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Inhibition of Nitric Oxide Synthase with Pyrazole-1-Carboxamidine and Related Compounds*

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ABSTRACT. Guanidines, amidines, *S*-alkylisothioureas, and other compounds containing the amidine function (—C(=NH)NH_2) have been described as inhibitors of the generation of nitric oxide (NO) by NO synthase (NOS). Here we report on the inhibition of the activity of NOS isoforms by compounds in which the amidine function is attached to a nitrogen of 1,2-diazo heterocycles to form *N*-carboxamidines and related compounds. 1*H*-Pyrazole-1-carboxamidine HCl (PCA) inhibited the activity of purified inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS) isoforms to a similar extent (IC_{50} = 0.2 μM). 3-Methyl-PCA and 4-methyl-PCA showed reduced potencies, but a preference for iNOS [IC_{50} = 5 and 2.4 μM, respectively; cf. N^G-methyl-L-arginine (NMA) IC_{50} = 6 μM]. Inhibition of purified iNOS by PCAs could be reversed completely by excess L-arginine, while their inhibition of NO production by stimulated RAW macrophages could be reversed by transfer to a drug-free medium. This suggests a competitive mode of inhibition. PCA caused potent concentration-dependent inhibition of the acetylcholine-induced, endothelium-dependent relaxations of precontracted rat thoracic aorta (IC_{50} = 30 μM). 4-Methyl-PCA inhibited the relaxations only at ≥300 μM. In contrast, 4-methyl-PCA was more effective than both PCA and NMA in restoring the *ex vivo* contractility of aortic rings taken from lipopolysaccharide-treated rats. PCA and NMA, but not 4-methyl-PCA, caused marked increases in mean arterial pressure when administered i.v. to anesthetized rats. In conclusion, PCA and related compounds caused potent inhibition of NOS. Substitution of the pyrazole ring reduced potency, but improved selectivity towards iNOS as exemplified by 4-methyl-PCA. BIOCHEM PHARMACOL **54**;3: 409–417, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. inhibition; nitric oxide synthase; L-arginine; pyrazole-1-carboxamidine; blood pressure

NO¶ is involved in the regulation of many physiological processes and in the pathophysiology of many disease states. Consequently, the pharmacological regulation of the NOS isoenzymes responsible for the generation of NO is of interest, not only in establishing the further roles of NO, but also for possible therapeutic intervention. NO from the inducible isoform (iNOS), following its induction in various cells and tissues, has been implicated in the pathogenesis of various forms of circulatory shock and inflammation [1, 2]. NO from the constitutive endothelial isoform (eNOS) is involved in, *inter alia*, the regulation of blood pressure and blood flow, and NO from the neuronal isoform (nNOS) is involved in neurotransmission, neurodegeneration, and, possibly, neuroinjury [3–5]. Thus, while inhibition of NOS may be beneficial in many instances, selective inhibition of only the relevant NOS isoform is preferred in order to leave the important physiological roles of the other isoforms undisturbed.

The generation of NO by NOS can be inhibited by analogs of the substrate L-arginine, but recently several classes of compounds containing the "amidine" function (—C(=NH)NH_2), such as *S*-alkylisothioureas [6–8], amidines [9], aminopyridines [10], and various guanidines [11–13], have been reported to inhibit the activity of NOS with potencies towards iNOS equal to or better than that of the arginine analogue N^G-methylarginine, some of them with selectivity towards iNOS [14].

The present study investigated the effects on NOS

* The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does the mention of any trade names, commercial product, or organizations imply endorsement by the U.S. Government.

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¶ *Abbreviations:* -CA, carboxamidine; DMEM, Dulbecco's modified Eagle's medium; IFN, γ-interferon; LPS, lipopolysaccharide; MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; iNOS, inducible NOS; eNOS, endothelial NOS; NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal NOS; NMA, N^G-methyl-L-arginine; and PCA, 1*H*-pyrazole-1-carboxamidine HCl.

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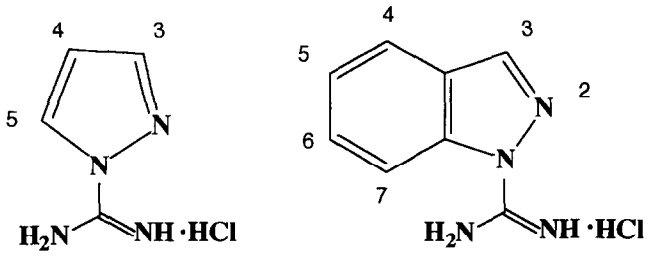

1H-pyrazole-1-carboxamidine 1H-indazole-1-carboxamidine

FIG. 1. Examples of mono- and bicyclic carboxamidines used in this study.

activity of PCA and related compounds, where the guanidine function is part of a 1,2-diazo heterocycle (Fig. 1).

MATERIALS AND METHODS

Materials

DMEM and fetal bovine serum were from Gibco (Grand Island, NY). NMA was from Calbiochem (La Jolla, CA). Thiopentone sodium was obtained from Abbott Laboratories (Chicago, IL). Murine IFN was from Genzyme (Cambridge, MA). Tetrahydrobiopterin was obtained from Cayman Chemical (Ann Arbor, MI). L-[2,3,4,5-^{3}H]-Arginine hydrochloride was obtained from DuPont/NEN (Boston, MA). Bacterial LPS (*Escherichia coli*, serotype No. 0127:B8) and all other reagents were obtained from the Sigma Chemical Co. (St. Louis, MO).

