Nitroprusside differentially inhibits ADP-stimulated calcium influx and mobilization in human platelets

Robert O. MORGAN and Andrew C. NEWBY

Department of Cardiology, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN, U.K.

1. The effect of nitroprusside on cGMP concn., cAMP concn., shape change, aggregation, intracellular free Ca^{2+} concn. (by quin-2 fluorescence) and Mn^{2+} entry (by quenching of quin-2) was investigated in human platelets incubated with 1 mM- Ca^{2+} or 1 mM-EGTA. 2. Nitroprusside (10 nM–10 μM) caused similar concentration-dependent increases in platelet cGMP concn. and was without effect on cAMP concn. in the presence of extracellular Ca^{2+} or EGTA. 3. In ADP (3–6 μM)-stimulated platelets, nitroprusside caused 50% inhibition of shape change at 0.4 μM (+ Ca^{2+}) or 1.3 μM (+ EGTA), aggregation at 0.09 μM (+ Ca^{2+}) and of increased intracellular Ca^{2+} at 0.02 μM (+ Ca^{2+}) or 2.1 μM (+ EGTA). Entry of 1 mM- Mn^{2+} (– Ca^{2+}) was inhibited by 80% by 5 μM -nitroprusside. 4. In ionomycin (20–500 nM)-stimulated platelets, nitroprusside (10 nM–100 μM) did not inhibit shape change or intracellular- Ca^{2+} -increase responses, and only partially inhibited aggregation. 5. In phorbol myristate acetate (10 nM)-stimulated platelets, neither shape change nor aggregation was inhibited by 5 μM -nitroprusside. 6. The data demonstrate that nitroprusside inhibits ADP-mediated Ca^{2+} influx more potently than Ca^{2+} mobilization. Nitroprusside appears not to influence Ca^{2+} efflux or sequestration and not to affect the sensitivity of the activation mechanism to intracellular Ca^{2+} concn. or activation of protein kinase C.

INTRODUCTION

In blood platelets and vascular smooth muscle, increases in both cAMP and cGMP cause negative regulation (Kukovetz *et al.*, 1981; MacIntyre *et al.*, 1985). This may be important in mediating additive, synergistic (Radomski *et al.*, 1987a) or differential (Radomski *et al.*, 1987b) inhibitory effects of the two endothelium-derived regulators prostacyclin and endothelium-derived relaxant factor, which may thus subserve antithrombotic and antispasmodic roles (Moncada, 1982; Furchgott, 1983; Furlong *et al.*, 1987).

Because of the importance of decreasing platelet aggregability and vasomotor tone in patients with coronary artery disease, there has been intense interest in the mechanism of action of cAMP- and cGMP-increasing agents. In platelets, increasing cAMP inhibits agonist-induced rises in intracellular Ca^{2+} concn. (Yamanishi *et al.*, 1983; Bushfield *et al.*, 1985; Pannochia & Hardisty, 1985; Sage & Rink, 1985), stimulates Ca^{2+} sequestration (Fox *et al.*, 1979; Kaser-Glanzmann *et al.*, 1979) and decreases the Ca^{2+} -sensitivity of the activation mechanism (Knight & Scrutton, 1984; Pannochia & Hardisty, 1985). Increasing cGMP is also known to inhibit agonist-induced increase in Ca^{2+} concn. (Kawahara *et al.*, 1984; MacIntyre *et al.*, 1985; Nakashima *et al.*, 1986), but it has been less extensively studied. In vascular smooth muscle, increasing cAMP may have a primary effect on the Ca^{2+} -sensitivity of the contractile proteins (Kerrick & Hoar, 1981; Morgan & Morgan, 1984), whereas increasing cGMP may decrease intracellular Ca^{2+} concn. by inhibiting Ca^{2+} influx and mobilization (Karaki *et al.*, 1984; Collins *et al.*, 1986) or stimulating extrusion (Itoh *et al.*, 1983; Suematsu *et al.*, 1984).

The present study was undertaken to define, more precisely, the sites of action of nitroprusside on human platelets and, in particular, to test whether only transduction of receptor-mediated activation was inhibited, rather than events distal to increase in intracellular Ca^{2+} concn. and diacylglycerol formation. Secondly, since ADP has been shown to trigger kinetically distinguishable Ca^{2+} -influx and Ca^{2+} -mobilization processes in platelets (Sage & Rink, 1986, 1987), we investigated whether nitroprusside was equally effective in inhibiting these processes.

