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# Inhibition of Human Platelet Aggregation by Diazeniumdiolates: Extent of Inhibition Correlates with Nitric Oxide Load Delivered

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**Abstract**

The profile of nitric oxide (NO) release from the diazeniumdiolate class of NO donors was evaluated using inhibition of platelet aggregation as a model.

At 37°C, the NO complexes (*Z*)-1-{*N*-methyl-*N*-[6-(*N*-methylammoniohexyl)amino]}-diazen-1-ium-1,2-diolate (dimethylhexanediamine complex), sodium (*Z*)-1-(*N,N*-diethylamino)diazen-1-ium-1,2-diolate (diethylamine complex), (*Z*)-1-{*N*-[3-aminopropyl]-*N*-[4-(3-aminopropylammonio)butyl]amino}diazen-1-ium-1,2-diolate (spermine complex), and (*Z*)-1-[*N*-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (diethylenetriamine complex) have half-lives of 1, 2 and 39 min, and 20 h, respectively. All the diazeniumdiolates caused concentration-dependent inhibition of platelet aggregation; IC50 values (values for which the effect was half the maximum) were 26·0 ± 24·1, 34·9 ± 24·0 and 14·9 ± 6·4 nM for dimethylhexanediamine complex, diethylamine complex and spermine complex, respectively, when pre-incubated with platelets for one half-life. Inhibition by all compounds was time-dependent. Pretreatment of platelets with spermine complex for 5 and 39 min resulted in IC50 values of 1·7 ± 0·85 μM and 19·7 ± 0·12 nM, whereas IC50 values for sodium nitroprusside were 27·3 ± 1·25 nM and 25 nM (average, n = 2), at 5 and 39 min, respectively. Pre-incubation of each diazeniumdiolate at a concentration of 100 nM for 5 min at 37°C, which resulted in the theoretical delivery of NO loads from 96·9% down to 0·3%, resulted in decreasingly efficacious inhibition of platelet aggregation. Linear regression analysis of the theoretical NO load delivered against the actual maximum inhibition (%) showed a strong correlation ($r^2 = 0·975$). All four diazeniumdiolates caused concentration- and time-dependent inhibition of agonist-stimulated elevation of intra-platelet $Ca^{2+}$ levels; IC50 values were, respectively, 8·7 ± 1·49 nM and 11·5 ± 1·36 nM for dimethylhexanediamine complex and diethylamine complex at their half-lives, and 176 ± 16·9 nM and > 100 μM, for spermine complex and diethylenetriamine complex at 2 min pre-incubation time. The respective nucleophiles not complexed with NO did not show anti-aggregatory properties or inhibition of agonist-induced elevation of intra-platelet $Ca^{2+}$ levels. The inhibitory effects of all diazeniumdiolates tested were attenuated by 10 μM haemoglobin.

These studies indicate that these compounds induce controlled, predictable release of NO at biological pH and temperature.

Nitric oxide (NO) has come to the forefront as an important biological mediator, playing a major role in the cardiovascular, immune and central and peripheral nervous systems. The importance of NO signalling in several systems has led to an explosion of research in clinical and experimental medicine for pharmacological agents that mimic or modulate the effects of NO. One difficulty, however, is that the myriad of biological effects of NO in the body makes systemic treatment with pharmacological agents decidedly non-specific. Most currently available NO donors release their NO load rapidly and release is often dependent on thioproteins (Needleman et al 1969; Ahlner et al 1991), which are plentiful in plasma in man (Lorber et al 1964). It would be particularly useful therapeutically to have agents that exhibit controlled

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release of NO that is independent of thioactivation. Such an agent could be properly titrated to reduce or eliminate side-effects and still give effective treatment over a prolonged period of time.

