDS).

Additions	Acidification	Alkalinization
Without pretreatment		
DIDS	0.03 $\pm$ 0.01	0.23 $\pm$ 0.05
α -Thrombin	0	0.16 $\pm$ 0.03
SITS	0	0.01 $\pm$ 0.01*
DTSSP	0	0.01 $\pm$ 0.02*
DIDS (20 μ M) after pretreatment with		
Oxymetazoline	0	0.00 $\pm$ 0.02*
Yohimbine	0	0.00 $\pm$ 0.02*
Indomethacin	0.05 $\pm$ 0.01	0.16 $\pm$ 0.04
8'-Bromo-cGMP	0	0.12 $\pm$ 0.05*
dbcAMP	0	0.01 $\pm$ 0.01*
PGI ₂	0	0.01 $\pm$ 0.01*
Staurosporine	0	0.01 $\pm$ 0.02*
H7	0	0.01 $\pm$ 0.01*
Calphostin C	0	0.01 $\pm$ 0.02*
EIPA	0.09 $\pm$ 0.02*	0
Fibrinogen	0.02 $\pm$ 0.02	0.20 $\pm$ 0.05
GRGDS + fibrinogen	0.03 $\pm$ 0.02	0.21 $\pm$ 0.04

showing a maximal response at 2 μ M DIDS (results not shown). A similar heterogeneity is known for adrenaline-induced platelet aggregation [33,34].

DIDS-induced signal generation

Next, we investigated whether the changes in pH_i upon addition of DIDS were a direct effect of the activated α_{2A} -AR or were mediated via any of the known signal-transducing pathways in platelets. Pretreatment of the cells with indomethacin (30 μ M) to abolish the production of prostaglandin endoperoxides and thromboxane A₂ failed to affect DIDS-induced changes in pH_i (Table 1). Addition of 8'-bromo-cGMP (1.5 mM), which directly raises the intracellular cGMP level, blocked acidification and decreased the alkalinization to about 50 % of control values. The alterations in pH_i were completely inhibited by preincubation with dbcAMP (2.5 mM) and PGI₂ (10 ng/ml), which both raise the cAMP concentration in platelets. A similar inhibition of both the fall in pH_i and the subsequent increase was found after preincubation with the PKC inhibitor staurosporine (1 μ M), indicating that activation of PKC is an essential step in pH_i changes induced by DIDS. Also a preincubation with either H7 (50 μ M) or calphostin C (1 μ M), both inhibitors of PKC [35,36], resulted in inhibition of DIDS-induced changes in pH_i . In addition, the effect of DIDS (20 μ M) on Na⁺/H⁺ exchange was studied in the presence of fibrinogen (200 μ M) or a combination of fibrinogen and GRGDS (4.9 μ g/ml), which is a synthetic peptide that blocks the binding of fibrinogen to the platelet [37]. Previously, Banga et al. [17] showed that adrenaline activates the antiporter only when fibrinogen binds to GPIIb/IIIa. However,

Figure 2 DIDS-induced activation of PKC

³²P-labelled platelets were stimulated at 37 °C with 1 unit/ml α -thrombin ($\square$) or 20 μ M DIDS, without ($\bullet$) or after a 2 min preincubation with oxymetazoline (500 μ M; ∇), 8'-bromo-cGMP (1.5 mM; $\circ$) or dbcAMP (2.5 mM; $\diamond$). SITS (20 μ M; $\blacktriangle$) and DTSSP (20 μ M; $\bullet$) were used as controls. Data are expressed as percentages of maximal α -thrombin (1 unit/ml)-induced increase in 47 kDa-protein phosphorylation.

the DIDS-induced activation of the Na⁺/H⁺ exchanger was not affected by either fibrinogen or GRGDS and fibrinogen (Table 1). Thus the activation of the Na⁺/H⁺ exchanger by DIDS appears to result from activation of PKC without apparent involvement of fibrinogen binding.

DIDS-induced activation of PKC

As illustrated in Figure 2, addition of DIDS (20 μ M) triggered a 3.5–4-fold increase in the phosphorylation of the 47 kDa protein, the major substrate of PKC in platelets. This increase was preceded by a lag phase of about 10 s, which fell in the period in which the pH_i decreased (Figure 1a). The 47 kDa-protein phosphorylation was transient and had returned to levels found in resting platelets after 5 min. A similar rise in 47-kDa-protein phosphorylation was found upon stimulation with α -thrombin (1 unit/ml), but here the rise was immediate and the phosphorylation remained high for at least 5 min.