Purification and Characterization of NOS Isoforms

iNOS was prepared from murine macrophage (RAW 264.7) cells following stimulation with IFN and LPS, using ADP-agarose [15, 16]. A typical preparation exhibited a specific activity of 0.2 to 0.6 μmol citrulline formed/min/mg protein as measured using the citrulline formation assay at 100 μM L-arginine and saturating amounts of relevant co-factors. Bovine pulmonary arterial eNOS was prepared and characterized as previously described [17]. These preparations of eNOS typically possessed a specific activity of 0.3 ± 0.1 μmol citrulline formed/min/mg protein at 100 μM L-arginine. An NOS preparation physically, kinetically, and immunologically identical to bovine nNOS, but containing substoichiometric quantities of bound BH_4 (0.15 mol BH_4/mol) was prepared from extracts of GH3 cells by adsorption to ADP-agarose and elution with NADPH [16]. Typically, the specific activity was approximately 0.6 μmol citrulline formed/min/mg protein as measured at saturating concentrations of L-arginine and co-factors. The respective K_m values of iNOS, nNOS, and eNOS for its arginine substrate were 12, 4.3, and 5 μM.

Measurements of NOS Activity of ADP-Agarose Purified Enzyme

NOS activities of iNOS, eNOS, and nNOS were measured by conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline using a modification of the procedure of Bredt and Snyder [18] as described previously [19]. Standard incubations for the measurement of citrulline formation by eNOS or nNOS contained 30 mM HEPES, pH 7.4, 1 mM dithiothreitol, 120 nM L-[^{3}H]arginine (a subsaturating concentration), 1 mM EGTA, 0.85 mM Ca^{2+}, 6 μM calmodulin, 100 μM NADPH, and 100 μM BH_4. Standard incubations for the measurement of iNOS contained 30 mM HEPES, pH 7.4, 1 mM dithiothreitol, 120 nM L-[^{3}H]arginine, 1 mM EGTA, 100 μM NADPH, and 300 μM BH_4. Assays were started by the addition of purified enzyme. Incubations were conducted at 30° for 30 min in duplicate, and the effect of various concentrations of agent on NOS activity was tested. Variability of values around the mean routinely averaged ± 3% of the mean. Routinely, assays were conducted at dilutions of enzyme that provided 5–10% of total substrate consumption. Under these conditions, product formation was linear over time with control rates for iNOS, nNOS, and eNOS of 81.8, 25.5, and 49 nmol citrulline formed/min/mg protein, respectively.

In separate experiments, iNOS activity was determined by the conversion of oxyhemoglobin to methemoglobin by NO formation as described by Noack *et al.* [20]. Standard incubations contained 50 mM HEPES, pH 7.4, 100 μM L-arginine, 1 mM EGTA, 100 μM NADPH, 2 μM BH_4, and 5 μM oxyhemoglobin. Reactions were initiated with ADP-agarose purified iNOS, and the difference in light absorbance at 401 nm was determined continuously. Methemoglobin formation is quantified using an extinction coefficient for methemoglobin of 38 mM^{-1} cm^{-1}. This assay is suitable for the detection of NO formation over shorter time periods (up to 10 min in our studies). Control rates (no inhibitor) were found to be 0.29 μmol NO/min/mg protein.

Measurements of iNOS Activity in Cultured Macrophages

iNOS activity in macrophages from the mouse macrophage cell line RAW 264.7 was measured (a) by the reduction of oxyhemoglobin to methemoglobin by NO, and (b) as the concentration of nitrite in the culture medium 24 hr after induction.

(A) KINETIC MEASUREMENT OF NO FORMATION RATE. RAW 264.7 cells were cultured in DMEM with 10% fetal bovine serum [15]. Cells were transferred and cultured in 6-well plates until confluent. Cells were treated with *E. coli* LPS (10 μg/mL) and murine IFN (50 U/mL) for 16 hr, and the medium was replaced with Ham's F-10 medium containing 100 μM arginine and 5 μM oxyhemoglobin. The rate of NO formation was determined by the rate of formation of

methemoglobin measured as the difference in light absorbance at 401 nm (methemoglobin absorbance maximum) and 411 nm (isosbestic point of methemoglobin and oxyhemoglobin) using the extinction coefficient used above. Values were corrected for auto-oxidation determined over identical time periods in 6-well plates containing cells not induced with cytokine. The wells were found to produce 0.6 nmol NO/min/mg protein with a rate that varied by less than 2% among the 6 wells. The medium was then replaced with fresh Ham's F-10 medium containing 100 μM arginine and 5 μM oxyhemoglobin with and without inhibitor, and NO formation was measured at 2-min intervals.

(B) END POINT DETERMINATION OF TOTAL CUMULATIVE NITRITE FORMATION. Cells from the mouse macrophage cell line RAW 264.7 were cultured in DMEM supplemented with 4 mM L-glutamine, penicillin (10,000 U/L), streptomycin (10,000 U/L), L-arginine (0.4 mM), and 10% fetal bovine serum [21]. Cells were cultured in 96-well plates until they reached 60–80% confluence. To induce iNOS, fresh culture medium containing *E. coli* LPS (10 μg/mL) was added. NOS inhibitors were added to the cells 6 hr after immunostimulation (to prevent potential action of the inhibitors on the process of iNOS induction [see Ref. 6]). Accumulation of nitrite in the cell culture medium (in the absence or presence of various inhibitors) was measured after 24 hr by the Griess reaction [6]. Standard curves were obtained with sodium nitrite (1–100 μM).