Nitric oxide has been shown to be a potent inhibitor of platelet aggregation (reviewed in Ahlner et al 1991; Moncada et al 1991). Using a model of agonist-stimulated platelet aggregation, this study tested the hypothesis that diazeniumdiolates, because of their first-order kinetic release of NO (Maragos et al 1991), would produce a time-dependent and predictable platelet inhibition. The study tested predictability by examining the correlation between inhibition observed using a series of diazeniumdiolates with their theoretical release of NO according to first-order kinetics. Also tested, as a measure of predictability, were the dose- and time-dependent properties of the diazeniumdiolates. The data indicate that diazeniumdiolates have a time-dependent, highly predictable profile of pharmacological activity, and show potential for therapeutic use.

## Materials and Methods

*Drugs*

The nitric oxide adducts of diethylamine (sodium (*Z*)-1-(*N*,*N*-diethylamino)diazen-1-ium-1,2-diolate), spermine ((*Z*)-1-{*N*-[3-aminopropyl]-*N*-[4-(3-aminopropylammonio) butyl] amino}diazen-1-ium-1,2-diolate), dimethylhexanediamine ((*Z*)-1-{*N*-methyl-*N*-[6-(*N*-methylammoniohexyl)amino]} diazen-1-ium-1,2-diolate), and diethylenetriamine ((*Z*)-1-[*N*]-(2-aminoethyl)-*N*-(2-ammonioethyl)-amino]diazen-1-ium-1,2-diolate; all diazenuimdiolates) were synthesized as described previously (Maragos et al 1991; Hrabie et al 1993) and were the generous gift of Dr Larry Keefer, NCI, Frederick, MD. Type I equine tendon collagen was from ChronoLog (Havertown, PA). Thrombin was generated by standard methods in this laboratory. It was tested against NIH standard thrombin and found to have a specific activity of 3000 Units $mg^{-1}$. Other chemicals were obtained from Sigma (St Louis, MO).

*Platelets*

Human blood, anti-coagulated with citrate/phosphate/dextrose/adenine (CPD-A1), was obtained from volunteer donors through American Red Cross Blood Services and was processed to platelet-rich plasma (PRP) within 3 h of collection.

*Platelet aggregation*

To furnish a more defined media for experimentation, platelets were washed as previously described (Tandon et al 1989) and resuspended at $2 \times 10^8$ platelets $mL^{-1}$ in Tyrode-HEPES buffer at pH 7·4. Inhibitors were dissolved in 10 mM NaOH, a solution sufficiently alkaline to make the release of NO from the complexes negligible (Keefer et al 1996). The appropriate concentration of inhibitor in 10 mM NaOH, or 10 mM NaOH as vehicle control, was added to the platelets as 1% of the total incubation volume. This addition of alkali did not affect the pH of the Tyrode-HEPES buffer. After addition of the inhibitor the platelets were pre-incubated at 37°C for the time indicated. The first-order release of NO from diazeniumdiolates begins in the presence of the pH 7·4 buffer. Appropriately pre-incubated platelets were transferred to an aggregation cuvette with a siliconized magnetic stirrer bar and placed in a Payton Dual Channel Aggregation Module (Buffalo, NY) at 37°C, stirring at 900 rev $min^{-1}$. Aggregation was stimulated with thrombin, collagen, ADP, or adrenaline to determine if these inhibitors were specific for any particular agonist. The shorter-acting inhibitors were incubated directly in the 37°C chamber of the aggregometer. It should be noted that platelets from individual donors differ greatly in their response to the various platelet agonists. To standardize the individual responses when examining in-vitro dose-response relationships of drugs in human volunteer plasma, we used the formula:

$$\text{Percent of maximum inhibition} = [AUC_{CONTROL} - AUC_{TREATED}]/[AUC_{CONTROL}] \times 100$$

where $AUC_{CONTROL}$ is the AUC (area under the aggregation–time curve) of the aggregation trace for vehicle-treated platelets, and $AUC_{TREATED}$ is the AUC of the aggregation trace for diazeniumdiolate-treated platelets.