MATERIALS AND METHODS

Preparation of resuspended and quin-2-loaded resuspended platelets

Blood was obtained by venepuncture from 17 healthy volunteers (10 male, 7 female) who had not recently taken drugs known to affect platelet function. Blood (20–40 ml) was mixed in the ratio 17:3 (v/v) with a solution containing 15 g of trisodium citrate/l, 15 g of citric acid/l and 20 g of D-glucose/l. The mixture was centrifuged at 700 g for 5 min at room temperature (23 °C) and the supernatant, platelet-rich plasma, was aspirated.

Resuspended and quin-2-loaded resuspended platelets were prepared as described by Hallam & Rink (1985a). Platelet-rich plasma was centrifuged at 900 g for 10 min in flat-bottomed tubes, and as much of the supernatant as possible was removed. The pellet was resuspended by aspiration into a plastic Pasteur pipette to twice the original volume with a 'resuspension buffer' containing 145 mM-NaCl, 5 mM-MgSO₄, 10 mM-Hepes/NaOH, pH 7.4, 10 mM-glucose, 1 mg of fatty-acid-free bovine serum

Abbreviations used: quin-2, 2-[[2-bis(carboxymethyl)amino-5-methylphenoxy]methyl]-6-methoxy-8-bis(carboxymethyl)aminoquinidine; PMA, phorbol 12-myristate 13-acetate; cAMP, cyclic AMP; cGMP, cyclic GMP.

albumin/ml, 0.1 unit (as defined by supplier) of apyrase/ml and 5 munits of hirudin/ml. Resuspension buffer was added to adjust the suspension to a concn. (determined by counting in a haemocytometer) of $(1-2) \times 10^8$ platelets/ml. To prepare quin-2-loaded resuspended platelets, platelet-rich plasma was supplemented with 0.4 unit of apyrase/ml and 20 munits of hirudin/ml, and then quin-2 acetoxymethyl ester was added to a final concn. of $15 \mu\text{M}$. The mixture was incubated for 30 min at room temperature with gentle agitation and then diluted with an equal volume of 'resuspension buffer'. EGTA was then added (final concn. 2 mM), and platelets were then resuspended as described above.

Measurement of shape change and aggregation

Aggregometry was performed on stirred (1100 rev/min) suspensions of resuspended platelets in silicone-treated glass tubes in a single-channel Born-type aggregometer at either 23°C or 37°C as indicated in the text. Transmission was set at 20% with resuspended platelets and 90% with resuspension buffer. Platelets were pretreated with 1 mM- Ca^{2+} (from a solution of 100 mM- CaCl_2) or 1 mM-EGTA (from a solution of 100 mM-sodium EGTA, pH 7.4) for 1-3 min before addition of agonists from solutions at 100 times their final concn.

Shape change was quantified by measuring the peak decrease in light transmission after adding agonists. In the absence of added fibrinogen, shape change in the resuspended platelets used here was rarely followed by aggregation, so that the nadir of light transmission was usually sustained for several minutes.

To measure aggregation, the platelet suspension was supplemented with 1 mg of fibrinogen/ml. Aggregation was quantified by measuring the maximum rate of increase and the peak increase in light transmission after adding agonist.

Measurement of intracellular Ca^{2+} concn.

Intracellular Ca^{2+} concn. was measured in stirred suspensions of quin-2-loaded resuspended platelets in a glass cuvette at room temperature by using a Perkin-Elmer (204A) spectrofluorimeter attached to a chart recorder. Excitation was at 339 nm and emission at 500 nm. Platelets were pretreated with 1 mM- Ca^{2+} or 1 mM-EGTA for 1-3 min, at the end of which basal fluorescence emission was noted. The peak fluorescence after adding agonist was also noted. Basal and stimulated intracellular Ca^{2+} concns. were calculated as described by Tsien *et al.* (1982) after observing fluorescence maxima after addition of $50 \mu\text{M}$ -digitonin (final concn.) and then fluorescence minimum in the presence of 25 mM-EGTA (final concn.).

Inhibition by nitroprusside

Inhibition by nitroprusside was measured against concns. of agonist just sufficient to give a maximal response. Owing to the ease with which platelets become desensitized to ADP during quin-2 loading, resuspension and incubations (Hallam & Rink, 1985a), the ADP-sensitivity of each batch of platelets was determined by using incremental concns. The just-maximal ADP concn. for shape change was 0.3 or $0.6 \mu\text{M}$, and that for aggregation or intracellular Ca^{2+} increase 3 or $6 \mu\text{M}$. Inhibition of shape change by nitroprusside was also investigated at the ADP concn. giving maximal

aggregation. Having determined the ADP sensitivity, samples from the same platelet suspension were preincubated with 1 mM- Ca^{2+} or 1 mM-EGTA for 2 min, and then different concentrations of nitroprusside were added for 1 min, followed by ADP. At the end of each series of experiments, the response to ADP in the absence of nitroprusside was redetermined, and, if different from the initial response by more than 20%, the results of the whole experiment were discarded.