Cell Respiration

Mitochondrial respiration, an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of MTT to formazan [6].

Hemodynamic Measurements

All animal experiments were performed in accordance with NIH guidelines and with the approval of the institutional review board of the Children's Hospital Research Foundation. Male Wistar rats (Charles River Laboratories, Wilmington, MA) were anesthetized with thiopentone sodium (120 mg/kg, i.p.) and instrumented as described [8]. The trachea was cannulated to facilitate respiration, and temperature was maintained at 37° using a homeothermic blanket. The right carotid artery was cannulated and connected to a pressure transducer for the measurement of phasic and mean arterial blood pressure (MAP) and heart rate, which were digitalized using a Maclab A/D converter (AD Instruments, Milford, MA). The left femoral vein was cannulated for the administration of drugs. Upon completion of the surgical procedure, cardiovascular parameters were allowed to stabilize for 10 min. For the dose–response curves with NOS inhibitors, cumulative doses of L-NMA, PCA, and 4-methyl-PCA (0.1, 0.3, 1, 3, and 10 mg/kg bolus injections) were injected 5 min apart, and readings were taken at 4.5 min after the injection of each dose.

Organ Bath Experiments

Rings of thoracic aortae from rats were mounted in organ baths (5 mL) filled with warmed (37°), oxygenated (95% O_2/5% CO_2) Krebs solution (pH 7.4) consisting of (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, and glucose 11.7, in the presence of indomethacin (10 μM). Isometric force was measured with isometric transducers (Kent Scientific Corp., Litchfield, CT). A tension of 1 g was applied, and the rings were equilibrated for 60 min, changing the Krebs solution every 15 min [22]. To study the effects of NOS inhibitors on endothelium-dependent relaxation (an indicator of eNOS activity), intact rings from normal animals were used. Rings were precontracted with noradrenaline (100 nM) and pretreated for 30 min with various concentrations of NOS inhibitors (0, 30, 100, and 300 μM). Relaxations in response to acetylcholine (1 nM–10 μM) were then recorded. The effects of inhibitors on endothelium-denuded rings from LPS-treated rats were studied as an indicator of the ability of these compounds to inhibit iNOS activity in the vascular smooth muscle. Thus, endothelium-denuded rings were prepared from aorta of rats previously treated with LPS (15 mg/kg for 3 hr). After incubation and wash-outs, the vessels were first pretreated (or not, for controls) with inhibitor, and then the effect of noradrenaline (1 nM–10 μM) was tested.

Synthesis of Cyclic N-Carboxamidines

PCA was synthesized by the reaction of pyrazole with cyanamide according to the method of Bernatowitz *et al.* [23]. The other compounds were prepared by similar routes with the exception of 3,5-dimethyl PCA and pyrazole, which were purchased from Aldrich (Milwaukee, WI). For example, equimolar amounts of 3-methylpyrazole, cyanamide, and HCl (added as a 4 M solution in dioxane or as gas) were refluxed in dry dioxane for 2 hr under nitrogen. Upon cooling, the mother liquor was tipped off, and the residual brown oil was induced to crystallize. Repeated recrystallization from ethanol/ether mixtures gave colorless crystals of 3-methyl-PCA as the hydrochloride salt (m.p. 150°). *N*-Benzyl-PCA was prepared from pyrazole and benzylcyanamide under similar conditions (benzylcyanamide was prepared from reacting two equivalents of benzylamine with cyanogen bromide in methanol at 0°). Purity was confirmed by reverse-phase HPLC (C18 column with gradient elution by acetonitrile in 50 mM phosphate buffer, pH 4.5, and detection at 254 nm) and by TLC on silica plates developed in dichloromethane:ethyl acetate:ethanol (± triethylamine) [7:2:1:1]. Spots were located by absorbance at 254 nm and by spraying with 2% sodium pentacyanoamine ferroate (PCAs give a bright yellow color). ^1H and ^{13}C NMR confirmed structure. All starting materials were from Aldrich.

TABLE 1. Inhibition of purified nitric oxide synthase isoforms by PCAs: IC_{50} values at 120 nM arginine

Compound	IC_{50} (μM)		
	iNOS	nNOS	eNOS
Aminoguanidine	20	280	480
N^G-Methyl-L-arginine (NMA)	6.0	6.0	12
Pyrazole	180	1000	>3000
Pyrazole-1-carboxamidine (PCA)	0.2	0.2	0.2
3-Methyl-PCA	5.0	8.0	15
4-Methyl-PCA	2.4	10	22
3,5-Dimethyl-PCA	1600	2500	ND*
N-Benzyl-PCA	>2000	85	ND
1,2,4-Triazole-CA	0.5	2.0	10
Indazole-CA	440	660	190
7-Nitroindazole-CA	4.3	3.0	ND
Benzotriazole-CA	300	64	ND
Indoline-CA	>1000	200	ND

* ND, not determined.