*Intracellular $Ca^{2+}$ measurement*

Changes in intracellular $Ca^{2+}$ concentrations were measured by a previously described method (Alessio et al 1993) using the fluorescent probe Fura-2. Briefly, platelets were labelled with 2·5 $\mu$M Fura-2-acetoxymethyl ester (Calbiochem, San Diego, CA) at 37°C for 30 min in PRP containing prostaglandin $E_1$ ($PGE_1$; 1 $\mu g\ mL^{-1}$), adjusted to pH 6·5 with citric acid to keep the platelets from autoactivating during the dye-loading process (Alessio et al 1993). To remove free fluorescent probe, platelets were washed by standard methods (Tandon et al 1989) and re-suspended in Tyrode-HEPES buffer at pH 7·4 to a concentration of $2 \times 10^8\ mL^{-1}$ and a final $Ca^{2+}$ concentration of 1 mM. Fluorescence was stimulated at an excitation wavelength of 339 nm and an emission wavelength of 500 nm (slit width 6 nm).

While the use of two excitation wavelengths has the advantage that it corrects for differences in instrument sensitivity and dye loading (Grynkiewicz et al 1985), single-channel recording has been used with satisfactory results from platelets (Sage & Rink 1987; Morgan & Newby 1989). All studies were completed within 2 h of labelling with Fura-2; during this time the leakage of Fura-2 was negligible. The measurements were performed in a water-jacketed instrument at 37°C. Inhibitors were added as described in the section on platelet aggregation. A maximum response was obtained by stimulating platelets with thrombin in the presence of vehicle. The thrombin response was then measured in platelets pre-incubated with inhibitors. Data were expressed as percent of maximum response. Platelet preparations for which the increase in $[Ca^{2+}]_i$ in response to thrombin was less than 600 nM were rejected from the study. Measurement and calibration of the Fura-2 responses were essentially the same as those of Tsien et al (1982), with modifications for the physicochemical properties of Fura-2.

*Data analysis*

Calculations of IC50 (i.e. values for which the effect was half the maximum) and linear regression analysis were determined using the iterative line- and curve-fitting programs of the GraphPad Prizm software.

## Results

*The effect of concentration on the anti-aggregatory efficacy of diazeniumdiolates*

The structures of the diazeniumdiolates are shown in Figure 1. The rates of NO release from these complexes at pH 7·4 have been determined previously and are listed in Table 1. To determine if the diazeniumdiolates have classical concentration–response characteristics, increasing concentrations of each complex (except diethylenetriamine complex) were pre-incubated with washed platelets at 37°C for one half-life and the aggregation response to 1 nM alpha thrombin was tested. Testing at the respective half-lives enables comparison of different complexes under conditions where the total NO load delivered is theoretically the same and where similar IC50 values would be predicted. Increasing concentrations of dimethylhexanediamine complex, diethylamine complex and spermine complex resulted in increasingly efficacious inhibition of thrombin-stimulated platelet aggregation (Table 2). The extent of inhibition by the different complexes was approximately the same at every concentration tested, with IC50 values of 14·9 ± 6·4 nM, 26·0 ± 24·1 nM, and 34·9 ± 24·0 nM for spermine complex, dimethylhexanediamine complex and diethylamine complex, respectively (n = three different platelet donors in duplicate). To determine if the inhibition by diazeniumdiolates was of a general nature, or was specific for thrombin-stimulated platelets, the