Measurement of platelet cGMP and cAMP concn.

The concentration of cGMP and cAMP were measured as described by MacIntyre *et al.* (1985), by using resuspended platelets. Portions of suspension (1 ml) were preincubated at 37°C for 3 min with either 1 mM- Ca^{2+} or 1 mM-EGTA. Sodium nitroprusside was then added from aqueous solution at 100 times the final concn. After further incubation for 1 min, reactions were terminated with 2 ml of ethanol, and the mixture was then centrifuged at 500 g for 10 min at 4°C . The deproteinized supernatant was removed, evaporated to dryness at 23°C and the residue was redissolved in 0.5 ml of 50 mM-sodium acetate buffer, pH 6.2. The concn. of GMP or cAMP was measured by radioimmunoassay.

Statistical methods

Values are given throughout as means $\pm$ S.E.M. for the numbers of observations given in parentheses. Means were compared by Student's *t* test, by using unpaired data unless otherwise indicated.

Materials

ADP (A9021), apyrase (A7646), hirudin (H7016), human fibrinogen (F4883) and phorbol myristate acetate (PMA; P8139) were obtained from Sigma Chemical Co., Poole, Dorset, U.K. The quin-2 acetoxymethyl ester used was obtained either from Lancaster Synthesis, Morecambe, Lancs., U.K. or from Sigma as indicated in the text. Ionomycin, a product of Calbiochem, was purchased from Cambridge Biosciences, Cambridge, U.K.

RESULTS

Effects of nitroprusside on platelet cGMP concn.

In platelets incubated with either 1 mM- Ca^{2+} or 1 mM-EGTA, nitroprusside caused a similar dose-dependent elevation of cGMP concn. (Fig. 1). The 30-40% elevation with 10 nM-nitroprusside in the presence of either 1 mM- Ca^{2+} or 1 mM-EGTA was significant ($P < 0.05$, paired *t* test). The approx. 2.5-fold rises with 100 nM-nitroprusside, approx. 4-fold with $1 \mu\text{M}$ -nitroprusside and approx. 8-fold with $10 \mu\text{M}$ -nitroprusside were all significant when paired or unpaired data were used. Nitroprusside (10 nM- $10 \mu\text{M}$) did not elevate platelet cAMP concn. in the presence of either extracellular Ca^{2+} or EGTA (Fig. 1). To increase the power of this analysis, normalized changes in cAMP concn. relative to the absence of nitroprusside computed from the data in Fig. 1 were plotted against the log concn. of nitroprusside. No significant correlation was found in the presence of either extracellular Ca^{2+} ($r = 0.20$) or EGTA ($r = 0.13$, 22 degrees of freedom in each case).

Fig. 1. Nitroprusside-induced elevation of platelet cGMP or cAMP concn.

Human platelets [(1–2) × 10⁸/ml] were preincubated in duplicate for 3 min at 37 °C with 1 mM-CaCl₂ (○) or 1 mM-EGTA (□). Sodium nitroprusside was then added for 1 min at the concns. shown, and the platelets were then extracted with ethanol and cAMP concn. (a) or cGMP concn. (b) was measured by radioimmunoassay. Values are means ± S.E.M. for six different batches of platelets. *P < 0.05 versus the absence of nitroprusside.

Effects of nitroprusside on platelet responses to ADP

ADP (0.3–0.6 μM) induced a similar shape-change response in platelets incubated in the presence of extracellular Ca²⁺ or EGTA (Fig. 2). The magnitude of the response was also similar when platelets were activated with 3–6 μM-ADP (Fig. 2). Nitroprusside (5 nM–5 μM) inhibited shape-change responses to low (0.3–0.6 μM) and high (3–6 μM) concns. of ADP (Fig. 2). In the presence of either extracellular Ca²⁺ or EGTA, nitroprusside was less potent against the higher concns. of ADP (Table 1). Nitroprusside was approx. equipotent against shape-change responses to 0.3–0.6 μM-ADP in the presence of extracellular Ca²⁺ or EGTA (Table 1), but was significantly less potent against shape-change responses to 3–6 μM ADP in the presence of extracellular EGTA than in the presence of extracellular Ca²⁺ (Table 1).