Statistical Evaluation

The IC_{50} values were calculated by linear regression after logit-log transformation of concentration–response curves. Unless stated, values in the figures and text are expressed as means ± SEM of N observations. Student's unpaired *t*-test was used to compare means between groups.

RESULTS

Effects of PCA and Related Compounds on the Activity of the Purified iNOS, eNOS and nNOS Enzymes

PCA (see Fig. 1) potently inhibited the formation of citrulline by purified iNOS, eNOS, and nNOS isoenzymes to a similar extent and with potencies (IC_{50} = 0.2 μM) of up to 30 times that of NMA (Table 1 and Fig. 2). Although the NOS isoforms originate from different species, PCA appears to lack any discrimination between isoforms. Substitution of PCA with a methyl group on the ring in the 3- or 4- position (see Fig. 1) reduced the potency against all NOS isoforms, but not uniformly. 4-Methyl-PCA showed a moderate preference (10-fold) for iNOS over eNOS. The replacement of the ring carbon at C-4 on PCA (see Fig. 1) with a nitrogen had little influence on its inhibition of iNOS, but reduced its effectiveness against constitutive isoforms, resulting in a 20-fold preference for iNOS over eNOS. This is in contrast to the unfavorable effects of a similar substitution in the ring of aminopyridine [10]. Substitution of an amidino nitrogen, as in *N*-benzyl PCA, resulted in virtually no inhibitory activity against iNOS, but a modest activity against nNOS was retained.

Of the bicyclic compounds tested, only 7-nitroindazole-CA showed inhibitory activity comparable to those of the monocyclic compounds (Table 1). This is contrast to indazole-CA itself, which was a poor inhibitor of the purified enzymes.

In addition to the above experiments, the inhibitory effect of PCA on iNOS activity was also examined by assessing the inhibition of NO formation, measured as the formation of methemoglobin from oxyhemoglobin. In these experiments, the presence of PCA in the enzyme incubation caused a concentration-dependent reduction in the rate of NO formation. When the addition of PCA was delayed until a few minutes after the initiation of NO production, the onset of inhibition was rapid and the (reduced) rate of NO production linear with time (Fig. 3).

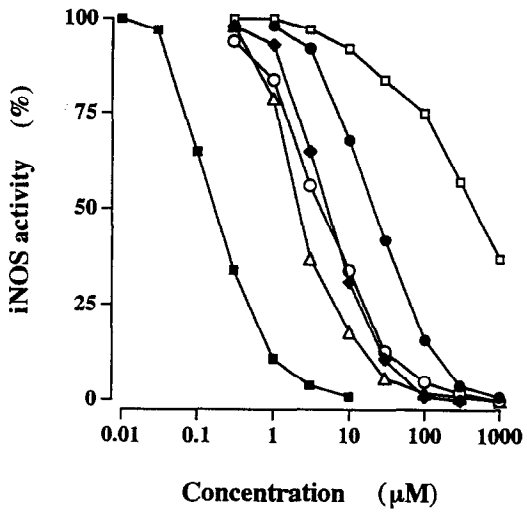

FIG. 2. Effect of N^G-methyl-L-arginine (◆), aminoguanidine (●), PCA (■), 4-methyl-PCA (△), 3-methyl-PCA (○), and indazole-carboxamidine (□) on the citrulline-forming activities of iNOS. Standard incubations contained 120 nM L-arginine and indicated concentrations of drug in a final volume of 150 μL. The control rate (no drug) was 81.8 nmol citrulline formed/min/mg protein.

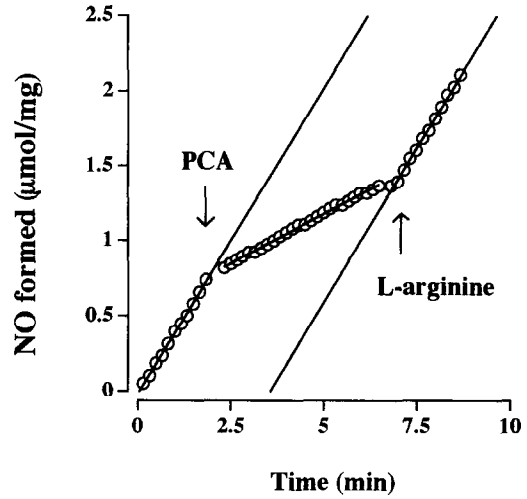

FIG. 3. PCA inhibition of NO formation by ADP-agarose purified murine macrophage iNOS and its reversal by L-arginine. Incubations contained 100 μM arginine, 5 μM oxyhemoglobin, and cofactors (see Materials and Methods). Reactions were initiated at zero time by the addition of 1 μg of purified murine macrophage iNOS. NO formation was assessed as the increase in absorbance at 401 nm accompanying the conversion of oxyhemoglobin to methemoglobin by NO. PCA (final concentration of 3 μM) was added at 2 min. At 6.5 min the concentration of arginine was increased to 3.3 mM. The control rate was 0.29 μmol NO formed/min/mg protein.

