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Comparative effects of several nitric oxide donors on intracellular cyclic GMP levels in bovine chromaffin cells: correlation with nitric oxide production

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1 Sodium nitroprusside, S-nitroso-N-acetyl-D,L-penicillamine, Spermine NONOate and DEA NONOate raised cyclic GMP levels in bovine chromaffin cells in a time and concentration dependent manner with different potencies, the most potent being DEA/NO with an EC_{50} value of 0.38 ± 0.02 μM.

2 Measurements of NO released from these donors revealed that DEA/NO decomposed with a half-life ($t_{1/2}$) of 3.9 ± 0.2 min. The $t_{1/2}$ for SPER/NO was 37 ± 3 min. SNAP decomposed more slowly ($t_{1/2} = 37 \pm 4$ h) and after 60 min the amount of NO produced corresponded to less than 2% of the total SNAP present. The rate of NO production from SNAP was increased by the presence of glutathione.

3 For DEA/NO and SPER/NO there was a clear correlation between nitric oxide production and cyclic GMP increases. Their threshold concentrations were 0.05 μM and maximal effective concentration between 2.5 and 5 μM.

4 For SNAP, threshold activation was seen at 1 μM, whereas full activation required a higher concentration (500–750 μM). The dose-response for SNAP increases in cyclic GMP was shifted nearly two orders of magnitude lower in the presence of glutathione. At higher concentrations an inhibition of cyclic GMP accumulation was found. This effect was not observed with either the nitric oxide-deficient SNAP analogue or other NO donors.

5 Although NO-donors are likely to be valuable for studying NO functions, their effective concentrations and the amount of NO released by them are very different and should be assessed in each system to ensure that physiological concentrations of NO are used.

Keywords: Cyclic GMP; nitric oxide donors; nitric oxide measurements; NONOates; SNAP; sodium nitroprusside

Abbreviations: AP-SS, N-acethyl-D,L-penicillamine disulphide; DAN, 2,3-diaminonaphthalene; DEA/NO, 2-(N,N-Diethylamino)-diazenolate-2-oxide; EC_{50}, half maximal stimulatory concentration; EDTA, ethilene dinitrilo tetra-acetic acid; e_{NO}, stoichiometry of NO released; GSH, glutathione; IBMX, 3-isobutyl-1-methylxanthine; NO, nitric oxide; sGC, soluble guanylate cyclase; SNAP, S-nitroso-N-acethyl-D,L-penicillamine; SPER/NO, (Z)-1-{N-[3-aminopropyl]-N-[4-(3-aminopropylammonio) butyl]-amino}-diazen-1-ium-1,2-diolate

Introduction

Nitric oxide (NO), is synthesized endogenously by many cell types and participates in several physiological and pathological processes (Moncada *et al*., 1991). The major signal transduction pathway for NO, when it is produced at submicromolar concentrations by constitutive nitric oxide synthase isoforms, is high-affinity binding to the ferrous haem cofactor of soluble guanylate cyclase (sGC) (Ignarro *et al*., 1982; Waldman & Murad, 1987). The equilibrium dissociation constant of NO for activating sGC has been estimated to be <250 nM, at 10°C, and a complete nitrosyl complex formation has been observed with as little as 500 nM NO. These values fall within the physiological range of NO, which is believed to be 100–500 nM (Malinski & Taha, 1992; Stone & Marletta, 1996). At high concentrations of NO, such as those that might be achieved with the inducible NO synthase (4–5 μM) according to Laurent *et al*. (1996) other signalling mechanisms participate in NO's biological action. Enzymes containing iron-sulphur groups bind NO, and their action is modified. Some of these enzymes perform critical roles in energy metabolism or are involved in nucleic acid synthesis (Henry *et al*., 1993). Thus it is likely that this may be one of the possible mechanisms by which NO signalling produces cell toxicity and death. Considering the normal function of NO and the wide range of pathological conditions in which it has been implicated when it is synthesized at different concentrations, measuring NO concentrations has received an enormous amount of attention (Laurent *et al*., 1996).

Compounds which release NO are useful tools for studying processes in which NO participates and the molecular mechanisms involved in them. Several types of NO-releasing agents are now available, including: sydnonimines, organic nitrites, sodium nitroprusside, nucleophyle-NO adducts and S-nitrosothiols. Of these, one of the most commonly employed is S-nitroso-N-acetyl-D,L-penicillamine (SNAP). Such compounds have shown diverse and remarkable biological effects (Chen & Schofield, 1993). In some cases these effects appear to be opposed to one another (Chen & Schofield, 1993; Desole *et al*., 1994), although differences in concentrations, incubation times or other experimental conditions might explain the apparent contradiction.

In many systems the action of NO is carried out through the activation of soluble guanylate cyclase and the production of cyclic GMP. Therefore NO is mediated in most of its biological effects by cyclic GMP as the second messenger (Murad, 1986).

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For this reason cyclic GMP is frequently used as a sensitive indirect measure of NO production. Nitric oxide can be exogenously supplied to tissues and cells by various NO generating compounds, each of them with its own specific properties.

In this study we have looked into four nitric oxide donors using bovine chromaffin cells. The NO/cyclic GMP signalling pathway has been characterized in these cells and plays a regulatory role in catecholamine secretion (Rodríguez-Pascual *et al*., 1995; 1996; Schwarz *et al*., 1998). The four NO donors selected were nitroprusside and SNAP, which are used widely, and two nucleophile-NO adducts, which are relatively novel and whose spontaneous NO release at 37°C and pH 7.4 has been accurately characterized (Maragos *et al*., 1991). Although Schmidt *et al*. (1997) have recently published a mathematical model for calculating nitric oxide concentrations from this type of compound in aerobic solutions, it is important to measure NO levels and the donor's capacity to increase cyclic GMP levels under the same experimental conditions and with the same cells in order to compare them with other donors. Thus, the main objective of this study was to compare the potencies of four NO donors in stimulating cyclic GMP accumulation by bovine chromaffin cells, and to analyse whether there is a correlation between this effect and NO generation by them.

