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Isolation of nitric oxide synthase from human platelets

Arumugam Muruganandam, Bulent Mutus *

Department of Chemistry and Biochemistry, University of Windsor, Windsor, Ontario, Canada

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Abstract

We are reporting a distinct constitutive isoform of nitric oxide synthase that has been purified to homogeneity from human platelet cytosolic fractions. Purification involved ultra centrifugation at $100000 \times g$ followed by two sequential affinity chromatography procedures: adenosine 2′,5′-bisphosphate (2′,5′-ADP)-Sepharose and calmodulin Sepharose 4B. Purified enzyme appeared as a single band (~ 80 kDa) under denaturing condition (SDS-PAGE). The native enzyme appears to be dimeric, since its molecular weight estimated by gel filtration was ~ 150 kDa. Enzyme activity was dependent on L-arginine, NADPH and (6R)-5,6,7,8-tetrahydro-L-biopterine. Partially purified platelet NOS ($100000 \times g$ supernatant) activity was sensitive to calmodulin antagonists and to the N^{ω}-Monomethyl-L-arginine, a substrate analog of L-arginine.

Key words: Nitric oxide; Nitric oxide synthase; Purification; Platelets; Calmodulin

1. Introduction

Nitric oxide, an extremely labile, short-lived endogenous paracrine substance, has been described as (i) a servoregulator due to its involvement in the regulation of enzymes, (ii) as a retrograde messenger for acting as an inter and intracellular signaling agent, and (iii) as a host protector for mediating cytotactic-cytotoxic effects on host cell invaders [1,2]. The endothelium derived NO had been shown earlier to regulate hemostatis, the dysaggregation of platelets and prevention of platelet adhesion [3,4]. Further, the neutrophil derived NO had been shown to antagonize platelet ATP secretion by activating the guanylate cyclase, thereby increasing the cyclic GMP levels [5]. Although in vitro studies have shown NO to be an inhibitor of platelet aggregation and platelet adhesion under static conditions, the ex vivo studies in humans have shown that endothelium derived NO is not sufficient to effect the platelet aggregation under flow conditions [6,7].

Endothelium derived NO in the blood vessel lumen is susceptible to hemoglobin binding which would reduce free [NO]. Hence, it is conceivable that it would be advantageous for platelets, which are free of hemoglobin, to synthesize its own NO for a more localized response. Consequently, platelet NO synthesis could be a determinant factor in certain platelet dysfunctions such as in the hyperaggregation of platelets. The existence of the Arg:Nitric oxide pathway and the involvement of a Ca^{2+}, NADPH dependent enzyme in the generation of NO in platelets has been reported [8,9]. The exact physiological role of the NO generated by the endogenous platelet NOS is not known, and the platelet nitric oxide synthase (NOS) has not been studied in detail. Megakaryocytes, parent cells to platelets, have been reported to posses both the constitutive and inducible forms of nitric oxide synthase [10]. We purified the NO producing constitutive enzyme from human platelets and consequently we report a Ca^{2+} CaM dependent isoform of platelet nitric oxide synthase which exhibits cofactor and kinetic characteristics similar to those from other cells and tissues but most interestingly a distinct molecular weight.

* Corresponding author. Fax: +1 5199737098. E-mail: MUTUSB@UCC.UWINDSOR.CA.

Nonstandard abbreviations: NOS, nitric oxide synthase; CaM, calmodulin; PRP, platelet rich plasma; L-MeArg, N^{ω}monomethyl-L-arginine; BH_4, (6R) -5,6,7,8-tetrahydro-L-biopterine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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2. Materials and methods

2.1. Materials

Antipain, leupeptin, pepstatin, phenylmethylsulfonyl fluoride, 2′,5′-ADP agarose, calmodulin agarose, Sepharose 4B, L-Arginine, NADPH, valine, hemoglobin; drugs: W5, (*N*-(6-aminohexyl) naphthalenesulfonamide hydrochloride), W7 (*N*-(6-aminohexyl)-5-chloro-2-naphthalene sulfonamide hydrochloride), W13 (*N*-4 aminobutyl)-5-chloro-naphthalene sulfonamide hydrochloride) and trifluoperazine dihydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO). (6R)-5,6,7,8-tetrahydro-L-biopterin dihydrochloride (BH_4) was purchased from Research Biochemicals, Natick, MA. All other chemicals used were reagent grade from BDH (Toronto, Ontario).