Higher concentrations of ADP (3–6 μM) were required to produce a maximal aggregation response than those

Fig. 2. Concentration-dependence of nitroprusside inhibition of ADP-induced shape change

Stirred human platelets [(1–2) × 10⁸/ml] were preincubated at 37 °C for 1 min with 1 mM-CaCl₂ (●, ■) or for 4 min with 1 mM-EGTA (○, □). Where shown, sodium nitroprusside was then added for a further 1 min, followed by 0.3–0.6 μM-ADP (○, ●) or 3–6 μM-ADP (□, ■). Shape change was measured on a chart recorder as peak decrease in light transmission (1 cm was equivalent to 4% transmission). Values are means ± S.E.M. obtained from 4–21 preparations of platelets. *P < 0.005 versus the absence of nitroprusside.

(0.3–0.6 μM) giving a maximal shape-change response (results not shown). Nitroprusside (10 nM–1 μM) inhibited peak aggregation responses to 3–6 μM-ADP of platelets incubated in the presence of extracellular Ca²⁺ and fibrinogen (Fig. 3) with a half-maximal concentration of 90 nM, which was significantly less than that needed to inhibit the shape-change response to the same concns. of ADP (Table 1). A similar effect was found on the maximum rate of aggregation (results not shown).

In platelets loaded with quin-2 obtained from Lancaster Synthesis, the resting intracellular Ca²⁺ concn. was not different in the presence of extracellular Ca²⁺ (37 ± 3 nM, *n* = 40) or EGTA (40 ± 5 nM, *n* = 14). Nitroprusside had no effect on resting intracellular Ca²⁺ concn. (Fig. 4b). ADP (0.3–6 μM) produced a dose-dependent increase in intracellular Ca²⁺ concentration (Fig. 4a). ADP (3–6 μM) elevated Ca²⁺ concentrations approx. 7-fold in the presence of extracellular Ca²⁺, but only approx. 2-fold in the presence of extracellular EGTA (Fig. 4b). Nitroprusside (10 nM–5 μM) inhibited ADP-induced Ca²⁺ concn. rises, and was significantly more potent in the presence of extracellular Ca²⁺ than in the presence of extracellular EGTA (Fig. 4b, Table 1).

In platelets loaded under the same conditions with quin-2 obtained from Sigma, resting intracellular Ca²⁺ concn. was 73 ± 6 nM (*n* = 12) in the presence of extracellular Ca²⁺, and ADP (3–6 μM) elevated this to 1.1 ± 0.2 μM (*n* = 12). In the presence of extracellular EGTA, resting and ADP-stimulated intracellular Ca²⁺ concns. were 64 ± 10 nM and 170 ± 30 nM (*n* = 9)

Table 1. Summary of inhibitory potencies of nitroprusside

Log(concn.)–response relationships were constructed as detailed in the legends to Figs. 2, 3 and 4(b) for the numbers of separate batches of platelets shown. For each batch the log(concn.) of nitroprusside giving 50% inhibition was estimated by interpolation and the antilog value calculated. Values shown are means $\pm$ S.E.M. Means were compared as log (concn.) by using an unpaired Student's *t* test: **P* < 0.005 versus shape change in the presence of extracellular Ca^{2+} ; †*P* < 0.001 versus elevation of intracellular Ca^{2+} concn. in the presence of extracellular Ca^{2+} .

Process	ADP concn. (μM)	Added agents	Nitroprusside concn. giving 50% inhibition (μM)	No. of observations
Shape change	0.3–0.6	Ca^{2+}	0.15 ± 0.03	20
	0.3–0.6	EGTA	0.20 ± 0.05	11
	3–6	Ca^{2+}	0.43 ± 0.08	5
	3–6	EGTA	$1.3 \pm 0.3^*$	7
Aggregation	3–6	Ca^{2+} , fibrinogen	$0.09 \pm 0.03^*$	10
Elevation of intracellular Ca^{2+} concn.	3–6	Ca^{2+}	0.016 ± 0.006	14
	3–6	EGTA	$2.1 \pm 0.8^\dagger$	7

Fig. 3. Effect of nitroprusside on ADP- or ionomycin-induced aggregation

Stirred human platelets $[(1-2) \times 10^8/\text{ml}]$ were preincubated for 1 min at 37°C in the presence of 1 mM-Ca^{2+} and 1 mg of fibrinogen/ml. Sodium nitroprusside was then added as indicated for 1 min, followed by $3-6\text{ }\mu\text{M-ADP}$ (○) or $0.2\text{ }\mu\text{M-ionomycin}$ (●). Aggregation was measured as the peak increase in light transmission and was related in each batch of platelets to the difference in light transmission between the unstimulated platelet suspension and platelet-poor plasma. Values are means $\pm$ S.E.M. obtained from 5–18 batches of platelets stimulated with ADP and 5–15 batches stimulated with ionomycin: **P* < 0.05 versus aggregation in the absence of nitroprusside.