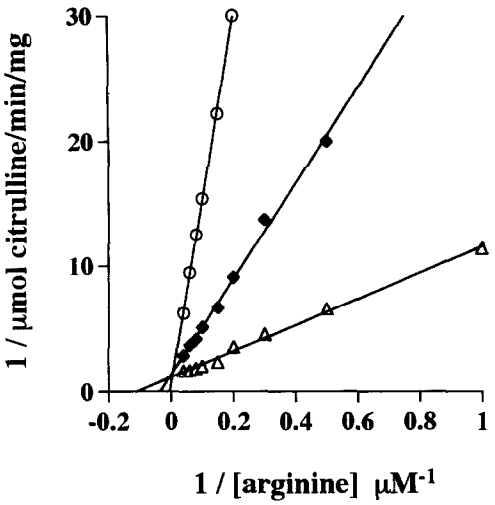

FIG. 4. Effect of PCA on the arginine concentration dependence of murine macrophage iNOS. Standard incubations were constructed containing the indicated concentrations of arginine ranging from 1 to 25 μM, without (△) or containing 0.3 μM (◆) or 1 μM (○) PCA. Reactions were initiated with 0.36 mg of affinity purified murine macrophage iNOS, as described in Materials and Methods. Values are plotted in double-reciprocal format as reciprocal μM arginine versus reciprocal μmol citrulline formed/min/mg protein.

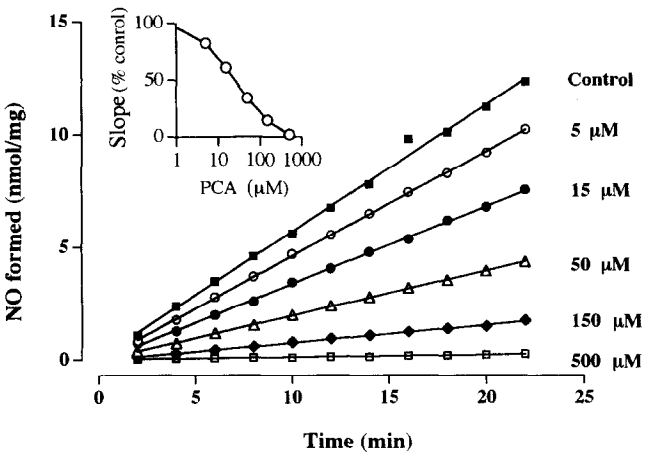

FIG. 5. Effect of PCA on NO formation by cytokine-induced RAW 264.7 cells. Confluent cells in 6-well plates were treated with IFN/LPS for 16 hr and then were incubated in fresh Ham's F-10 medium containing 100 μM arginine, 5 μM oxyhemoglobin, and the indicated concentrations of PCA. Control NO formation (without PCA) was 0.56 nmol/min/mg protein (variation < 4%). Total cumulative NO formation rates were determined at 2-min intervals. The slopes of the plots represent the rates of NO formation at the indicated concentration of PCA. These are shown in the inset as percent of control slope (rate) and plotted against [PCA]. An IC_{50} value of 25 μM was determined. The results depicted are representative of those obtained from two independent experiments.

Furthermore, the addition of excess L-arginine completely reversed this inhibition (Fig. 3). Similar patterns of arginine dependent inhibition were also observed for 3-methyl-PCA and for 4-methyl-PCA (data not shown). The IC_{50} values were estimated as 2, 50, and 25 μM for PCA, 3-methyl-PCA, and for 4-methyl-PCA, respectively, in the presence of 100 μM extracellular arginine.

The nature of the inhibition of iNOS was investigated further for PCA by measuring citrulline formation at various concentrations of L-arginine and PCA. As expected from the above, the data reveal that PCA acts as a competitive inhibitor versus arginine substrate, altering the apparent K_m of iNOS for its arginine substrate, but not the maximal velocity obtainable in the presence of drug (Fig. 4). For a competitive inhibitor the apparent K_m in the presence of drug = the K_m in the absence of drug × (1 + $[i]/K_i$). In the absence of drug, the K_m of iNOS for arginine substrate was observed to be 12 μM, while in the presence of 0.3 μM PCA the apparent K_m for arginine was raised to 33 μM. Using the above expression, we calculate the K_i value of iNOS for PCA to be 0.17 μM, a value very similar to the IC_{50} for PCA (0.2 μM) measured at a concentration of arginine of 0.12 μM (see Table 1).

Effects of PCA and Related Compounds on iNOS Activity in Immunostimulated RAW Cells

PCA and related compounds concentration-dependently inhibited iNOS activity in stimulated RAW macrophages. PCA (Fig. 5) and 1,2,4 triazole-CA reduced NO formation by RAW cells with IC_{50} values of about 25 and 300 μM, respectively (in 100 μM extracellular arginine). The diminished rates of NO formation were linear over the 30 min studied, indicating a time-independent inhibition of NO formation consistent with a reversible inhibition of intracellular iNOS activity. The onset of inhibition following addition of PCA to the medium of stimulated cells was rapid (Fig. 6), which implies a ready uptake of PCA by this cell line. Similarly, replacement of the medium with PCA-free medium resulted in a rapid, but partial, restoration of the pretreatment NO production rate. In contrast to the arginine reversal experiments on purified iNOS, some residual inhibition remained that could not be "washed out." Only 56 and 52% of control activity was recovered following wash-out of 50 and 500 μM PCA, respectively (Fig. 6). When the treated cells were lysed and the lysate was measured for ability to catalyze the conversion of arginine to citrulline, only approximately 50% of the iNOS activity of untreated (control) cells was observed. The treated cells were still viable as measured by trypan blue exclusion.