Methods

Isolation and culture of bovine chromaffin cells

Chromaffin cells were obtained after digestion of bovine adrenal glands with collagenase (EC 3.4.24.3) in retrograde perfusion as previously described by Rodriguez-Pascual *et al*. (1995). Briefly, glands supplied by a local slaughterhouse were trimmed of fat, cannulated through the adrenal vein and washed with Ca^{2+}/Mg^{2+}-free saline buffer, containing (in mM) NaCl 154, KCl 5.6, $NaHCO_3$ 3.6, glucose 5, and HEPES 5, pH 7.4. Digestion was performed with a 0.2% collagenase plus 0.5% bovine serum albumin solution in the above medium. After digestion, glands were halved, soft medulla were removed, minced and dispersed cells filtered through a nylon mesh. Cells were purified through an Urografin density gradient (Wilson, 1987). Of the collected cells >90–95% were chromaffin cells, as they were massively and clearly stained by neutral red. Purified chromaffin cells were suspended in Dulbecco's modified Eagle's medium (DMEM), containing 10% heat-inactivated foetal calf serum and standard antibiotics. Cells were plated in collagen-treated 6- or 24-well Costar cluster dishes at a density of 5×10^6 and 10^6 cells $well^{-1}$ respectively in culture medium supplemented with 10 μM cytosinearabinofuranoside and 10 μM fluorodeoxyuridine, maintained at 37°C in 5% CO_2/95% air, these cells were used for 3–5 days following cell isolation.

Intracellular cyclic GMP measurements

Cells were serum-deprived for 24 h before cyclic GMP measurement and then washed twice with Locke's solution, pH 7.4 (composition in mM): NaCl 140, KCl 4.4, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 4, glucose 5.6, EDTA 0.01 and HEPES 10. After a 30-min pre-incubation at 37°C in Locke's solution containing 0.5 mM IBMX, the cells were stimulated with agents or vehicle in 0.5 ml of Locke's solution for the indicated times. Incubations were terminated through aspiration of the medium and the addition of 300 μl of 6% trichloroacetic acid. Cells were then scraped out of the wells and centrifuged. The supernatant fractions were neutralized with 3 M KOH plus 1.5 M TEA, the cyclic GMP content was determined in the crude extracts by using commercial [^{3}H]-cyclic GMP radioimmunoassay kit (Amersham) as described previously (Rodríguez-Pascual *et al*., 1996).

Nitric oxide measurements

Two different methods have been used to determine NO release by NO donors.

Haemoglobin reaction This technique is based on the quantitative oxidation of oxyhaemoglobin (HbO_2, oxyHb) to methaemoglobin (metHb) in aqueous solution by nitric oxide (Feelich & Noack, 1987):

$$HbO_2 + NO \rightarrow MetHb + NO_3^-$$

The formation of NO can be monitored under initial rate conditions because the oxidation of HbO_2 with NO occurs at a rate that is faster than the reaction between molecular oxygen and NO (Hevel & Marletta, 1994).

Commercial oxyhaemoglobin (ferrous human A_0) was dissolved in 100 mM HEPES (pH 7.5) as a 25 mg ml^{-1} solution (300 μM) and quickly frozen at −80°C in small aliquots, following the method recommended by Hevel & Marletta (1994). The concentration of HbO_2 was calculated prior to each experiment based on the equation:

$$[HbO_2]\ (\mu M) = [1.013\ (A_{576}) - 0.3269\ (A_{630}) - 0.7353\ (A_{560})] \times 10^2$$

Reaction was monitored by absorbance measurements as an increase at 401 nm or a decrease at 576 nm when low or high concentrations of NO donors were used, respectively, (Hevel & Marletta, 1994). In both cases the extinction coefficient was determined prior to the experiments, and found to be $54{,}346 \pm 1727\ M^{-1}\ cm^{-1}$ at 401 nm ($n = 13$), and $13{,}237 \pm 2580\ M^{-1}\ cm^{-1}$ at 576 ($n = 6$). Nitric oxide released from NO donors was measured both in the absence and in the presence of 10^6 cells ml^{-1}. In the absence of cells, measurements of nitric oxide were carried out in a cuvette in a final volume of 500 μl of Locke's solution, containing 10 or 70 μM HbO_2 and the absorbance at 401 or 576 nm read. Then, NO donors from a 100 fold concentrated solution or vehicle were added, the mixture was kept at 37°C for the required times and the absorbance read at the appropriate wavelength. To measure NO from donors at different concentrations in the presence of cells, bovine chromaffin cells plated on 24-well costar cluster dishes were serum-deprived for 24 h and washed twice with Locke's solution. Then 1.5 ml of the same solution containing HbO_2 were added, 500 μl were taken out immediately and the absorbance read at $t = 0$ min; at this time NO donors or vehicle were added to give the required concentration, and after 10 min (DEA/NO) or 15 min (SNAP and SPER/NO) at 37°C, 500 μl were removed and the absorbance read. The oxidation of HbO_2 to metHb in the absence of any NO donors was subtracted, in order to minimize the possible interference of other cellular mechanism, including basal NO release (Schwarz *et al*., 1998). When NO produced by NO donors was measured as a function of time, 5×10^6 cells plated on 6-well costar cluster dishes were serum deprived 24 h and washed twice with Locke's solution. Then 5.5 ml of the same buffer containing 10 or 70 μM HbO_2 were added, 500 μl immediately removed and the absorbance read ($t = 0$), at this time NO donor or vehicle was added and 500 μl

aliquots removed at the required experimental times. HbO_2 oxidation as a function of time in the absence of NO donor was analysed and subtracted from other measurements.