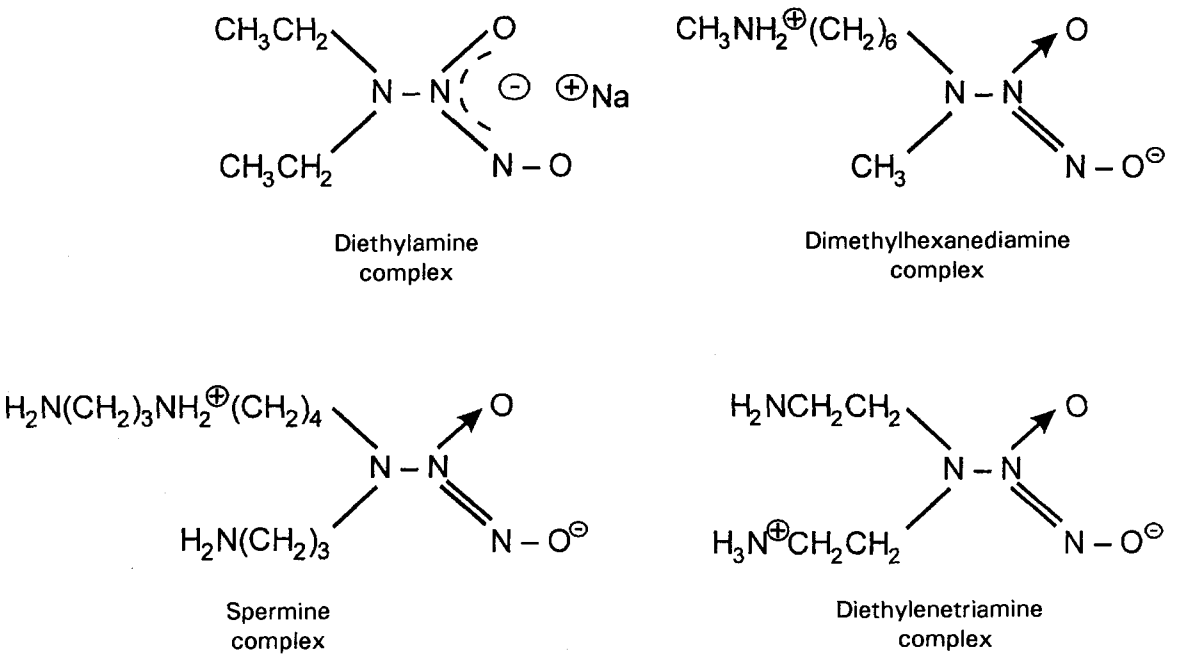


Figure 1. The two-dimensional structures of the nucleophile-NO complexes. The diethylamine complex exists as a sodium salt; the other complexes are zwitterionic. The resonant structure of the anionic portion of the diethylamine complex is depicted schematically.

Table 1. Half-lives of nitric oxide release from nucleophile-NO complexes at 37°C and pH 7·4.

| Nucleophile-NO complex | Half-life* |
|---|---|
| Dimethylhexanediamine complex | 1 min |
| Diethylamine complex | 2 min |
| Spermine complex | 39 min |
| Diethylenetriamine complex | 20 h |

*From Keefer et al (1996).

effect was tested using collagen, ADP and epinephrine. Similar concentration-dependent inhibition was observed using diazeniumdiolates with platelets stimulated by these agonists. Results for adrenaline, for which different methodology is needed to test aggregation (Raulli et al 1994), and ADP are not shown. Experiments using collagen are shown below. Spermine, *N,N'*-dimethylhexanediamine and diethylamine not complexed with NO did not inhibit platelet aggregation at concentrations up to 10 $\mu$M and for incubation periods up to 1 h. This indicates that the observed inhibition by diazeniumdiolates is the result of released NO and not some pharmacological effect of the nucleophile. Incubation of platelets alone for periods up to 1 h at 37°C had no effect on platelet aggregation. Incubation with nitrate or nitrite ions had no effect on platelet activation (data not shown).

*The effect of pre-incubation time on the anti-aggregatory potency of spermine complex*

If the diazeniumdiolates deliver NO according to first-order kinetics, the potency of each complex should be time-dependent. For experimental expedience spermine complex was chosen for its favourable half-life relative to the experimental protocol. The time-dependent differences in potency of platelet inhibition by spermine complex are demonstrated in Figure 2. Spermine complex and sodium nitroprusside, added as a time-independent control, were pre-incubated with washed