To investigate whether the DIDS-induced activation of PKC was an α_{2A} -AR-mediated event, the platelets were preincubated with oxymetazoline (500 μ M). This treatment completely inhibited the phosphorylation of the 47 kDa protein such in parallel with the abolishment of increased Na⁺/H⁺ exchange. No activation of PKC was observed when DIDS was replaced by SITS (20 μ M; Figure 2) or DTSSP (20 μ M), which is in concert with the effect of these agents on the Na⁺/H⁺ exchanger (Figure 1).

To identify the pathways that play a role in the coupling of the α_{2A} -AR to PKC, platelets were pretreated with indomethacin (30 μ M), 8'-bromo-cGMP (1.5 mM) or dbcAMP (2.5 mM). Indomethacin failed to affect the 47 kDa-protein phosphorylation patterns triggered by DIDS (results not shown), but pretreatment with 8'-bromo-cGMP (1.5 mM) prolonged the lag phase to about 20 s. Thereafter, the phosphorylation proceeded undisturbed (Figure 2). Elevation of cAMP by dbcAMP (2.5 mM) completely blocked DIDS-induced 47 kDa-protein phosphorylation. As expected, no increase in phosphorylation of the 47 kDa protein was seen in platelets that had been treated with stauro-

Figure 3 Formation of $[^{32}\text{P}]\text{PA}$

^{32}P -labelled platelets were stimulated at 37°C with 1 unit/ml α -thrombin ($\square$) or $20\ \mu\text{M}$ DIDS, without ($\circ$) or after a 1 min preincubation with $1\ \mu\text{M}$ staurosporine ($\bullet$). Data are expressed as percentages of resting values.

Figure 4 DIDS-induced ^{125}I -fibrinogen binding

Gel-filtered platelets were stimulated in the presence of ^{125}I -fibrinogen ($1\ \mu\text{M}$) at 22°C with DIDS ($20\ \mu\text{M}$), without ($\bullet$) or after preincubation with either oxymetazoline ($500\ \mu\text{M}$; ∇) or 8'-bromo-cGMP ($1.5\ \text{mM}$; $\circ$). SITS ($20\ \mu\text{M}$; $\blacktriangle$) was used as a control. A preincubation with staurosporine ($1\ \mu\text{M}$) completely inhibited DIDS-induced ^{125}I -fibrinogen binding (results not shown). Platelets were also stimulated with α -thrombin ($1\ \text{unit/ml}$) ($30\ \text{nM}$ D-phenylalanyl-L-prolyl-L-arginylchloromethane was added 3 min after stimulation to prevent coagulation) ($\square$).

sporine ($1\ \mu\text{M}$). These results support the concept that DIDS activates Na^+/H^+ exchange via α_{2A} -AR-mediated activation of PKC.

Formation of $[^{32}\text{P}]\text{PA}$

Since PKC is generally considered to be activated by diacylglycerol, which is derived from $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis by PLC, we next investigated whether DIDS activated the metabolism of polyphosphoinositides. Measurements of ^{32}P -labelled platelets stimulated with DIDS revealed only minor changes in $[^{32}\text{P}]\text{PtdIns}(4,5)\text{P}_2$ (results not shown) and $[^{32}\text{P}]\text{PA}$ (Figure 3), when compared with effects of α -thrombin ($1\ \text{unit/ml}$). When platelets were pretreated with staurosporine ($1\ \mu\text{M}$), the minor increase in $[^{32}\text{P}]\text{PA}$ seen after addition of DIDS was completely

Figure 5 DIDS-induced aggregation and 5-hydroxy $[^{14}\text{C}]$ tryptamine secretion

Gel-filtered platelets were stirred (900 rev./min) at 37°C in the presence of fibrinogen ($1\ \mu\text{M}$), except for α -thrombin, to prevent coagulation. The following agonists were added: α -thrombin ($1\ \text{unit/ml}$; a), DIDS ($20\ \mu\text{M}$), without (b) or after a preincubation with indomethacin ($30\ \mu\text{M}$; c), 8'-bromo-cGMP ($1.5\ \text{mM}$; d), oxymetazoline ($500\ \mu\text{M}$; e), PGI_2 ($10\ \text{ng/ml}$; f) and staurosporine ($1\ \mu\text{M}$; g). SITS ($20\ \mu\text{M}$; h) and DTSSP ($20\ \mu\text{M}$; i) were added as controls. The insert shows 5-hydroxy $[^{14}\text{C}]$ tryptamine secretion at 37°C after stimulation with DIDS ($20\ \mu\text{M}$), SITS ($20\ \mu\text{M}$) or α -thrombin ($1\ \text{unit/ml}$), and expressed as percentages of α -thrombin ($5\ \text{units/ml}$)-induced secretion.

abolished, indicating that activation of PKC precedes activation of PLC (Figure 3).