Nitrite determination Nitrites, which are stable end-products of nitric oxide, were determined using the method of Misko *et al*. (1993), which is based upon the reaction of nitrite with 2,3-diaminonaphthalene (DAN) under acidic conditions so as to form 1-(H)-naphthotriazole, a fluorescent derivative product. For quantification of nitrites, 1 ml of Locke's solution containing NO donors or nitrite standard at different concentrations was mixed with 100 μl of freshly prepared DAN (0.025 mg ml^{-1} in 0.62 M HCl). After a 15 min incubation period at 37°C the reaction was stopped with 50 μl of 2.8 N NaOH. Formation of the fluorescent 2,3-diaminonaphthotriazole was measured using a Perkin Elmer LS-50 fluorimeter. The excitation and emission wavelengths were set to 375 and 415 nm, respectively. The DAN reagent was protected from light. Nitrite standards (greater than 98% pure from SIGMA) were routinely made fresh, dissolved in double-deionized water and kept on ice prior to use.

NO-donors preparations

SNAP and AP-SS were dissolved in DMSO and kept in darkness. SPER/NO and DEA/NO were prepared according to the procedure described by Keefer *et al*. (1996). A concentrated stock solution was prepared every day in dilute alkali (0.1 N NaOH) and kept cool. We carefully followed the storage recommendations for these products, and routinely flushed the reagent bottle with dry nitrogen before each closing and then sealed the joint between the bottle and the cap with plastic in order to avoid moisture entering. The initial NONOate concentrations were based on spectrophotometric measurements at 250 nm using molar extinction coefficients of 8000 and 6500 M^{-1} cm^{-1} for spermine and diethylamine NONOates, respectively (Ramamurthi & Lewis, 1997). SNP was dissolved in the buffer and kept in darkness.

Drugs and reagents

Culture media and heat-inactivated foetal calf serum were obtained from GIBCO (Uxbridge, U.K.). Culture plates were obtained from Costar (Cambridge, MA, U.S.A.). Collagen A was from Biochrom KG (Berlin, Germany). Collagenase A (EC 3.4.24.3) from Clostridium hystoliticum was purchased from Boehringer Mannheim (Mannheim, Germany). Urografin was from Schering España (Madrid, Spain). [^{3}H]-cGMP radioimmunoassay kit, was purchased from Amersham (Buckinghamshire, U.K.). IBMX, 2,3-diaminonaphthalene (DAN), nitrite standard, ferrycianure, SNP, and oxyhaemoglobin were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). DEA NONOate, Glutathione (GSH), Spermine NONOate, S-nitroso-N-acetyl-D,L-penicillamine (SNAP), N-

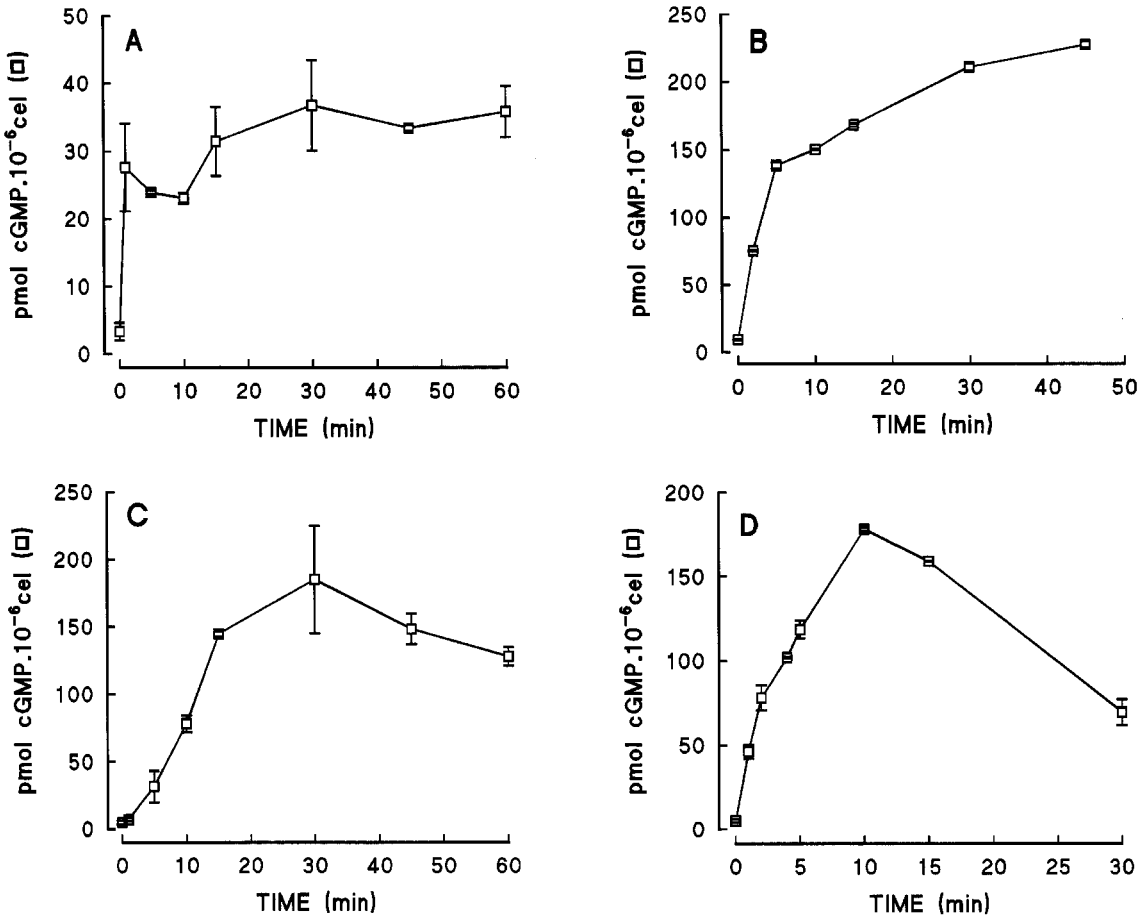

Figure 1 Effects of different NO donors on cyclic GMP levels in bovine chromaffin cells as a function of time. Cyclic GMP levels were determined in bovine chromaffin cells (10^6 cells $dish^{-1}$) pre-incubated for 30 min with Locke's solution plus 0.5 mM IBMX. Cyclic GMP in the cells was assayed as described in Methods. (A) 250 μM SNAP; (B) 100 μM SNP, (C) 5 μM SPER/NO and (D) 2.5 μM DEA/NO. Data (means ± s.e.mean) were obtained from four different experiments performed in triplicate and are expressed as pmol 10^6 $cells^{-1}$.