Incubation of LPS-stimulated RAW macrophages with carboxamidines for 18 hr, and the subsequent measurement of cell respiration, revealed cytotoxicity of the bicyclic compounds (Table 2). Some of the bicyclic compounds were toxic at around 100 μM, so that inhibition of NOS activity as measured by nitrite accumulation could not be reasonably determined nor could IC_{50} values be accurately established. For the monocyclic compounds, which showed no signs of adversely affecting cell viability, the rank order of potency on nitrite production by RAW cells was similar to that seen with purified iNOS, with some notable

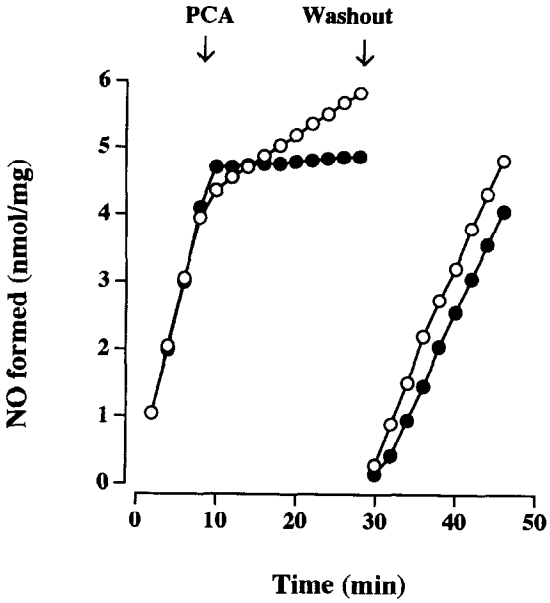

FIG. 6. Effect of PCA on the time–course of NO formation by cytokine-induced RAW 264.7 cells. NO formation was measured as detailed in the legend to Fig. 5. PCA was added to the cell medium after 8 min to give a final concentration of either 50 μM (○) or 500 μM (●). Fifteen minutes later, the medium containing the PCA was removed, and the cells were washed and incubated with PCA-free medium. A control rate of 0.56 nmol/min/mg protein (0–8 min) was observed prior to the addition of PCA, and rates of 0.28 nmol/min/mg protein (56% of control value) and 0.26 nmol/min/mg protein (52% of control value) were observed following restoration to PCA-free medium. The results depicted are each representative of those obtained from two independent experiments.

exceptions. Most noticeably, PCA and its methyl derivatives, while being more potent than NMA on purified enzyme, were only equivalent to or weaker than NMA at reducing nitrite accumulation by these cells.

TABLE 2. Effects of compounds on nitrite formation by stimulated macrophages and on cell viability

Compound	Inhibition of nitrite, EC_{50} (μM)	Reduced viability at concentration (μM)
N^G-Methyl-L-arginine (NMA)	105	>3000
Pyrazole	>3000	>3000
Pyrazole-1-carboxamidine (PCA)	110	>3000
3-Methyl-PCA	2000	>3000
4-Methyl-PCA	1000	>3000
3,5-Dimethyl-PCA	>1000	>3000
N-Benzyl-PCA	>3000	>3000
1,2,4-Triazole-CA	420	>3000
Indazole-CA	>3000	1000
7-Nitroindazole-CA	(100)	100
Benzotriazole-CA	(120)	100
Indoline-CA	>1000	200

Cells were incubated for 6 hr with LPS, and then for a further 18 hr with drug, before nitrite concentrations and viability were assessed by MTT assay.