DIDS-induced fibrinogen binding

PKC plays a central role in platelet signal transduction, since among many other steps it regulates Na^+/H^+ exchange and the exposure of fibrinogen-binding sites on GPIIb/IIIa [5,6]. Stimulation of platelets with DIDS ($20\ \mu\text{M}$) in the presence of ^{125}I -fibrinogen ($1\ \mu\text{M}$) at 22°C resulted in a maximal binding of ^{125}I -fibrinogen of 81072 ± 7293 ($n = 3$) molecules bound per platelet after 30 min (Figure 4). The velocity and extent of the binding were comparable with those induced by α -thrombin ($1\ \text{unit/ml}$; Figure 4). The DIDS-induced ^{125}I -fibrinogen binding was not affected by a preincubation with indomethacin ($30\ \mu\text{M}$; 30 min, 37°C ; results not shown), but was completely blocked by oxymetazoline ($500\ \mu\text{M}$; Figure 4), yohimbine ($1\ \text{mM}$; results not shown) and staurosporine ($1\ \mu\text{M}$; results not shown). Preincubation with 8'-bromo-cGMP ($1.5\ \text{mM}$) delayed the fibrinogen binding for about 5 min (22°C) and decreased the maximal number of exposed binding sites to 72142 ± 5218 ($n = 3$; Figure 4). This lag phase was not detected when the binding studies were performed at 37°C . At this temperature the exposure of fibrinogen-binding sites is very rapid, and fibrinogen binding is already measured after a 2 min incubation. In control studies SITS ($20\ \mu\text{M}$; Figure 4) and DTSSP ($20\ \mu\text{M}$; results not shown) failed to induce ^{125}I -fibrinogen binding.

DIDS-induced aggregation

When gel-filtered platelets were stimulated with α -thrombin ($1\ \text{unit/ml}$), a rapid irreversible aggregation was observed (Figure 5a). Addition of DIDS ($20\ \mu\text{M}$) in the presence of $1\ \mu\text{M}$ fibrinogen also induced platelet aggregation (Figure 5b). The

Figure 6 Single-platelet disappearance induced by adrenaline and DIDS

Gel-filtered indomethacin-treated platelets were stimulated at 37 °C with adrenaline (10 μM; ■ and □) or DIDS (20 μM; ● and ○) in the presence of fibrinogen (1 μM). Agonists were added after a preincubation with various concentrations of yohimbine (□ and ○) or oxymetazoline (■ and ●). The curves shown were obtained within one experiment, but are representative of three similar experiments. The data are expressed as a percentage of the α -thrombin (1 unit/ml)-induced single-platelet disappearance (means $\pm$ S.D.; $n = 3$).

DIDS-induced aggregation was not preceded by shape change, which is also observed after adrenaline-induced aggregation [11]. Preincubation with indomethacin (30 μM; 30 min; 37 °C) did not affect DIDS-induced aggregation (Figure 5c), but preincubation with 8'-bromo-cGMP (1.5 mM) decreased the aggregation velocity (Figure 5d). When platelets were preincubated with oxymetazoline (500 μM; Figure 5e), PGI₂ (10 ng/ml; Figure 5f) or staurosporine (1 μM; Figure 5g), the aggregation was completely blocked. SITS (20 μM) and DTSSP (20 μM) failed to induce aggregation (Figures 5h and 5i respectively).

Platelet aggregation induced by DIDS occurred in the absence of ATP secretion (results not shown). The absence of dense-granule secretion was confirmed by experiments using 5-hydroxy[¹⁴C]tryptamine-labelled platelets (Figure 5, insert), which revealed that only $2.8 \pm 0.1\%$ of the total secretable 5-hydroxytryptamine was released after 5 min stimulation with DIDS. Also, in studies in the presence of apyrase, a scavenger of ADP, no inhibition of DIDS-induced aggregation was found, indicating that ADP was not released and did not contribute to platelet activation upon stimulation by DIDS.

Figure 6 illustrates in more detail the inhibition by oxymetazoline of DIDS-induced aggregation, measured by the disappearance of single platelets at 2 min after stimulation. Oxymetazoline dose-dependently inhibited DIDS-induced aggregation, showing an EC₅₀ of about 70 μM and more than 90% inhibition at 500 μM. Yohimbine was a less potent inhibitor than oxymetazoline, with an EC₅₀ of about 700 μM and complete inhibition at 1 mM. These values are 10–100 times those for inhibition of adrenaline-induced single-platelet disappearance.