acetyl-D,L-penicillamine disulphide (AP-SS), were purchased from Alexis Corporation (San Diego, CA, U.S.A.). SNAP was also purchased from Calbiochem (San Diego, CA, U.S.A.) and from Molecular Probes (Eugene, OR, U.S.A.). Inorganic salts were from Merck (Germany).

Analysis of data

Experiments were performed in triplicate. Cyclic GMP and NO concentration results are expressed as pmol 10^6 cells^{-1} and μM $\pm$ s.e.mean, respectively. Concentration-response relationships were fitted to a sigmoidal model of the form log-concentrations versus response. The curve-fitting was done with the program Parameter Fitter (Biosoft).

Results

Cyclic GMP production by chromaffin cells in response to different NO donors

Cyclic GMP levels in bovine chromaffin cells were measured in the presence of the non-specific phosphodiesterase inhibitor IBMX at 0.5 mM. Pre-incubation for 30 min with IBMX caused a 2 fold increase in the levels of cyclic GMP. The mean basal cyclic GMP content of cultured bovine chromaffin cells was 1.9 ± 0.1 pmol 10^6 cells^{-1} ($n = 24$). Figure 1 shows the time course of cyclic GMP accumulation produced by 250 μM SNAP (Figure 1A), 100 μM SNP (Figure 1B), 5 μM SPER/NO (Figure 1C) and 2.5 μM DEA/NO (Figure 1D). In the presence of SNAP the levels of cyclic GMP rose until they reached a stable value after about 15 min. In the presence of SNP cyclic GMP content increased rapidly over 10 min and increased slowly thereafter. In both cases cyclic GMP levels remained high while the stimulatory agents were present, indicating continuous NO production by these agents. However SPER/NO caused a continuous increase in cyclic GMP, reaching a maximum after 15–30 min and diminishing slowly thereafter, and in the presence of DEA/NO intracellular cyclic GMP content rose rapidly in the first 10 min and the values dropped at longer experimental times.

The effects of the four NO-releasing compounds were concentration-dependent and their maximal effect was comparable (Figure 2). However their relative efficacies were very different, as can be seen in Table 1, which shows the concentrations of the four NO donors for threshold and maximum increases of cyclic GMP levels in chromaffin cells, and the corresponding NO concentrations generated. The table also includes the calculated half-life and the EC_{50} values for the four compounds employed. DEA/NO was the most potent, with an EC_{50} of 0.38 ± 0.02 μM. A significant increase in cyclic GMP level was observed at 0.1 μM DEA/NO and the maximum was achieved at 2.5 μM. Moreover, this maximum