2.2. Human platelet preparation

Platelets were obtained from the Metropolitan hospital of Windsor. Then they were spun at $200 \times g$ for 20 min to prevent erythrocyte contamination. The platelet rich plasma (PRP), free from erythrocyte, was again centrifuged at $2000 \times g$ for 20 min. The platelet pellet obtained was washed free of plasma contamination by their repeated ($2\times$) suspension in phosphate buffered saline, pH 7.4. The harvested platelets were then stored at -80°C [11].

2.3. Isolation of platelet NOS

Purification of NOS from platelets was carried out as previously described [12]. Briefly, platelet was homogenized in extraction buffer [10 mM Hepes, pH 7.4, 1 mM DTT, 0.32 M sucrose, antipain (1 mg/l), leupeptin (1 mg/l), pepstatin (1 mg/l), phenylmethylsulfonyl fluoride (100 mg/l)] using a polytron homogenizer at 4°C. The homogenate was clarified by centrifugation (Sorvall RC5C) at $20.000 \times g$ for 30 min, and the supernatant was again centrifuged (Beckman L5–65) at $100000 \times g$ for 1 hr. The supernatant was decanted and mixed with the 2′,5′-ADP agarose that had been equilibrated with buffer A (50 mM Tris, pH 7.4, 1 mM DTT). The agarose was then transferred to a column (25×7 mm, about 2.5 ml) and the unbound protein was washed with buffer A containing 0.5 M NaCl and then with a salt-free buffer. The active fraction containing NOS was eluted using 2 mM NADPH. The ADP eluate was subsequently applied to a calmodulin agarose high affinity column (20×7 mm about 1.8 ml) made up of calmodulin agarose and non-labelled Sepharose 4B (1:8) in buffer A, supplemented with 1 mM $CaCl_2$. After washing with buffer A containing 0.3 M NaCl, the pure NOS was eluted with 5 mM EGTA.

2.4. NOS assay

NOS activity was measured spectrophotometrically by following the oxidation of oxyhemoglobin to methemoglobin by NO [13]. The assay was carried out at 37°. The NO production was monitored by observing the time-dependent increase in absorbance at 405 nm. A typical assay mixture consisted of 15 mM Hepes pH 7.2, 200 μM. $CaCl_2$, 1 mM $MgCl_2$, 100 μM L-Arg, 100 μM NADPH, 5 mM Valine, 1.6 μM oxyhemoglobin, 12 μM (6R)-5,6,7,8-tetrahydro-L-biopterin dihydrochloride (BH_4). The reaction was initiated by adding the platelet protein.

2.5. Electrophoresis and molecular mass determination

The homogeneity of the purified enzyme and the molecular mass of the enzyme subunits were determined by SDS-PAGE, as described by Lammeli [14] using a 7.5% polyacrylamide gel and followed by silver stain. The gels were stained with coomassie brilliant blue R-250. The native molecular mass of the platelet NOS was determined by gel filtration, using Sephadex G-200 column (1.5×80 cm) equilibriated with buffer A at a flow rate of 0.3 ml/min.

2.6. Protein estimation

Protein concentrations were determined according to the method of Bradford with bovine serum albumin as the standard [15].

2.7. Data analysis

IC_{50}S was estimated from the fit to a simple saturation function where percentage activity = 100–100 [antagonist]/(IC_{50+}[antagonist]). The steady-state kinetic parameters K_M and V_{max} were estimated from a non-linear regression (Marquardt-Levenberg algorithm) fit of the initial rate data to the Michealis-Menten-Henri equation. The rate of NO formation was calculated using the absorption coefficient of hemoglobin for the wavelength pair 405 nm and 413 nm, which was found to be 83441 $M^{-1} \cdot cm^{-1}$ in the Shimadzu UV-160 spectrophotometer.