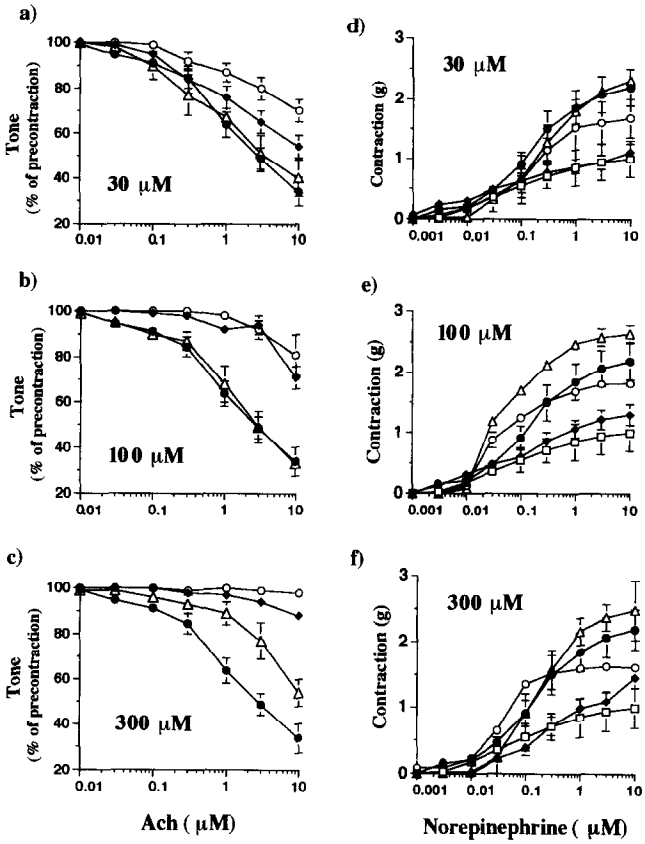

FIG. 7. (a–c) Effects of PCA (○), NMA (◆), and 4-methyl-PCA (△) at concentrations of (a) 30, (b) 100, and (c) 300 μM on the relaxations elicited by acetylcholine (ACh, 10 nM–10 μM) on rat thoracic aortic rings with intact endothelium (precontracted with noradrenaline). Acetylcholine-induced relaxations also were studied under control conditions (●). (d–f) Effects of pretreatment with PCA (○), NMA (◆), and 4-methyl-PCA (△) at concentrations of (d) 30, (e) 100, and (f) 300 μM on the contractions elicited by norepinephrine on endothelium-denuded vessels prepared from aortae of rats previously treated with LPS. Responses to noradrenaline in the absence of drug (LPS control, □) and to rings from normal animals (●) are also shown. Results are means ± SEM of N = 6–8 rings from 3–4 animals.

Vascular and Hemodynamic Effects of PCA and 4-Methyl-PCA

PCA and 4-methyl-PCA were selected for vascular and *in vivo* studies, and NMA, a non-isoform selective NOS inhibitor, was used as a reference compound. Both PCA and NMA caused concentration-dependent inhibition of the acetylcholine-induced, endothelium-dependent relaxations of precontracted rat thoracic aorta (Fig. 7, a–c). PCA was the more effective in this respect, showing inhibition at the lower concentration (30 μM). 4-Methyl-PCA showed inhibition of the relaxations only at concentrations of 300 μM. Conversely, 4-methyl-PCA was more effective than PCA in restoring the *ex vivo* contractility of aortic rings taken from LPS-treated rats (Fig. 7, d–f). PCA was more effective than NMA. The reduced contractile responses to norepinephrine of aortic rings prepared from LPS-treated animals, when compared with tissues taken

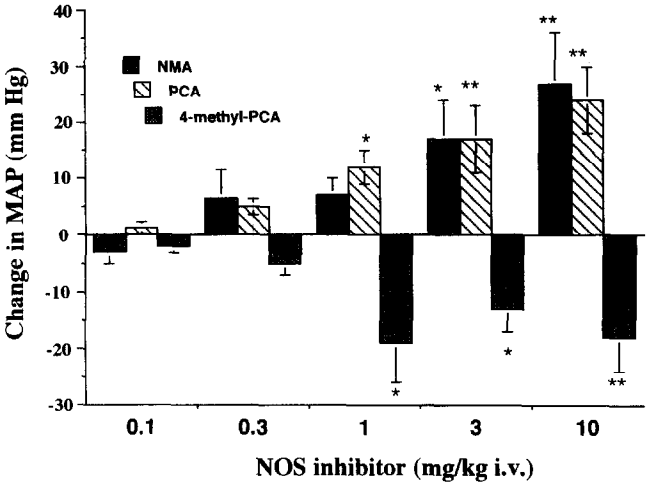

FIG. 8. Effects of 4-methyl-PCA, PCA, and NMA (at 0.1 to 10 mg/kg bolus doses) on mean arterial blood pressure (MAP) in anesthetized rats. Readings were taken at 4.5 min after the administration of each dose of the inhibitors. Key: (*) $P < 0.05$; and (**) $P < 0.01$ represent significant changes in MAP. Data represent means ± SEM of 6 rats in each group.

from normal animals, is a result of induction of iNOS in the aortic smooth muscle cells [see Ref. 2]. Consequently, restoration of the response to norepinephrine is a measure of effectiveness of the agent against iNOS. NMA and PCA caused dose-dependent increases in mean arterial pressure when given as a bolus to anesthetized rats. 4-Methyl-PCA, however, did not raise blood pressure even when given at doses up to 10 mg/kg, i.v., but caused a decrease in MAP (Fig. 8). This hypotensive effect is unlikely to be related to inhibition of NOS.

DISCUSSION

The pyrazole-*N*-carboxamidines tested inhibited isolated iNOS with potencies equivalent to or greater than that of NMA. Isoform selectivity was variable, with the simplest *N*-carboxamide, PCA, showing virtually no preference and others showing 10- to 20-fold differences in potency between isoforms. The substitution of the pyrazole ring of PCA altered the relative affinities of the derivative for iNOS and the constitutive isoforms, increasing selectivity but decreasing potency.

Monitoring of NO production by purified enzyme, using the hemoglobin assay, clearly showed that the effects of PCA, 3-methyl-PCA, and 4-methyl-PCA could be reversed completely by adjustment to a 33-fold excess of arginine substrate (Fig. 3). In addition, perfectly linear rates of formation of NO in the presence of PCA (Figs. 3–5) or analogues (3-methyl-PCA, 4-methyl-PCA, and 1,2,4-triazole-CA, data not shown) were seen for both purified enzyme and stimulated cells over periods of up to 30 min. These observations imply that the binding of PCA or its analogues to the arginine binding site of NOS is reversible. If irreversible inhibition occurs at all, its rate of onset is very slow by comparison with other irreversible inhibitors or mechanism-based inactivators.

The effects of PCA within intact cells could be reversed quickly and substantially simply by transferring to a PCA-free medium. However, in this instance, reversal was only partial. This may be due to as yet undefined effects of PCA on cells, but no toxicity was evident from measurement of either cell respiration (measured by MTT assay after 18 hr, Table 2) or from trypan blue exclusion (data not shown).