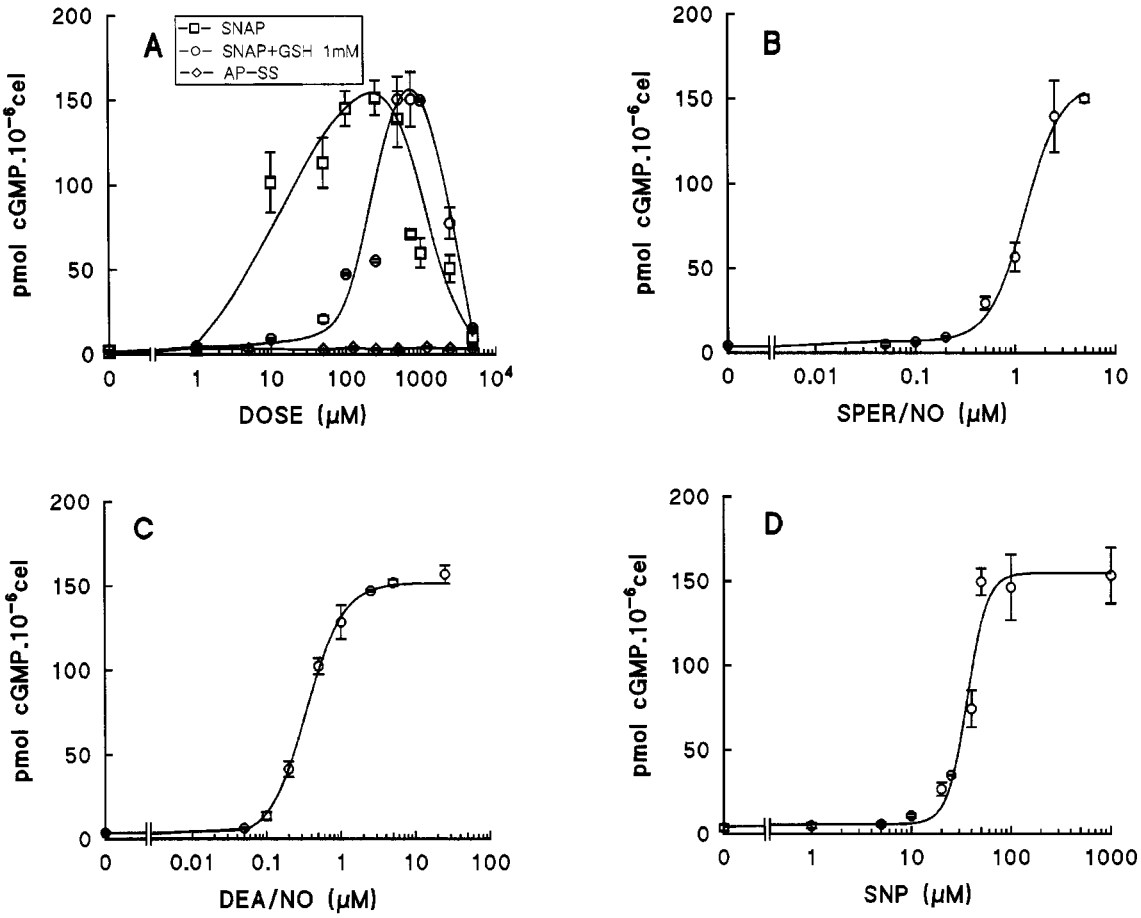

Figure 2 Concentration-dependence curves for cyclic GMP accumulation induced by NO donors. Cells (10^6 cells dish^{-1}) were preincubated for 30 min with 0.5 mM IBMX. After preincubation, they were incubated for 15 min with either SNAP or AP-SS, or SNAP plus 1 mM GSH (A), SNP (D) or SPER/NO (B) or for 10 min with DEA/NO (C). Cyclic GMP was measured as described in Methods and is expressed as pmol 10^6 cells^{-1}. Experiments were performed in triplicate and data are means $\pm$ s.e.mean from three different cellular preparations.

Table 1 Concentrations of NO-donors and NO produced by them, that causes threshold, half-maximal and maximal accumulation of cyclic GMP

		Activation threshold		*EC_{50}*		*Maximum activation*	
Donor	*$t_{1/2}$* (min)	*Donor* (μM)	*NO* (μM)	*Donor* (μM)	*NO* (μM)	*Donor* (μM)	*NO* (μM)
SNAP	$2.2\times10^3\pm0.24\times10^3$	1	0.006	317 ± 42	1.8 ± 0.2	750	3.8 ± 0.4
SPER/NO	37 ± 3	0.05	0.02	1.12 ± 0.46	0.5 ± 0.2	5	2.4 ± 0.2
DEA/NO	3.9 ± 0.2	<0.05	<0.05	0.38 ± 0.02	0.5 ± 0.1	2.5	2.2 ± 0.5
SNP	N.D.	5	N.D.	36.3 ± 0.02	N.D.	100	N.D.

Half-life ($t_{1/2}$) were calculated from semi-logarithmic plot of the data from Figure 4, considering that the stoichiometry for DEA/NO was 1.2, for SPER/NO was 2 and 1 for SNAP. EC_{50} values were calculated from Figure 2 data. Nitric oxide produced by each NO donor concentration was measured by the haemoglobin method in the presence of chromaffin cells; incubation times were fixed to that of maximum effect on cyclic GMP levels for the different agents (data not shown): 15 min for SNAP, SNP and SPER/NO, and 10 min for DEA/NO.

level was not modified at higher DEA/NO concentrations (25 μM).

The dose-response curve for SNAP was bell-shaped. A significant increase (1.7 fold) was observed at 1 μM and a maximum at 500 μM to 1 mM ($EC_{50}=317\pm42$ μM, $n=9$). Above 1 mM SNAP the cyclic GMP accumulation fell markedly to just 10% of the highest response at 5 mM. As is shown in Figure 2, the presence of GSH at 1 mM in the incubation medium shifted the curve nearly two orders of magnitude to the left, and even the inhibitory effect was observed at lower SNAP concentration. Since this fall in cyclic GMP levels at supramaximal donor concentration was only observed with SNAP, the effect of the NO-deficient SNAP (N-acetyl-D,L-penicillamine, AP-SS) on basal or SNP-stimulated cyclic GMP levels was analysed to investigate whether inhibition or desensitization of soluble guanylate cyclase was due to an effect relating to SNAP itself or rather to the disulphide product formed during its decomposition. As is shown in Figures 2A and 3, NO-deficient SNAP did not modify basal levels of cyclic GMP at any of the concentrations tested. Figure 3 also shows that 15 min stimulation with 100 μM SNP produces a 75 fold increase of cyclic GMP levels and simultaneous treatment with SNP and N-acetyl-D,L-penicillamine (5 mM) did not modify this response. Nevertheless, this response was markedly diminished when cells were simultaneously stimulated with 100 μM SNP and 5 mM SNAP, and the increase in cyclic GMP was equal to that produced by 5 mM SNAP alone. In both cases the increase in cyclic GMP correspond to a 25% of the response produced by 100 μM SNP. Since SNAP was dissolved in DMSO, the effect of DMSO (2.8% final concentration) was also tested, and as it is shown in Figure 3, DMSO did not modify either the basal or SNP-stimulated cyclic GMP levels. We also studied the effect of 5 mM SNAP on cyclic GMP levels at different times, and the amounts of cyclic GMP measured at times above and below 15 min were similar to those shown in Figures 2A and 3 (Results not shown).

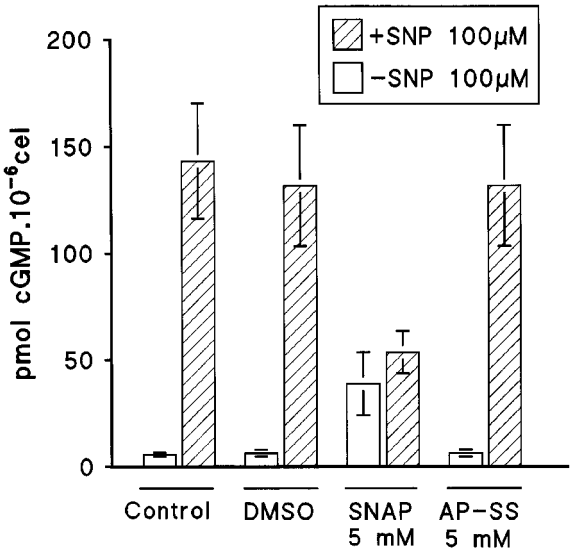

Figure 3 Effects of high concentrations of SNAP, AP-SS or DMSO on basal or SNP-stimulated cyclic GMP accumulation in bovine chromaffin cells. Cells were preincubated for 30 min with 0.5 mM IBMX. After pre-incubation the effect of 5 mM SNAP, 5 mM AP-SS and DMSO (2.8% final concentration) was tested on basal cyclic GMP levels or SNP-stimulated cyclic GMP levels. Addition of SNAP, AP-SS or DMSO plus SNP (100 μM) was simultaneous, and incubations were performed for 15 min. Cyclic GMP was assayed as described in Methods. Experiments were performed in triplicate and data are means$\pm$s.e.mean from four cellular preparations. Control means basal or SNP stimulated cyclic GMP levels in the absence of SNAP, DMSO or AP-SS.