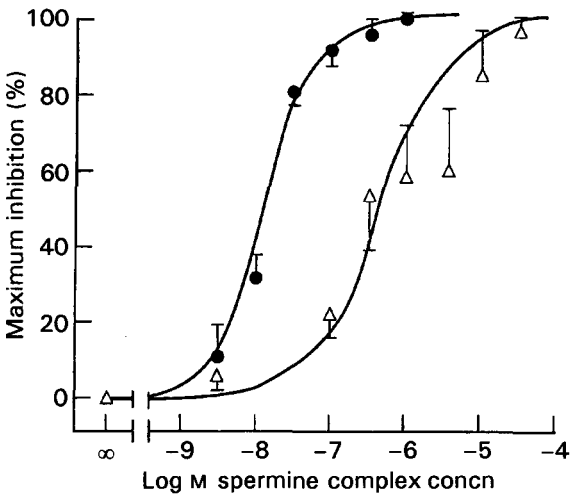


Figure 2. The effect of pre-incubation time on the spermine complex concentration–response curve. Different concentrations of the spermine complex were pre-incubated with washed platelets from man for 5 (△) and 39 (●) min and assayed for collagen-stimulated (2·5 $\mu$g mL$^{-1}$) aggregation. Each point represents the mean and s.e.m. of results from three individual platelet donors, each experiment being performed in duplicate. IC50 values for spermine complex and sodium nitroprusside for the two time-points are given in Table 3.

platelets at 37°C for 5 and 39 min. At each pre-incubation time spermine complex led to concentration-dependent inhibition of collagen-stimulated platelet aggregation that reaches saturation (Figure 2, n = three different platelet donors in duplicate). The difference in potency between 5- and 39-min pretreatment with spermine complex is approximately two orders of magnitude. IC50 values are given in Table 3, as are those of sodium nitroprusside. As expected, sodium nitroprusside did induce concentration-dependent inhibition of platelet aggregation (not shown), although time-dependent inhibition was not evident (Table 3). These data demonstrate time-dependent inhibition of platelet aggregation by spermine complex.

Table 2. Inhibition of platelet aggregation by different concentrations of nucleophile-NO complexes.

| Complex | Percent of maximum inhibition for complex concentration of (log M): | | | |
|---|---|---|---|---|
| | −9 | −8 | −7 | −6 |
| Dimethylhexanediamine | 7·35 ± 2·88 | 24·87 ± 15·01 | 36·13 ± 24·08 | 81·15 ± 6·28 |
| Diethylamine | 10·47 ± 3·66 | 2·24 ± 6·63 | 64·92 ± 18·15 | 80·10 ± 3·66 |
| Spermine | 12·56 ± 4·71 | 40·14 ± 2·44 | 63·87 ± 4·71 | 94·24 ± 3·70 |

Nucleophile-NO complexes were pre-incubated for their respective half-lives at the concentrations indicated with washed platelets from man. Platelets were then assayed using thrombin to stimulate aggregation. At no concentration did any of the nucleophile-NO complexes induce inhibition significantly different from that induced by other complexes at that concentration.

Table 3. IC50 values for spermine complex and sodium nitroprusside at 5 and 39 min pre-incubation times.

| Inhibitor | IC50 values (M) | |
|---|---|---|
| | 5 min | 39 min |
| Spermine complex | $1{\cdot}70 \pm 0{\cdot}85 \times 10^{-6}$ | $1{\cdot}97 \pm 0{\cdot}12 \times 10^{-8}$ |
| Sodium nitroprusside | $2{\cdot}73 \pm 1{\cdot}25 \times 10^{-8}$ | $2{\cdot}50 \times 10^{-8}$ (n = 2) |

Platelets ($2 \times 10^8$ mL$^{-1}$) were pre-incubated with different concentrations of spermine complex or sodium nitroprusside for 5 or 39 min and stimulated with 2·5 $\mu$g mL$^{-1}$ collagen. The data are the means ± s.e.m. of results from at least three separate experiments (except for sodium nitroprusside, 39 min).