respectively. Nitroprusside ($10\text{ nM}-1\text{ }\mu\text{M}$) was again more potent in inhibiting Ca^{2+} -concn. rises in the presence of extracellular Ca^{2+} rather than EGTA (Fig. 5). As with the data in Fig. 4(b), the lowest significantly effective concentration of nitroprusside was $30-50\text{ nM}$ in the presence of extracellular Ca^{2+} and $1\text{ }\mu\text{M}$ in the presence of EGTA. Moreover, 50% inhibition of the Ca^{2+} concn. rises took place at similar nitroprusside concns. (Fig. 4b, cf. Fig. 5).

Addition of ADP ($6\text{ }\mu\text{M}$) to platelets incubated without added extracellular Ca^{2+} transiently increased quin-2 fluorescence (Fig. 6d) to an extent similar to that in the presence of extracellular EGTA (results not shown). Nitroprusside ($5\text{ }\mu\text{M}$) inhibited the ADP-induced increase in quin-2 fluorescence (Fig. 6a). When platelets were preincubated with 1 mM-Mn^{2+} in the absence of added Ca^{2+} , ADP first induced a transient increase in quin-2 fluorescence and then an accelerated decrease to a steady value below that before the addition of ADP (Fig. 6b). As shown by Hallam & Rink (1985b), this decrease is due to quenching of quin-2 during transient ADP-induced stimulation of Mn^{2+} entry. Nitroprusside ($5\text{ }\mu\text{M}$) inhibited both the initial ADP-induced rise in quin-2 fluorescence and the subsequent fall (Fig. 6c). In a series of such experiments the decrease in quin-2 fluorescence after addition of ADP ($6\text{ }\mu\text{M}$) to platelets incubated with 1 mM-Mn^{2+} amounted to $36 \pm 4\%$ (8) of the value before addition of ADP. In platelets pretreated for 1 min with $5\text{ }\mu\text{M-nitroprusside}$, the ADP-induced decrease in quin-2 fluorescence was only $7 \pm 3\%$ (8) of its initial value (*P* < 0.001).

Effects of nitroprusside on platelet responses to ionomycin

Ionomycin (20 nM) induced a shape-change response in platelet suspensions stirred at 37°C in the presence of 1 mM-Ca^{2+} , which was similar in magnitude ($2.1 \pm 0.3\text{ cm}$, $n = 6$) to that seen with ADP (cf. Fig. 2). After preincubation for 1 min with $1\text{ }\mu\text{M-nitroprusside}$, shape-change response to ionomycin was unaltered ($1.8 \pm 0.2\text{ cm}$, $n = 7$), as it was with $100\text{ }\mu\text{M-nitroprusside}$

Fig. 4. Elevation of intraplatelet Ca^{2+} concn. by ADP, and its inhibition by nitroprusside

(a) Stirred suspensions of platelets loaded with quin-2 obtained from Lancaster Synthesis $[(1-2) \times 10^8/\text{ml}]$ were incubated for 1 min at room temperature (23°C) with 1 mM- CaCl_2 and then ADP was added at the concns. shown. The peak increase in fluorescence was measured and used to calculate the peak intracellular Ca^{2+} concns. in accordance with Tsien *et al.* (1982). Values are means $\pm$ S.E.M. obtained from 4-22 batches of platelets: * $P < 0.05$ versus unstimulated platelets. (b) Stirred quin-2

Fig. 5. Inhibition by nitroprusside of intra-platelet Ca^{2+} concn. in quin-2 (Sigma)-loaded platelets

Stirred quin-2 (Sigma)-loaded platelets were preincubated for 1 min at 23°C in the presence of 1 mM- Ca^{2+} (●) or 1 mM-EGTA (○). ADP ($3-6 \mu\text{M}$) was then added, and the peak increase in fluorescence was measured and used to calculate the peak intracellular Ca^{2+} concn. The peak increases in intracellular Ca^{2+} concn. in the presence of nitroprusside were then related, for each batch of platelets, to those obtained in its absence. Values are means $\pm$ S.E.M. ($n = 6$): * $P < 0.05$ versus the absence of nitroprusside (Student's *t* test with paired values).