3. Results and discussion

This is the first report of the purification of nitric oxide synthase to homogeneity from platelet cytosolic fractions. Over the course of the purification several interesting observations were made. The crude platelet preparation $20000 \times g$ supernatant demonstrated enzyme activity in the absence of cofactors. The addition of Ca^{2+}, NADPH and (6R)-BH_4 only enhanced the enzyme activity in the crude supernatant, but it was

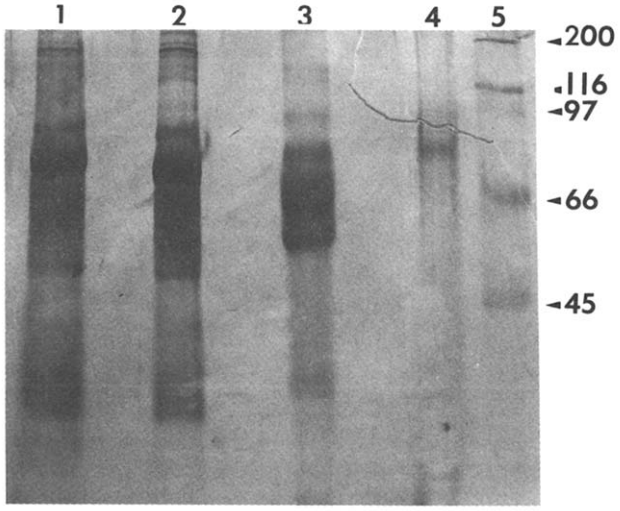

Fig. 1. SDS-PAGE of platelet Nitric oxide synthase: Silver staining of the 7.5% polyacrylamide gel. Lane 1: 5 μg of the 20000 $\times$ g crude supernatant. Lane 2: 5 μg of 100000 $\times$ g supernatant, Lane 3: 3 μg of ADP agarose eluate. Lane 4: 0.8 μg of the CaM agarose eluate. Lane 5: high molecular weight reduced protein standards (Bio Rad) myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa).

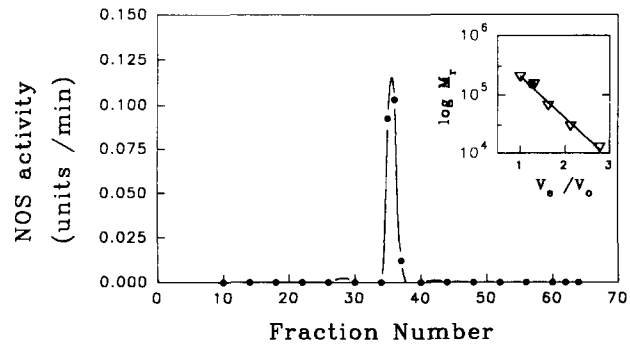

Fig. 2. Elution pattern of platelet NOS from an Sephadex G-200 gel filtration chromatography. The platelet pure NOS (4.5 μg) was loaded on to a G-200 column (1.5 $\times$ 80 cm) previously equilibriated with buffer A, and 1.4 ml fractions were collected at a flow rate of 0.3 ml/min. The elution profile of the NOS activity is shown. The molecular standard marker proteins used for calibration were β amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome C (12.4 kDa) (inset).

essential in the further purification steps. Besides cofactors, addition of 2 μM of calmodulin (CaM) was required for demonstrating pure nitric oxide synthase enzyme activity. The crude platelet preparation 20000 $\times$ g supernatant was subjected to ultra centrifugation at 100000 $\times$ g, followed by two sequential affinity chromatography procedures: adenosine 2′5′-bisphosphate (2′,5′-ADP) agarose and calmodulin Sepharose 4B.

The constitutive platelet NOS enzyme which bound to CaM agarose was eluted as a single peak exhibiting a specific activity of 920.1 U/mg protein. This CaM-dependent enzyme was purified 582-fold with a final yield of 2.4% from the total platelet crude NOS which may be comprised of both the constitutive and inducible forms of the enzyme activity (Table 1). However, there is no report to date indicating the presence of the inducible form of NOS in platelets. The homogeneity of the enzyme was confirmed with a 7.5% polyacrylamide slab gel run under reduced conditions in the presence of SDS. (Fig. 1). Silver enhancement of the gel showed one stained band and extrapolation of the mobilities of the reduced high molecular weight standards yielded a molecular mass of about 80 kDa. The native molecular mass of the platelet NOS was determined using an sizing column Sephadex G-200. NOS activity was eluted from the column with a V_e/V_o corresponding to a molecular mass of ~ 150 kDa indicating that platelet NOS is dimeric in its native state (Fig. 2).