Table 4. Reversal of diazeniumdiolate-induced inhibition by haemoglobin.

| Complex | Percent of maximum inhibition | |
|---|---|---|
| | Control | +10 $\mu$M haemoglobin |
| Dimethylhexanediamine | 70·29 ± 2·15 | 44·32 ± 11·65* |
| Diethylamine | 61·45 ± 2·97 | 32·54 ± 17·56* |
| Spermine | 20·89 ± 3·12 | 3·75 ± 2·51** |
| Diethylenetriamine | 3·70 ± 3·50 | – |

Washed platelets from man were pre-incubated with diazeniumdiolates (100 nM) for 5 min in the absence and presence of 10 $\mu$M haemoglobin, and assayed for aggregation stimulated by collagen (2·5 $\mu$g mL$^{-1}$). Data are means ± s.e.m. of results from at least three individual platelet donors, each experiment being performed in duplicate. *$P < 0{\cdot}05$, **$P < 0{\cdot}01$, signifi-

*The effect of haemoglobin on the inhibitory efficacy of diazeniumdiolates at isomolar concentrations*

To confirm that the platelet inhibition observed was because of released NO and not a structural pharmacophore of the diazeniumdiolate complexes, the ability of the NO-scavenger haemoglobin to reverse the inhibitory effects of NO was tested. Dimethylhexanediamine complex, diethylamine complex, spermine complex and diethylenetriamine complex (100 nM) were pre-incubated with washed platelets for 5 min in the presence and absence of 10 $\mu$M haemoglobin and the mixture was subsequently stimulated with 2·5 $\mu$g mL$^{-1}$ collagen (Table 4). Haemoglobin at 10 $\mu$M significantly reversed the inhibition of platelet aggregation induced by diazeniumdiolates, but had no effect on platelet aggregation alone. Attenuation of the inhibitory effect of diazeniumdiolates by haemoglobin indicates that the observed inhibition is the result of released NO.

*Correlation of theoretical NO load delivered with observed percent inhibition*

Of note in Table 4 is that the extent of inhibition for each nucleophile-NO complex was inversely proportional to its half-life, the order of efficacy being dimethylhexanediamine complex > diethylamine complex > spermine complex > diethylenetriamine complex. These data are consistent with the theoretical NO load delivered. Comparison of the actual inhibition observed with each inhibitor with the theoretical amount of NO released is shown in Table 5. A plot of these data points yields a line

Table 5. Theoretical NO load delivered (%) and actual inhibition by 100 $\mu$M nucleophile-NO complex (%).

| Complex | Theoretical amount of NO delivered (%) | Percentage of maximum inhibition of platelets |
|---|---|---|
| Dimethylhexanediamine | 96·9 | 70·3 ± 2·15 |
| Diethylamine | 82·4 | 61·5 ± 2·97 |
| Sperminex | 8·5 | 20·9 ± 3·12 |
| Diethylenetriamine | 0·3 | 3·7 ± 3·50 |

Platelets were pre-incubated for 5 min with nucleophile-NO complexes, and stimulated with 2.5 $\mu$g mL$^{-1}$ collagen (Table 4). The theoretical amount of NO delivered (%) was calculated using first-order kinetic equations. The decay constant (k) was calculated from the half-life, and the theoretical amount of NO delivered (%) was calculated by use of the formula $2{\cdot}3 \log [N]_0/[N] = kt$, where k is the decay constant, t is the time from $t_0$, $[N]_0$ is the concentration of diazeniumdiolate at $t_0$, and [N] is the concentration of diazeniumdiolate at time t. The actual amount of inhibition shown (%) is the mean ± s.e.m. of results from three separate experiments, using blood from different donors, each experiment being performed in duplicate.

with a correlation coefficient of 0·975. Thus, there is strong correlation between the amount of NO released and the extent of inhibition of agonist-stimulated platelet aggregation, indicating a direct concentration–effect relationship between released NO and platelet inhibition. Moreover, these data further support the predicable nature of these inhibitors.