(2.4 ± 0.2 cm, $n = 5$). Ionomycin (20 nM) also induced a similar shape change in the presence of 1 mM-EGTA (2.5 ± 0.2 cm, $n = 7$). This was again unaffected by $1 \mu\text{M}$ -nitroprusside (2.3 ± 0.3 cm, $n = 16$).

Ionomycin ($0.5 \mu\text{M}$) induced aggregation in platelets incubated in the presence of 1 mM- Ca^{2+} and 1 mg of fibrinogen/ml (Fig. 3). The peak aggregation response was inhibited by approx. 30% by pretreatment with $1 \mu\text{M}$ -nitroprusside, and by approx. 40% with $10 \mu\text{M}$ -nitroprusside (Fig. 3). A similar inhibitory effect of nitroprusside was seen on the maximum rate of aggregation (results not shown).

(Lancaster Synthesis)-loaded platelets were preincubated for 1 min at 23°C in the presence of 1 mM- CaCl_2 (●, ■) or 1 mM-EGTA (○). Sodium nitroprusside was then added, and the final Ca^{2+} concns. in unstimulated platelets was assessed after 5 min (■; $n = 4-10$). Alternatively ADP ($3-6 \mu\text{M}$) was added after 1 min (●, $n = 7-12$; ○, $n = 6-8$) and the peak increase in fluorescence was measured and used to calculate the peak intracellular Ca^{2+} concns. Values are means $\pm$ S.E.M.: * $P < 0.05$ versus the absence of nitroprusside.

Fig. 6. Quenching of quin-2 fluorescence by Mn^{2+} and its inhibition by nitroprusside

Stirred quin-2 (Lancaster Synthesis)-loaded platelets (P) were preincubated at 23 °C with (a, b) or without (c, d) 1 mM- $MnCl_2$ (M) and with (a, c) or without 5 μM -sodium nitroprusside (N) for 1 min before addition of 6 μM -ADP (A). After the fluorescence had been monitored for at least 4 min, 50 μM -digitonin (D) was added together with 1 mM- $MnCl_2$ if not previously present, and fluorescence minimum was subsequently measured. A representative is shown of similar experiments on eight batches of platelets.

Table 2. Effect of nitroprusside on ionomycin-induced elevation of cytosolic Ca^{2+} concn.

Stirred quin-2 (Lancaster Synthesis)-loaded platelets $[(1-2) \times 10^8/\text{ml}]$ were preincubated for 2 min at 23 °C with either 1 mM- $CaCl_2$ or 1 mM-EGTA, and then sodium nitroprusside was added for 1 min at the concns. shown. After a further 1 min ionomycin was added, and the peak of fluorescence was recorded and used to calculate the peak intracellular Ca^{2+} concn. in accordance with Tsien *et al.* (1982). Values are means $\pm$ S.E.M. for n batches of platelets: * $P < 0.05$ versus the absence of ionomycin; †not significantly different from in the absence of nitroprusside.

Addition	Ionomycin concn. (μM)	Nitroprusside concn. (μM)	Peak cytosolic Ca^{2+} concn. (μM)
1 mM- Ca^{2+} ($n = 6$)	0	0	24 ± 8
	0	5	$20 \pm 5^\dagger$
	0.05	0	$64 \pm 10^*$
	0.05	5	$54 \pm 12^\dagger$
	0.5	0	$620 \pm 150^*$
	0.5	5	$720 \pm 140^\dagger$
1 mM-EGTA ($n = 5$)	0	0	15 ± 4
	0	1	$16 \pm 4^\dagger$
	0.5	0	$37 \pm 5^*$
	0.5	1	$34 \pm 3^\dagger$

In the presence of extracellular Ca^{2+} , ionomycin produced a concn.-dependent increase in intracellular Ca^{2+} concn. (Table 2). Nitroprusside (5 μM) was without effect on the ionomycin-induced elevation in intracellular Ca^{2+} concn. (Table 2), when this was either less than or greater than that produced by 3–6 μM -ADP (cf. Fig. 4b). Ionomycin also produced a small increase in intracellular Ca^{2+} in platelets incubated with extracellular EGTA (Table 2). This rise was not inhibited by 5 μM -nitroprusside (Table 2).

Effect of nitroprusside on platelet responses to phorbol ester

PMA (10 nM) caused a slow shape change and aggregation response in platelets incubated with Ca^{2+} . No phase of this response was inhibited by 5 μM -nitroprusside. Thus the magnitude of the shape-change response to 10 nM-PMA was 1.5 ± 0.3 cm ($n = 8$) in the absence and 1.6 ± 0.3 cm ($n = 8$) in the presence of 5 μM -nitroprusside. The maximum rate of aggregation was 0.9 ± 0.2 cm/min ($n = 9$) in the absence and 0.7 ± 0.1 cm/min ($n = 9$) in the presence of 5 μM -nitroprusside.