We find platelet NOS differs in subunit molecular weight from the CaM dependent NOS reported from various other sources. NOS molecular weight heterogeneity has been previously reported. For instance, the NOS M_r from bovine brain [16] and human cerebellum [17] has been reported to be 150 kDa and 160 kDa, respectively. In addition, polyclonal antibodies raised against the rat constitutive NOS detected isoforms of NOS in different human tissues with molecular weights of ~ 160 kDa in brain, cerebellum, and kidney medulla, ~ 140 kDa in placenta, ~ 125 kDa in lung and ~ 100 kDa in liver [18]. Controversy also exists as to the quaternary structure of NOS from rat cerebellum between two groups as either a dimer with a molecular weight of 155 kDa/subunit [12] or a monomer [19].

Table 1
Purification of platelet nitric oxide synthase
Purification of the NOS from the crude cytosolic fraction (20000 $\times$ g) involved ultra centrifugation at 100000 $\times$ g followed by two sequential affinity chromatography procedures: adenosine 2′,5′-bisphosphate (2′,5′-ADP)-Sepharose and calmodulin Sepharose 4B

Steps	Volume (ml)	Total mg protein	Total act (units)	Spec. act. U/mg	Fold	Yield %
Crude	27	169	267.84	1.58	–	100
100000 $\times$ g	23.5	34.54	64.77	1.87	1.18	24
ADP-Seph	22.5	0.048	19.35	403.1	255	7.2
CaM-Seph	0.607	0.007	6.44	920.1	582	2.4

The data represents a single typical purification procedure. The 2′5′-ADP agarose and CaM agarose eluate were concentrated and washed with centricon-30 microconcentrators and assayed for enzyme activity as mentioned in the text.

That the purified NOS from platelets had high enzyme activity and was not a proteolytic degradation product as the homogenization buffers contained protease inhibitors is evidence for a NOS with a novel molecular weight.

To further confirm the CaM dependence of the purified NOS, we modified the method of Billingsley et al. [20] for detecting CaM binding proteins. Protein from the SDS-polyacrylamide gel were transferred to nitrocellulose membrane using Bio-Rad mini trans blot cell and after blocking for 2 hours with polyvinylpyrrolidone (2% PVP, 50 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 1 mM $CaCl_2$) the membrane was incubated for 1 hr with the pure bovine brain calmodulin (Sigma) 60 μg dissolved in buffer B (50 mM Tris · HCl, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$). After washing extensively, the membrane was probed for the presence of CaM with the anti-bovine brain CaM polyclonal antibody (1 : 500) with an alkaline phosphatase conjugated rabbit anti-goat IgG (1 : 1000) detection system. Fig. 3 illustrates the CaM binding properties of the platelet NOS where the protein band (lane 1–4) corresponding to a molecular weight of 80 kDa was detected by anti CaM polyclonal antibody. Lane 1 and 2 contains other platelet CaM binding proteins besides NOS, which are also detected by this technique.

To further characterize the inhibitory and kinetic properties of platelet NOS the 100 000 × g supernatant was used. The inactivation of partially purified platelet NOS by the potent competitive inhibitor N^{ω}-Monomethyl-L-Arginine (L-MeArg) confirmed the presence of platelet NOS (IC_{50} 5.4 ± 0.5 μM). NOS Ca^{2+} dependence was assessed in the presence of 200 μM $CaCl_2$ and increasing increments of EGTA. The chelation of $CaCl_2$ by the EGTA inactivated the enzyme in a concentration dependent manner. It was found that 0.5 ± 0.07 μM of EGTA was required to reduce the NOS activity to 50%. (Fig. 4).

Kinetic characterization of the partially purified enzyme was performed after passing the 100 000 × g supernatant over a Dowex 50 W (Na^+ form) to remove endogenous L-arginine. The reaction was initiated by adding 0.085 mg of protein with the L-arginine concentration varied between 0.1 μM and 20 μM. The apparent K_M and V_{max} values for arginine at 37°C and pH 7.4 are 0.18 ± 0.011 μM and 3.25 ± 0.052 μmol/min/mg protein, respectively. The data are based on three experiments, each in triplicate, in mean ± SD.