DISCUSSION

The results presented in this paper show that DIDS activates the Na⁺/H⁺ exchanger, thereby inducing an increase in pH_i. This action has all the properties of a specific reaction involving the α_{2A} -AR, since it is abolished by α_{2A} -AR antagonists and appears to be independent of two other properties of DIDS, that are the inhibition of Cl⁻/HCO₃⁻ exchange and cross-linking of membrane glycoproteins. Both SITS, which is equally effective as DIDS as a blocker of Cl⁻/HCO₃⁻ exchange, and DTSSP, which,

similarly to DIDS, is a non-cell-permeant cross-linking reagent that spans 1.2 nm [31], failed to activate the Na⁺/H⁺ exchanger. Therefore DIDS appears a suitable tool with which to investigate signal processing via the α_{2A} -AR in platelets and possibly also in other cells.

The first question to address was whether the activation of the Na⁺/H⁺ exchanger was directly caused by the binding of DIDS to the α_{2A} -AR or whether other cell signalling pathways were involved. Banga et al. [17] postulated that binding of adrenaline to α_{2A} -AR on platelets directly activates the Na⁺/H⁺ exchanger and that activation of PLA₂ is a secondary event. Our observation that inhibition of prostaglandin endoperoxides and thromboxane A₂ formation fails to affect DIDS-induced pH_i changes and subsequent responses is in line with this model. The lack of a specific inhibitor for PLC-mediated signal transduction made it difficult to evaluate whether activation of the α_{2A} -AR triggered the activation of PLC. When DIDS was added to platelets, only minor changes in ³²P-labelled PtdIns(4,5)P₂ and metabolites were observed, which disappeared completely after treatment with staurosporine. Thus the minor activation of PLC seen after stimulation with DIDS occurs secondary to activation of PKC. Also, Fura-2 fluorescence remained in the range of that for unstimulated cells, indicating that DIDS did not initiate Ca²⁺ mobilization. Pretreatment with 8'-bromo-cGMP, which directly raises the cGMP content in the cell [38], partially inhibited the DIDS-induced alkalinization. We interpret these data as a direct effect of cGMP on the α_{2A} -AR or on the mechanisms that couple this receptor to the Na⁺/H⁺ exchanger.

An alternative pathway for formation of diacylglycerol is via phospholipase D (PLD)-mediated liberation of PA, followed by a dephosphorylation step. This would provide a second mechanism for activation of PKC. The observation that activation of PLD is secondary to an increase in PLC activity [39], and the fact that we failed to detect a major increase in [³²P]PA in the present experiments (Figure 3), argue against a role of PLD in DIDS-induced platelet activation.

DIDS-induced pH_i changes were completely abolished by raising the level of cAMP. Wachtfogel et al. [40] have shown that an increase in cAMP in platelets leaves the number of α_{2A} -AR and the K_d undisturbed in studies using [³H]yohimbine as a ligand. The binding of DIDS to the α_{2A} -AR may therefore be equally insensitive to alterations in the cAMP concentration, and the inhibition of Na⁺/H⁺ exchange by cAMP must be due to interference with the mechanisms that control the Na⁺/H⁺ exchanger upon activation with DIDS.

Previous studies have demonstrated that direct activation of PKC with phorbol ester or 1-oleoyl-2-acetyl-glycerol induced cytosolic alkalinization, due to activation of the Na⁺/H⁺ exchanger [41]. These findings indicate that phosphorylation of the Na⁺/H⁺ exchanger by PKC is an important mechanism for pH_i control. Recently Pouyssegur and colleagues cloned the human Na⁺/H⁺ exchanger and found phosphorylation of the antiporter by growth factor and phorbol ester [42,43]. Our present data indicate that the Na⁺/H⁺ exchanger in platelets is also regulated by a phosphorylation step. When platelets were preincubated with staurosporine, a widely used inhibitor of PKC, the DIDS-induced Na⁺/H⁺ exchange was completely inhibited. Although staurosporine has a number of side effects [44–46], a role for PKC in Na⁺/H⁺ exchange control is also indicated by the changes in phosphorylation of the 47 kDa protein, which varied in parallel with the changes in pH_i. When platelets were treated with 8'-bromo-cGMP, both 47 kDa-protein phosphorylation and the cytosolic alkalinization were delayed, and a preincubation with dbcAMP prevented both responses. It is uncertain whether the effects of 8'-bromo-cGMP and dbcAMP illustrate a direct