Differences in potency observed with purified iNOS were reflected in intact cells: 3-methyl- and 4-methyl-PCAs were about 10–20 times less potent than PCA itself. However, 1,2,4-triazole-CA was weaker against nitrite production by macrophages than would be expected from the *in vitro* results. The reason for this is as yet unclear. Although the effects of PCA on NO formation by whole cells were potent and rapid in those experiments where NO was measured using the hemoglobin assay (Fig. 6), there appears to be a discrepancy between the relative potencies of PCAs and NMA against purified iNOS and nitrite production by stimulated cells. This may be a consequence of the time-dependent inactivation of iNOS [24] by NMA within the cells, which, based on its inactivation rate constant, occurs largely within the first hour. In the presence of PCA, however, NO formation continues throughout the prolonged incubation period used to measure total NO accumulation. Such a discrepancy may also be a reflection of different mechanisms of uptake of the drugs by the cells, although uptake of both NMA and PCA is rapid. This underscores the difficulty in comparing reversible inhibitors with irreversible inactivators when different times of incubation are employed.

The bicyclic molecules were found to be relatively ineffective inhibitors of the purified enzymes as may have been expected from hydrophobic and steric considerations, in particular the substitution of the carbon at C5. In addition to the weaker effects against NOS, these compounds are toxic to cells and are not as promising as the monocyclic compounds. The exception is 7-nitroindazole-CA, which showed inhibitory activity comparable to that of the monocyclic compounds. This may be attributable to the potential of the 7-nitroindazole portion of the structure to interact with the heme iron as has been identified previously for 7-nitroindazole itself [15, 25].

The potent inhibition of isolated eNOS by PCA and NMA is reflected in their effects on isolated tissue and on MAP *in vivo*, since both the increased MAP and impaired relaxation to acetylcholine in the presence of NOS inhibitors are attributable to inhibition of eNOS in endothelial cells. Similarly, the weak effect of 4-methyl-PCA on the endothelium-dependent relaxations of rings suggests that it has only a modest effect on eNOS activity in endothelial cells. However, the demonstration of a hypotensive effect of 4-methyl-PCA when given *in vivo* raises the possibility that 4-methyl-PCA may relax smooth muscle and mask its effects on acetylcholine-induced relaxations. However, when tested on aortic rings taken from LPS-treated ani-

mals, 4-methyl-PCA restored the contractile response to norepinephrine to a greater extent than did either NMA or PCA. Any relaxant action of 4-methyl-PCA on smooth muscle would be expected to antagonize this effect. Since the reduced contractile responsiveness to norepinephrine is attributable to iNOS in the aortic smooth muscle cells, it appears that 4-methyl-PCA displays considerable efficacy against iNOS and is readily taken up by these cells. It is possible that differential cell uptake and/or recognition by cytokine-induced transporters, in addition to its intrinsically weak inhibition of eNOS, may contribute to the apparent *in vivo* selectivity of 4-methyl-PCA. The reason for the hypotensive effect of 4-methyl-PCA *in vivo* is unclear, but, as discussed above, there was no obvious evidence for such an effect on isolated tissues. Similar effects have been observed *in vivo* for amidines, guanidines, and other related compounds, regardless of their ability to inhibit NOS (see Ref. 14 and references therein). Consequently, this depressor effect of 4-methyl-PCA is probably unrelated to NOS.

PCA contains a carboxamidine function attached to a ring nitrogen, which effectively results in a 1,1-disubstituted guanidine. While such guanidines *per se* are not necessarily expected to be inhibitors of NOS (although some are), the two adjacent ring nitrogens in PCA may allow for additional interactions with portions of the arginine binding site. Conversely, the fact that pyrazole itself binds only weakly to NOS, as based on its inhibitory potency, suggests that the amidine of the PCAs plays the predominant role in binding.

Analogy of PCA to a substituted aminoguanidine (containing —N—N—C(=N—)NH_2), although superficially attractive, is not reflected in the mechanism of inhibition: aminoguanidine is an iNOS selective, mechanism-based, irreversible inactivator of NOS, which produces a time-dependent loss of enzyme activity [16], whereas PCA is a reversible, competitive antagonist of L-arginine binding to all three isoforms. In this respect, PCA is similar to cyclic amidines and isothioureas such as aminothiazolines [7, 8], aminopyridines [10], and iminopiperidine [9].

It is likely that other heterocyclic carboxamidines will show effects against NOS activity. 1,2,4-Triazole-CA, for example, was only slightly weaker than PCA in its effects on NOS, but thiomorpholine-4-carboxamidine was very weak (unpublished data). Another cyclic aminoguanidine analogue, 3-amino-1,2,4-triazole, is a very weak, but apparently irreversible inhibitor of iNOS [26]. The lack of inhibitory activity of *N*-benzyl PCA against iNOS (purified or in cells), but modest activity against nNOS, suggests that the binding site on nNOS (but not on iNOS), can accommodate large substituents on one of the amidine nitrogens. This would be in line with other models of nNOS inhibition.

The few examples of this class of compounds tested contain potent inhibitors of NOS as well as compounds that show selectivity both *in vitro* and *in vivo*. This suggests that further structural modifications and examination of the binding of these compounds to NOS may provide useful tools for the investigation of the mechanism of NOS in disease.

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