*The effect of diazeniumdiolates on reduction of agonist-stimulated elevation of intra-platelet $Ca^{2+}$ levels*

The capacity of diazeniumdiolates to inhibit the thrombin-stimulated increase in $[Ca^{2+}]_i$ was tested to determine the possible effects of these drugs on intracellular signalling. Washed platelets were loaded with Fura-2 as described, pre-incubated for 1 min (dimethylhexanediamine complex) or 2 min (others) at 37°C , then stimulated with 1–10 nM thrombin. All four diazeniumdiolates caused a concentration-dependent reduction of $[Ca^{2+}]_i$ elevation in platelets stimulated by thrombin. Basal levels of intracellular $Ca^{2+}$ were unaffected by diazeniumdiolates. The IC50 values for dimethylhexanediamine complex and diethylamine complex were 8·7 ± 1·49 and 11·5 ± 1·36 nM, respectively. The similar IC50 values reflect the fact that each compound was incubated for one half-life, when the NO load delivered was theoretically the same. The IC50 value for spermine complex was 176 ± 16·9 nM, whereas the value for diethylenetriamine complex was > 100 μM. The IC50 value for sodium nitroprusside was 1·1 μM (n = 2, data not shown). Nucleophiles not complexed with NO did not affect basal or thrombin-stimulated intracellular $Ca^{2+}$ levels at concentrations up to 10 μM and pre-incubation times up to 10 min, nor did nitrate or nitrite ions (10 mM; 10 min). These data imply that diazeniumdiolates have concentration-dependent effects on agonist-stimulated $[Ca^{2+}]_i$ elevation, with predictably similar IC50 values when inhibitors were pre-incubated at their half-lives.

## Discussion

These data, using aggregation of platelets from man as a model to test the inhibitory potency of nucleophile-NO complexes, are consistent with many studies showing the anti-platelet effect of NO or of agents that release NO (Ahlner et al 1991; Moncada et al 1991); the data are also consistent with a preliminary report of the use of this class of NO donor (Diodati et al 1993). All the diazeniumdiolates caused concentration- and time-dependent inhibition of thrombin-stimulated $[Ca^{2+}]_i$ elevation, without affecting basal levels. Nucleophiles that were not complexed with NO did not inhibit platelet aggregation or reduce agonist-stimulated $[Ca^{2+}]_i$ elevation, indicating that the nucleophiles were devoid of anti-platelet effects. The study demonstrated attenuation of platelet inhibition by addition of the NO-scavenger haemoglobin (Martin et al 1986), indicating that the inhibition is a result of released NO and is not caused by a structural pharmacophore inherent in the nucleophile-NO complexes. These data also indicate that the anti-platelet effect of these complexes is mediated by released NO.

A key finding implying controlled, predictable NO release from these compounds is that the extent of inhibition strongly correlates with the calculated NO load released by each complex (Tables 4 and 5). A similar release–activity correlation has also been demonstrated for diethylamine complex, spermine complex and other derivatives by use of aortic rings (Maragos et al 1991). A strong correlation between NO load delivered and efficacy indicates a direct relationship between released NO and platelet inhibition.

Interestingly, the anti-aggregatory potency of diazeniumdiolates is very similar to their potency in reducing $[Ca^{2+}]_i$ elevation (Figure 3), being within half an order of magnitude for both diethylamine complex (IC50 values of 34·9 and 11·5 nM