modulation of PKC activity. There is little information on the sensitivity of PKC to alterations in the level of cGMP. With regard to cAMP, conflicting reports have been published, with some authors describing inhibition of 47 kDa-protein phosphorylation in platelets activated by phorbol 12-myristate 13-acetate (PMA) or 1-oleoyl-2-acetyl glycerol [47] and others finding no interference [48]. We were repeatedly unable to show inhibition of 47 kDa-protein phosphorylation by dbcAMP (2.5 mM) in platelets stimulated with PMA (results not shown). We therefore propose that the sensitivity to cAMP and, possibly, to cGMP of the DIDS-induced PKC activation is related to a distinct step that mediates the interaction between the α_{2A} -AR and PKC. By analogy with the studies on DIDS-induced Na^+/H^+ exchange, the changes in PKC activity were an α_{2A} -AR-mediated event, since oxymetazoline completely inhibited the phosphorylation of the 47 kDa protein.

Previous studies have revealed a close correlation between the phosphorylation of the 47 kDa protein and the exposure of binding sites for fibrinogen on GPIIb/IIIa [5]. The present data show a similar relation when DIDS is the activating agent. Again, the binding of ^{125}I -fibrinogen was completely prevented by oxymetazoline and yohimbine, confirming the participation of the α_{2A} -AR. Preincubation with indomethacin had no effect, whereas 8'-bromo-cGMP induced a similar delay to that seen with measurements of pH_i and 47 kDa-protein phosphorylation. Not surprisingly, staurosporine inhibited all specific ^{125}I -fibrinogen binding. Also an increase in the cAMP level (by dbcAMP or PGI_2) completely inhibited the exposure of fibrinogen-binding sites induced by DIDS. This inhibition is also seen when platelets are stimulated with agonists such as ADP, α -thrombin and adrenaline [49].

It is apparent that the platelet responses induced by DIDS and adrenaline have much in common. Both agonists activate platelets via the α_{2A} -AR, resulting in the activation of the Na^+/H^+ exchanger [17], independent of PLC [17] or changes in cytosolic Ca^{2+} content [11,12]. They also induce fibrinogen binding and aggregation without shape change and secretion [12,50]. A notable difference is the activation of PKC. Whereas adrenaline induces little or virtually no activation of PKC [51,52], the DIDS-induced PKC activation is comparable with that seen in α -thrombin-treated platelets. However, there is some evidence that adrenaline activates PKC in the presence of fibrinogen [17] and that it enhances PKC activity in the presence of the Ca^{2+} ionophore A23187 [52] or ADP [51]. The recent observation that adrenaline induces fibrinogen binding via a staurosporine-sensitive mechanism [5] also supports the concept that the α_{2A} -AR is coupled to PKC.

Human platelets are a prototypic tissue for α_{2A} -adrenergic receptors. The receptor consists of 430 amino acids and contains seven putative transmembrane spanning domains, thus placing this receptor in the family of G-protein-coupled receptors [53]. The different transmembrane domains serve in the binding of agonists and antagonists. The fourth and seventh domains are involved in the binding of adrenaline and noradrenaline [54,55], whereas the third and seventh take part in the binding of yohimbine [56,57]. Oxymetazoline probably binds to other domains, since point mutations in the second and third transmembrane domains of the α_{2A} -AR differently affect the binding of yohimbine and oxymetazoline [56].

The affinity of α_{2A} -adrenergic receptors for agonists as well as antagonists is regulated by Na^+ ions, which bind to the second transmembrane domain. Mutation of aspartate-79 in this domain abolishes the Na^+ -sensitivity of agonists as well as antagonists [58]. Oxymetazoline probably binds to the Na^+ -binding site or to an adjacent region, since it contains an imidazole nucleus [59].

DIDS may bind to the same site, since the Na^+ -sensitivity of [^3H]yohimbine binding is completely abolished by 1 mM DIDS [19]. Thus differences in binding of yohimbine and oxymetazoline may explain why oxymetazoline is a much more potent antagonist of DIDS-induced Na^+/H^+ exchange and platelet aggregation than is yohimbine. The sensitivity of DIDS-induced platelet aggregation to oxymetazoline as well as to yohimbine is much lower than that of adrenaline-induced responses. Probably the difference in binding sites of these components affects the sensitivity to these antagonists. Thus, although both adrenaline and DIDS appear to activate platelets via the α_{2A} -AR, their interaction with the receptor may show considerable differences.

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