As our purified enzyme was CaM dependent, we studied the effect of selective calmodulin antagonists on the platelet NOS. The drugs W5, (*N*-(6-aminohexyl) naphthalenesulfonamide hydrochloride), W7 (*N*-(6-aminohexyl)-5-chloro-2-naphthalene sulfonamide hydrochloride), W13 (*N*-4 aminobutyl)-5-chloro-naphthalene sulfonamide hydrochloride) and trifluoperazine dihydrochloride inhibited NOS activity with respective IC_{50} values of 28.8 ± 3.6 μM, 45.6 ± 3.1 μM, 24 ± 0.76 μM and 18.3 ± 1.7 μM (Fig. 5). The sensitiv-

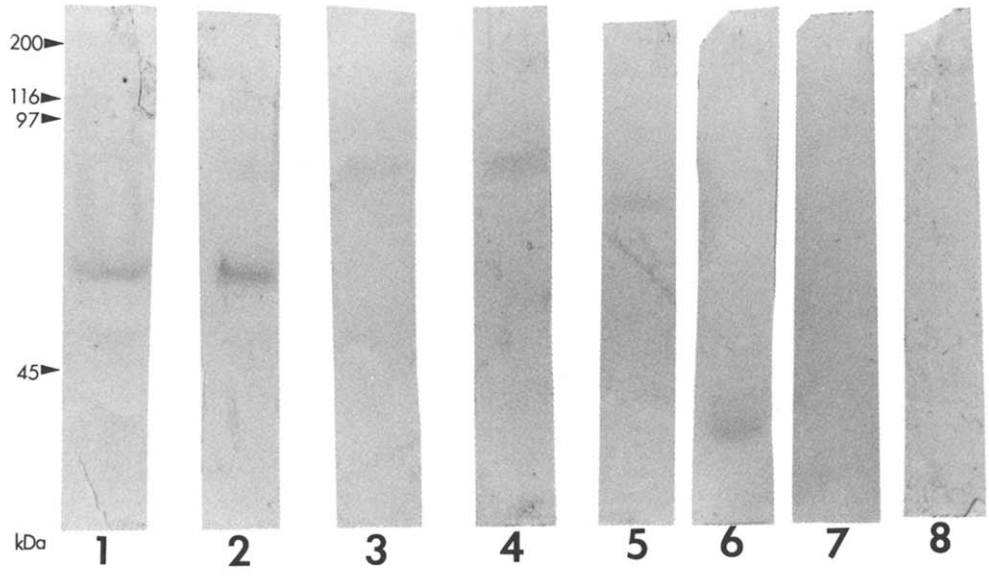

Fig. 3. SDS-PAGE and Calmodulin binding properties of NOS: Platelet proteins from the SDS gel was transferred to the nitrocellulose membrane and after incubation with Calmodulin (60 μg), the membranes were probed with polyclonal anti-CaM antibody (1, 500) and anti goat IgG alkaline phosphatase (1, 1000). Arrow indicates the high molecular weight reduced protein standards (Bio Rad) myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), ovalbumin (45 kDa). Lane 1, 15 μg of the 20 000 × g crude supernatant. Lane 2: 15 μg of 100 000 × g supernatant, Lane 3: 6 μg of ADP agarose eluate. Lane 4: 1 μg of the CaM agarose eluate. Lane 5: positive control 2 μg of Cyclic AMP phosphodiesterase. Lane 6: reagent control, 2 μg of bovine brain CaM. Lane 7: 15 μg of the 20 000 × g crude supernatant (minus CaM). Lane 8: 15 μg of the 20 000 × g crude supernatant (minus anti-CaM antibody).

ity to the CaM antagonists and the affinity to CaM revealed by the immunoblotting technique positively identifies, that the platelet NOS is only a constitutive form and not an inducible form, although both have been reported in the parent megakaryocytes.

To summarize, this study establishes that the isolated NOS from human platelets is a constitutive isoform with a distinct molecular weight, but has similar cofactor and kinetic characteristics to the other constitutive isoforms reported earlier. As platelets have only limited capacity to synthesize proteins, this distinct isoform of the enzyme could have been the product of the same gene but post transcriptionally modified in the parent megakaryocyte.