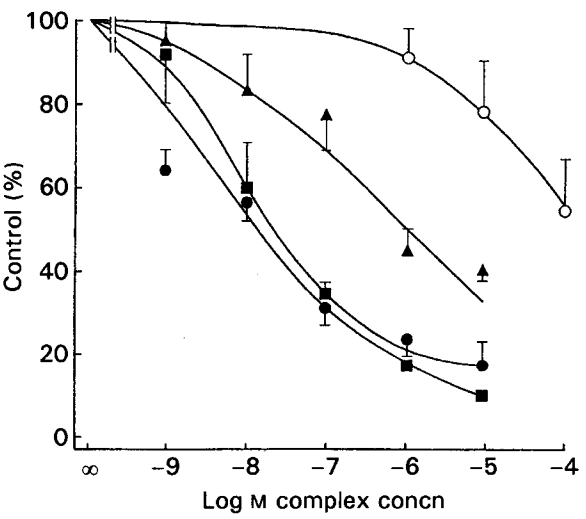


Figure 3. Inhibition of thrombin-induced intra-platelet calcium elevation by diazeniumdiolates. Diazeniumdiolate, uncomplexed nucleophile or vehicle was pre-incubated with platelets directly in the 37°C water-jacketed block of the instrument for the prescribed time. Maximum response was determined by stimulating with thrombin in the presence of vehicle. The thrombin response from inhibitor-treated platelets was expressed as a percentage of the maximum response. Each point represents the mean and s.e.m. of results from four individual platelet donors, each experiment being performed in duplicate. ●, Dimethylhexanediamine; ■, diethylamine; ▲, spermine; ○, diethylenetriamine.

for platelet inhibition and reduction of $[Ca^{2+}]_i$ levels, respectively) and dimethylhexanediamine complex (IC50 values of 26·0 and 8·7 nM for platelet inhibition and reduction of $[Ca^{2+}]_i$ calcium levels, respectively). That there are not large differences in the potency of diazeniumdiolates when comparing anti-aggregatory potency with reduction of agonist-elevated $[Ca^{2+}]_i$ suggests a singular mechanism
of inhibitory action. The effects observed with diazeniumdiolates are in contrast with the effects of sodium nitroprusside, for which potencies are different for inhibition of shape change, inhibition of platelet aggregation or reduction of intracellular calcium levels (Morgan & Newby 1989). Furthermore, an elegant study of sydnonimine derivatives showed that these drugs are two to three orders of magnitude more potent at inhibiting platelet aggregation than at inhibiting increases in intracellular calcium levels (Ivanova et al 1993). Such a large disparity in the anti-aggregatory potency of sodium nitroprusside and sydnonimines compared with their potency to inhibit $[Ca^{2+}]_i$ elevation suggests different inhibitory mechanisms of action for the two responses.

One possible explanation of the difference observed in this study compared with others is that the diazeniumdiolates do not require the presence of thiol groups to release their NO. It has been shown that diazeniumdiolates induce first-order kinetic release of NO in aqueous buffer solution (Maragos et al 1991). Sydnonimines, sodium nitroprusside and organic nitrate esters all require activation by thiols, resulting in the generation of free NO or the formation of *S*-nitrosylated donor proteins (Needleman et al 1969; Ignarro et al 1981; Stamler et al 1992). One could postulate a mechanism whereby the thiol-requiring NO donor *S*-nitrosylates critical platelet membrane thioproteins involved in aggregation, resulting in the deactivation of these proteins and inhibition of platelet aggregation. A similar theory has been proposed for neurons, where *S*-nitrosylation of a protein sub-unit of the NMDA receptor by *S*-nitrosocysteine, resulted in a functional blockade of neurotransmission (Lei et al 1992; Lipton et al 1993). In platelets, whose membranes are rich in thioproteins (Robey et al 1979), it has been demonstrated that reduction of thiol sites blocks the aggregatory response to ADP (Macfarlane & Mills 1981). Furthermore, myosin, which is known to be a critical protein in platelet degranulation (Fox & Phillips 1982), can react with nitrogen oxides to form a nitrosothiol (Kubberod et al 1974). Thus, given the numerous and important thiol-containing proteins in platelets, it is possible that thiol-requiring NO donors can nitrosylate, and possibly deactivate, key membrane proteins involved in platelet aggregation. This postulated mechanism is independent of NO-stimulated cGMP formation and might explain the large differences in anti-aggregatory potency compared with the potency for reduction of $[Ca^{2+}]_i$ levels observed with the thiol-requiring NO donors. Although this theory requires further study, the data from this study support it.

In summary, diazeniumdiolates show a concentration- and time-dependent profile of NO release, as demonstrated by their anti-aggregatory effects on platelets. Their unique temporal properties enable controlled and predictable release of NO and the ability to titrate a pharmacological effect over time. These controlled-release properties combined with the lack of a bioactivation requirement, distinguish these agents from other NO donors and suggest that these complexes can be useful pharmacological agents.

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