Nitric oxide production by NO-releasing compounds

Nitric oxide production at various NO donor concentrations was measured in the presence of chromaffin cells. Since the maximal effect of NO donors on cyclic GMP accumulation was observed at 10 min when cells were treated with DEA/NO and at 15 min when they were treated with SNAP or SPER/NO, NO production by these agents was measured at these times and in the same conditions as cyclic GMP was previously measured. The most relevant concentrations of NO donors relating to their effects on chromaffin cells cyclic GMP levels and the NO concentration produced by them in the presence of cells are summarized in Table 1. As is shown in Table 1, there is a good correlation between sGC stimulation and NO concentration when the two NONOates were employed. However, a higher NO concentration was required to produce half-maximal or maximal cyclic GMP accumulation when it was supplied by SNAP.

Nitric oxide was also measured over different times by recording the oxidation of HbO_2. With this technique what is measured at each time is the accumulated metHb. Figure 4 shows the NO concentration reached at each experimental time (measured as metHb formed) when 500 μM SNAP, 5 μM SPER/NO or 5 μM DEA/NO were in the medium in the presence of cells. As the metHb formed is stable over the experimental time the values shown in Figure 4 represent the NO accumulated over the period. It was not possible to measure NO production from SNP under these experimental conditions because of the presence of other reactions taking place. As is shown in the figure, addition of 5 μM DEA/NO produced a very rapid increase in metHb, indicating a rapid decomposition of this product, a steady-state was reached at

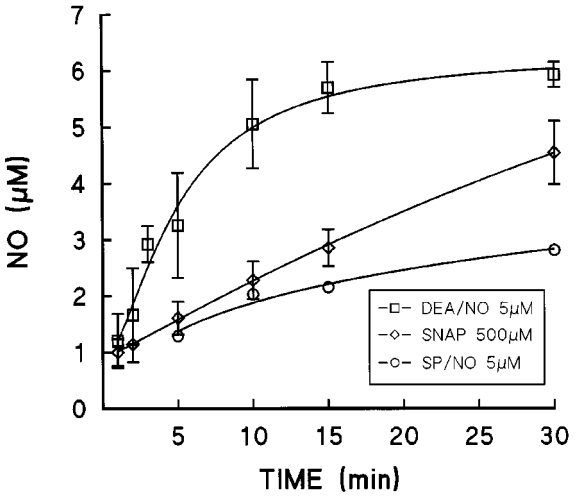

Figure 4 Time courses of NO production from different donors. NO released from 5 μM DEA/NO, 5 μM SPER/NO or 500 μM SNAP was measured at the indicated times by the haemoglobin reaction in the presence of chromaffin cells as described in Methods. Data are means $\pm$ s.e.mean from three different determinations and are expressed as NO concentration in μM reached at each time.

Table 2 Production of NO and nitrites by NO donors

	NO-donor (μM)	*Nitrite* (μM)	*NO* (μM)	*Stoichiometry*
SNAP	10	0.22 $\pm$ 0.03	0.19 $\pm$ 0.03	N.D.
	25	0.44 $\pm$ 0.20	0.47 $\pm$ 0.09	N.D.
	50	1.08 $\pm$ 0.03	0.78 $\pm$ 0.07	N.D.
	100	2.06 $\pm$ 0.05	2.28 $\pm$ 0.11	N.D.
	250	4.82 $\pm$ 0.14	5.8 $\pm$ 0.17	N.D.
DEA/NO	1	1.15 $\pm$ 0.20	–	1.15
	2.5	3.03 $\pm$ 0.34	–	1.20
	5	6.27 $\pm$ 0.81	–	1.25
SPER/NO	1	1.40 $\pm$ 0.30	–	1.40
	2.5	4.30 $\pm$ 0.07	–	1.72
	5	9.67 $\pm$ 0.38	–	1.94
	7.5	14.37 $\pm$ 0.50	–	1.92

Nitrite levels were measured by a fluorimetric assay as described in Methods. NO formation was determined by the haemoglobin method. These values were determined in the absence of cells. Since the decomposition of DEA/NO and SPER/NO was complete in the acidic medium present, the values of nitrites obtained, represent the total NO produced by these compounds, and the stoichiometry for NO production was calculated. As this is not the case for SNAP, the nitrite formation and NO released during incubation time (15 min) are indicated for this donor.

15 min, thus no more NO was released after this time. The half-life ($t_{1/2}$) calculated from the semi-logarithmic plot of these data was 3.9 $\pm$ 0.2 min. SPER/NO at 5 μM caused a more slowly and continuous increase in metHb accumulation over the experimental time and no steady-state was observed at these times, indicating that NO was continuously released. The half-life value calculated for decomposition of SPER/NO was 37 $\pm$ 3 min. When NO was measured from SNAP at 500 μM NO, production was linear over the time and the amount of NO after 60 min represented less than 2% of the total SNAP present. The calculated half-life for SNAP decomposition was 37 $\pm$ 4 h indicating that under our experimental conditions this compound was very stable.