Earlier studies have indicated that platelet CaM plays a dual regulatory role in the platelet release reaction, and it is thought to be analogous to a threshold sensitive gate. Recently we reported that CaM from diabetic platelets is nonenzymatically glycated [21]. The glycation of CaM may perturb the normal functioning of the CaM dependent enzymes such as

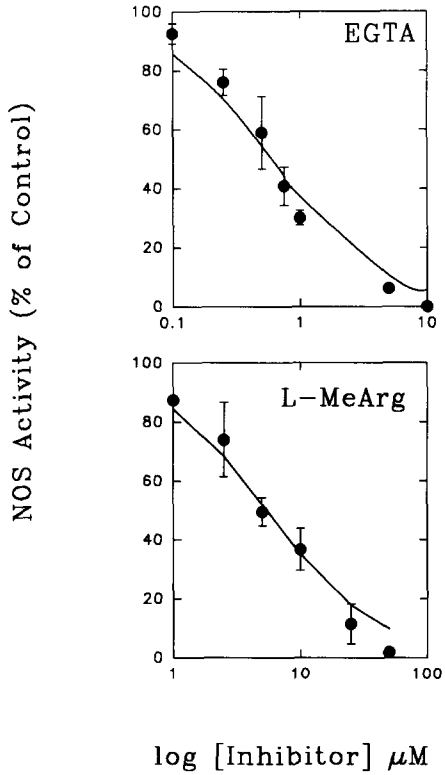

Fig. 4. Calcium dependence and Inactivation of platelet NOS by L-MeArg. The vertical axis is the percentage of residual activity of NOS to that of control. The partially purified platelet NOS (100000 $\times g$ supernatant) activity was assayed in the presence of 100 μM L-Arg with increasing concentrations of L-MeArg (IC_{50} 5.4±0.5 μM). NOS Ca^{2+} dependence was assessed in the presence of 200 μM $CaCl_2$ and increasing increments of EGTA in the presence of L-arginine, NADPH and BH_4 as described in the text. 0.5±0.07 μM of EGTA was required to reduce the NOS activity to 50%. The data are based on two experiments, each in triplicate, in mean±SD.

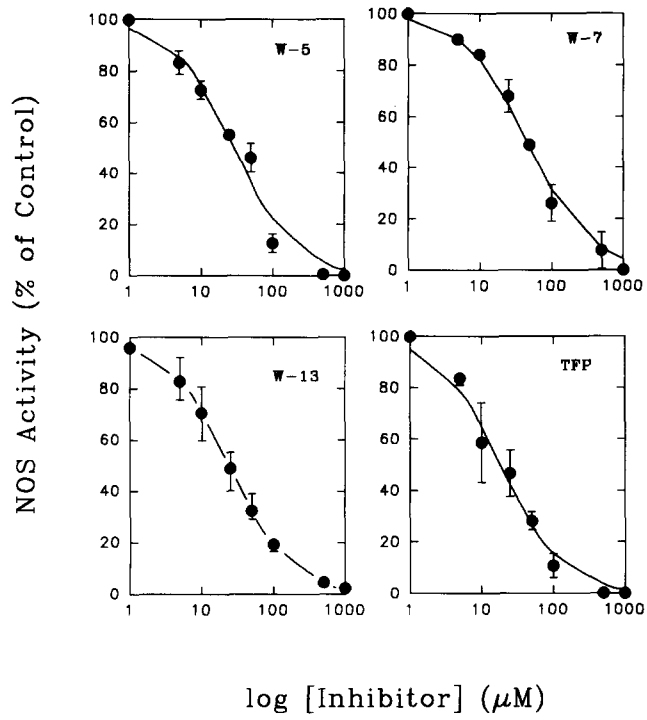

Fig. 5. Effect of Calmodulin antagonists on platelet NOS: The IC_{50} values for W5, W7, W13, Trifluoperazine were determined using the partially purified platelet NOS (100000× g supernatant) in the presence of 0.2 mM $CaCl_2$ and other other agents added as indicated in the text. The different panels shows the inhibition of platelet NOS with respective IC_{50} values of 28.8±3.6 μM, 45.6±3.1 μM, 24±0.76 μM and 18.3±1.7 μM. The data are based on two experiments, each in triplicate, in mean±SD.

NOS in platelets, and leading to platelet dysfunction. Attempts are underway to test the regulatory effect of normal versus glycated CaM on platelet nitric oxide synthase.

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