DISCUSSION

We confirmed the observation by Hallam & Rink (1985a) that ADP induced a small rise in intracellular Ca^{2+} concn. in quin-2-loaded platelets incubated with extracellular EGTA, and a much larger rise in the presence of extracellular Ca^{2+} . This suggested that both Ca^{2+} influx and mobilization from intracellular stores were activated by ADP. Success in distinguishing these processes probably depends on the ability of quin-2, at these levels of loading, to buffer the resulting rises in intracellular Ca^{2+} concn., since in fura-2-loaded platelets, ADP-induced Ca^{2+} mobilization is sufficient almost to saturate the dye, and hence fluorescence in the presence and absence of extracellular Ca^{2+} is similar (Sage & Rink, 1986, 1987). The consistent differences in resting and ADP-stimulated intracellular Ca^{2+} concentration obtained by us when loading with quin-2 from two different sources suggest differing degrees of buffering. Nonetheless, in both cases Ca^{2+} influx and mobilization could be quantified. To confirm that ADP triggered bivalent-cation influx into platelets, Hallam & Rink (1985b) showed that ADP stimulated the quenching of intracellular quin-2 by extracellularly applied Mn^{2+} , and we confirmed this observation also.

Nitroprusside inhibited ADP-induced increases in intracellular Ca^{2+} concn. in the presence of both extracellular Ca^{2+} and EGTA. Nitroprusside also

inhibited the ADP-stimulated quenching of quin-2 by extracellular Mn^{2+} , confirming inhibition of ADP-stimulated bivalent-cation influx. Nitroprusside inhibited Ca^{2+} influx 100 times more potently than it did Ca^{2+} mobilization, despite causing similar rises in intra-platelet cGMP concn. in the presence of extracellular Ca^{2+} or EGTA. The differential effects of nitroprusside were apparent under both conditions of loading of quin-2. These data provide further evidence that ADP-induced Ca^{2+} influx and Ca^{2+} mobilization are independent processes, as suggested by the rapid kinetic studies of Sage & Rink (1986, 1987). Indeed, a Ca^{2+} channel directly stimulated by ATP has been detected in isolated arterial myocytes (Benham & Tsien, 1987), although its relationship, if any, to the ADP-activation mechanism in platelets has yet to be established. Nitroprusside has been previously shown to inhibit both Ca^{2+} influx and Ca^{2+} mobilization during platelet responses to thrombin (Kawahara *et al.*, 1984; Nakashima *et al.*, 1986) or platelet-activating factor (MacIntyre *et al.*, 1985), but difference in potency against the two processes have not been noted. With thrombin and platelet-activating factor, rapid kinetic analysis (Sage & Rink, 1986, 1987) showed that both processes were triggered almost simultaneously, rather than Ca^{2+} influx clearly preceding Ca^{2+} mobilization as was the case for ADP. There is therefore no conclusive evidence that thrombin-stimulated Ca^{2+} influx and Ca^{2+} mobilization are independent processes.

Nitroprusside also exhibited different potency when inhibiting the various platelet responses to ADP measured, and differences in sensitivity to intracellular Ca^{2+} may have accounted partly for this. Aggregation took place only at 3–6 μM -ADP, and therefore had a higher apparent intracellular Ca^{2+} threshold than for shape change (cf. Hallam & Rink, 1985a). That aggregation was readily antagonized by nitroprusside may be explained if only a relatively small fall in intracellular Ca^{2+} concn. was required. Shape change in response to 0.3–0.6 μM -ADP took place with only a marginal increase in intracellular Ca^{2+} concn. (Fig. 4a; cf. Hallam & Rink, 1985a; Simpson *et al.*, 1986), and was also readily inhibited by nitroprusside, possibly for the same reason. Shape change in response to 3–6 μM -ADP was accompanied by a supramaximal rise in intracellular Ca^{2+} concn., and was less potently inhibited by nitroprusside, consistent with the need for a greater decrease in intracellular Ca^{2+} concn. However, nitroprusside probably exerts an effect additional to that on intracellular Ca^{2+} concn. In platelets stimulated with 3–6 μM -ADP in the presence of extracellular Ca^{2+} , for example, the concn. of nitroprusside that caused 50% inhibition of shape change (0.4 μM , Table 1) decreased intracellular Ca^{2+} concn. to approx. 110 nM (Fig. 4b), which was more than that accompanying maximal shape change in response to 0.3–0.6 μM -ADP in the absence of nitroprusside (Fig. 4a). Inhibition of diacylglycerol production by nitroprusside has been demonstrated in thrombin-stimulated platelets (Takai *et al.*, 1981), and might also therefore contribute to its effect on responses to ADP. It has been reported, however (Fisher *et al.*, 1985), that ADP does not stimulate phosphoinositide hydrolysis in human platelets. Rao & Kowalska (1987) have presented evidence that ADP affects shape change and elevation of Ca^{2+} concn. through different binding sites. This observation might also explain the existence of more than one nitroprusside-sensitive activatory pathway