In order to validate the NO values obtained with the haemoglobin method, nitric oxide was also determined by measuring nitrite formation by a fluoriometric assay, based upon the reaction of nitrite with DAN under acidic conditions. The values of NO concentration produced by different concentrations of SNAP found with this method were similar to those obtained using the haemoglobin method (Table 2). It was not possible to measure NO generated by higher SNAP concentrations due to the interference that large quantities of SNAP produces with regard to fluorimetric measurements. As it is shown in Table 2, NO concentration values measured in the form of NO or nitrites produced by SNAP over the same time and in the absence of cells are similar. These values were increased 4 fold when GSH 1 mM was present in the medium (data not shown). The values of the NO concentration in the absence of cells were higher to those obtained with the haemoglobin method in the presence of cells. In the case of SNAP the values obtained in the presence of cells corresponded to a 25% of the values obtained in the absence of cells. The reaction for nitrite determination was carried out under acidic conditions (pH $<$ 5) and NONOates decompose instantaneously at low pH, thus, the values of NO produced by these compounds reflect the NO concentration reached after their complete decomposition. The concentrations of NO produced by different NONOates concentrations and the stoichiometry are summarized in Table 2.

Production of NO by very high SNAP concentrations was determined using the haemoglobin method, measuring the decrease in absorbance at 576 nm. After 15 min at 37°C, 1 mM SNAP produced 27.5 and 4.4 μM of NO in the absence and the presence of chromaffin cells, respectively. SNAP at 3 mM produced 66.2 and 12.3 μM of NO in the absence and the presence of cells, respectively. As mentioned before the values of NO concentration measured in the presence of cells were significantly lower to those obtained in the absence of cells.

Discussion

The results of this study demonstrate that the four NO-releasing compounds used have very different potencies when it comes to producing comparable levels of cyclic GMP response in chromaffin cells. These differences might be explained by their specific decomposition rates and mechanisms by which they produce nitric oxide. The rank order of potencies of the four compounds in stimulating cyclic GMP accumulation was DEA/NO $>$ SPER/NO $>$ SNP $>$ SNAP. With an EC_{50} of about 0.4 μM, DEA/NO was the most potent and this value correlates well with those reported for endothelial cyclic GMP accumulation (Mayer *et al.*, 1995) and for bovine lung purified sGC activation (Friebe *et al.*, 1996). Under our experimental conditions the EC_{50} for SNAP-induced cyclic GMP accumulation was one order of magnitude higher than that obtained for SNP. The opposite order of potencies for these two compounds have been reported in cardiomyocytes (Davis *et al.*, 1997) and in mouse neuroblastoma clone NIE-115 cells (Lipton *et al.*, 1993), and rat cerebellar slices (Southam & Garthwaite, 1991). This apparent discrepancy may be due to the cell type or to the fact that the NO production from SNAP varies with the medium composition, and so for this reason in the presence of GSH (which increases NO release from nitrosothiols) the EC_{50} value for SNAP-induced cyclic GMP accumulation dropped by nearly two orders of magnitude.

Nitric oxide released from these donors was assessed in order to check if differences in amount of NO produced by

them might explain their relative potency in stimulating cyclic GMP in bovine chromaffin cells. Nitric oxide or its stable end-product (nitrite) were measured by two different methods. The formation of methaemoglobin was monitored at 401 or 576 nm. Although this method requires very careful experimental analysis in order to verify that NO is being measured accurately, it is very sensitive (the theoretical detection limit for NO is 1.3 μM) and allows a continuous measurement of NO (Murphy & Noack, 1994). This method has previously been employed to quantify NO production by SNP, however the values of NO obtained are always very low (Ioannidis *et al*., 1996). In our study, it was impossible to perform these measurements. In the presence of cells, where SNP releases NO, the absorbance at 401 nm increased for the first 15 min and diminished thereafter, thus indicating that another redox reaction might be occurring. However, in line with several previous reports, it may be conceded that SNP releases small amounts of NO over long periods (Bates *et al*., 1992; Rao *et al*., 1991). With this experimental approach no NO production was observed with SNP in the absence of cells. This fact was not unexpected, as although the mechanism of NO release from SNP is poorly understood, it is known not to decompose spontaneously but only after photolysis, addition of reducing agents or metabolic transformation (Bates *et al*., 1992; Murphy & Noack, 1994). When SPER/NO and DEA/NO were used their half-life and the NO concentrations produced were equal to those previously reported under similar experimental conditions (Ramamurthi & Lewis, 1997), including those expected by applying the theoretical mathematical model reported by Schmidt *et al*. (1997). The stoichiometry (e_{NO}) calculated for SPER/NO at 37°C and pH 7.4 was 1 : 1.7 – 1.9, i.e. similar to that previously described (e_{NO}, of 1.7 (Ramamurthi & Lewis, 1997) and e_{NO} of 2 (Keefer *et al*., 1996)). For DEA/NO the stoichiometry was 1 : 1.2. This value was within the range reported by several groups (e_{NO} of 1 (Schmidt *et al*., 1997) and 1.5 (Ramamurthi & Lewis, 1997)). Nevertheless, a wide range of concentrations of NO produced by the same SNAP concentrations have been reported by different groups (Matthews *et al*., 1996; Singh *et al*., 1996). Similar values of NO were measured in this study using two different methods, indicating that NO is being determined accurately under these experimental conditions. These values are similar to those obtained by some authors using the same method (Läfars & Gyllenhammar, 1995), but are lower than those determined by others (Ioannidis *et al*., 1996). The mechanism of nitric oxide release from nitrosothiols is not at all obvious and has been previously shown to be highly dependent on the components of a given system, particularly with regard to the concentration of reduced thiol and transition metals present (Dicks *et al*., 1996; Gordge *et al*., 1996; Singh *et al*., 1996). A transient release of nitric oxide from SNAP, amounting to less than 0.1% of the total SNAP present, has been observed in a phosphate buffer solution containing the non-redox-active metal chelating agent DTPA at physiological pH and temperature (Haddah *et al*., 1994). Under our experimental conditions a linear release of NO from SNAP over the time was measured, and the amount of NO after 60 min corresponded to less than 2% of the SNAP present, giving a very long half-life for SNAP. As expected the NO concentration after 15 min incubation in the presence of GSH was considerably higher. The fact that levels of NO produced by SNAP in the absence of cells (Table 2) (measured either as nitrite or NO) were four times higher than in the presence of cells, can be explained by differences in system composition as the presence of cells also contributes to the total metal ion content, thus affecting the release of NO from SNAP, together with a more rapid NO inactivation in the presence of cells demonstrated to be faster for high NO concentration (Kharitona *et al*., 1994; Goldstein & Czapski, 1995). Supporting this idea it has also been shown that several NO donors are two orders of magnitude more potent in stimulating guanylyl cyclase preparations than in stimulating cyclic GMP production in cerebellar slices (Knowles *et al*., 1990; Southam & Garthwaite, 1991).