in ADP-stimulated platelets. The final inhibitory potency of nitroprusside against platelet responses may therefore be determined by a complex function of its action on these pathways.

Elevation of cGMP concn. (Kawahara *et al.*, 1984; MacIntyre *et al.*, 1985; Nakashima *et al.*, 1986) appears to have a similar effect to elevation of cAMP concn. (Bushfield *et al.*, 1985; Sage & Rink, 1985, 1987) on Ca^{2+} influx and mobilization in response to thrombin or platelet-activating factor, although intracellular cGMP appears approx. 100 times more potent (MacIntyre *et al.*, 1985). This difference, and the observation that nitroprusside does not elevate platelet cAMP concn. (cf. MacIntyre *et al.*, 1985), suggest that the effect of cGMP is specific. This conclusion is further strengthened by our observation that increasing cGMP has a greater effect on ADP-induced Ca^{2+} influx whereas increasing cAMP appears to inhibit Ca^{2+} mobilization more readily (Sage & Rink, 1987).

Elevation of platelet cGMP concn. by nitroprusside did not prevent platelet responses to ionomycin or phorbol ester. This suggests that increasing cGMP in response to nitroprusside inhibits only the generation of second messengers in response to ADP, and not their action. Increasing platelet cAMP, by contrast, inhibits ionomycin-induced aggregation (Pannochia & Hardisty, 1985; Sage & Rink, 1985) and, partially, shape change (Sage & Rink, 1985). Although less fully studied, increased cAMP may also partially inhibit aggregation responses induced by phorbol esters (Ware *et al.*, 1985). In seeking to explain these differences, it may be important that cAMP, but not cGMP, decreases the sensitivity to Ca^{2+} concn. of stimulated 5-hydroxytryptamine release in permeabilized platelets (Knight & Scrutton, 1984). Indeed, Pannochia & Hardisty (1985) and Bushfield *et al.* (1985) conclude that decreasing the sensitivity to intracellular Ca^{2+} of the activatory mechanism may be the most important inhibitory mechanism for increasing cAMP.

Elevation of cGMP concn. did not affect ionomycin-induced rises in intracellular Ca^{2+} concn., even when these were less than or greater than responses to ADP. There was therefore no evidence for cGMP-dependent regulation of Ca^{2+} extrusion or sequestration. Elevation of cAMP concn. also fails to affect ionomycin-induced rises in Ca^{2+} concn. (Pannochia & Hardisty, 1985; Sage & Rink, 1985). There is, however, evidence from studies with permeabilized platelets (Yoshida *et al.*, 1988) that increased cAMP increases Ca^{2+} sequestration. The effect of increased cGMP should be tested in this context.

The present work emphasizes some important similarities and differences between the cyclic nucleotide regulation of platelet and smooth-muscle function. Elevation of cGMP concn. by nitroprusside appears to inhibit Ca^{2+} influx and Ca^{2+} mobilization in both platelets and vascular smooth muscle (cf. Karaki *et al.*, 1984; Collins *et al.*, 1986), and may therefore have a common mechanism. A further similarity is that increased cAMP, but not increased cGMP, decreases the Ca^{2+} -sensitivity for activation of both cell types (cf. Hathaway *et al.*, 1985). On the other hand, increased cAMP inhibits rises in intracellular Ca^{2+} concn. in platelets, but not in vascular smooth muscle (cf. Morgan & Morgan, 1984), and both cAMP and cGMP effect Ca^{2+} extrusion in vascular smooth muscle (cf. Itoh *et al.*, 1983; Suematsu *et al.*, 1984), but probably not in platelets. The basis for

these similarities and differences should be studied further, as they might be exploited for selective pharmacological intervention.

In conclusion, this work identifies two processes, receptor-mediated Ca^{2+} influx and mobilization, controlled by cGMP in human platelets. Attention should now be focussed on these processes to understand the molecular basis for their regulation.

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