A good correlation between NO production and cyclic GMP increases was observed when both NONOates were employed. In order to compare the time-courses for NO donors-stimulating cyclic GMP increases and their decomposition to release NO, the fact that the haemoglobin reaction method is a cumulative technique should be borne in mind. Moreover, the very high affinity of haemoglobin for NO causes it to be rapidly trapped, inhibiting a possible inactivation of NO. Thus, although in the case of DEA/NO a constant concentration of NO was reached after 15 min, these data mean that after this time there was no more NO released from this compound. When the time course for DEA/NO-stimulating cyclic GMP levels was analysed a significant fall in cyclic GMP levels was observed during that period, indicating that there was no more cyclic GMP synthesis. The amounts of NO produced by both NONOates at concentrations that caused half-maximal (EC_{50}) and maximal cyclic GMP accumulation are higher (2 or 3 fold) than the reported values for the equilibrium dissociation constant of NO for activating sGC and the concentration of NO that produced maximal nitrosyl complex, respectively (Stone & Marletta, 1996). However, this difference could be explained by the fact that we have measured the accumulated NO over the period and in the absence of haemoglobin it is expected that inactivation of NO by cells occurred, because although at low concentration, NO seems to be very stable and its inactivation is faster in cells- or tissues-containing media (Knowles *et al*., 1990; Southam & Garthwaite, 1991). Nevertheless the behaviour of SNAP is more complicated and a lack of correlation was observed between SNAP-increasing cyclic GMP levels and NO production, and higher NO concentrations were required to produce half-maximal and maximal increase in cyclic GMP. The time-course experiments revealed that whereas cyclic GMP levels follow the pattern of NO production for NONOates and even for SNP if we assume a continuous release of NO, a steady state of cyclic GMP levels was reached after 15 min when SNAP was used although the concentration used did not cause maximal stimulation of cyclic GMP synthesis and we have observed a lineal NO production over the time. On the other hand, in the dose-response experiments a biphasic effect was observed only when SNAP was employed as NO source. A similar SNAP profile has been obtained in rat cerebellar slices (Southam & Garthwaite, 1991). A biphasic effect has also been observed when purified soluble guanylate cyclase was treated with peroxinitrites in the presence of GSH (Friebe *et al*., 1996). The non NO-releasing SNAP analogue did not modify the basal or SNP-stimulated cyclic GMP levels, thus the inhibitory effect seems to be due to a mechanism in which SNAP participates. In the presence of GSH 1 mM the inhibitory effect was observed even at lower SNAP concentration. These results together with the fact that only SNAP at high concentrations produced a fall in cyclic GMP levels, might indicate that SNAP may have produced a modification of the enzyme, and depending on the extent of this modification, a desensitization or even an inhibition could be observed. While our study has not investigated the mechanism by which sGC desensitization is produced, other studies have found that it can result from an oxidation or

nitrosylation of critical free thiol groups of the enzyme (Davis *et al*., 1997). Nitrosylation has recently been reported to modulate the function of several proteins including, membrane-bound, cytosolic and nuclear proteins, and this mechanism is favoured by the presence of S-nitrosothiols (Lipton *et al*., 1993; Singh *et al*., 1996; Kaye *et al*., 1997).

All four compounds tested can clearly raise intracellular cyclic GMP to very high levels in bovine chromaffin cells, indicating the big capacity of these cells to respond to NO in this way. In previous studies we have shown that most of chromaffin cells express NOS I and α- and β-subunits of sGC (Schwarz *et al*., 1998) and that these cells responded to acetylcholine with transitory increases of both NOS activity and cyclic GMP levels (Rodríguez-Pascual *et al*., 1995; Schwarz *et al*., 1998). Since the physiological response is transitory short-lived NO donors such as DEA/NO which release NO during short times at low concentrations can better mimic the activation of the endogenous NOS/sGC pathway in these cells. In general it is well known that while constitutive isoforms of NOS, when activated, release small amounts of NO for short periods, the inducible isoform produces a long-lasting generation and large amounts of NO (Förstermann *et al*., 1995). Thus short-lived NO donors at low concentrations may be the most precise approach to mimic the behaviour of endogenously synthesized NO by a constitutive isoform while long-lived compounds would better mimic activation of inducible NOS.

Conclusion

The data reported here support the idea that different NO donors show very different potencies in stimulating guanylate cyclase, and that this correlates well with their capacities in releasing NO, except in the case of SNAP where a more complex mechanism is likely to be involved. It should keep in mind that very low NO concentrations such as those reported here are necessary to activate sGC. When the involvement of cyclic GMP in NO signalling is to be analysed small amounts of NO should be used because at higher concentrations other signalling mechanisms participate in the biological effect of NO. Thus, the appropriate dose of NO-releasing compounds and the amount of NO produced from it should be determined in each experimental condition in order to verify whether tissues or cultured cells are exposed to physiologically important NO concentrations.

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