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# MAXADILAN BINDS TO MEMBRANE FRACTIONS OF BRAIN TISSUE

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Summary: Maxadilan is a potent vasodilator peptide isolated from salivary glands extracts of the hematophagous sand fly. Besides effects on the cutaneous vasculature, it has also been shown to relax rabbit aortic rings while elevating levels of cAMP. As a result of the effects on the skin and aorta, it was elected to undertake an examination of the tissue distribution of binding sites for maxadilan. In addition to specific binding in rabbit aorta and spleen, binding was detected in brain from various species including human, bovine, rabbit, rat and mouse with a kD of between 85 and 201 pM. Competitive displacement of [$^{125}$I] maxadilan by a number of known vasoconstrictor peptides, vasodilator peptides and small molecule receptor ligands did not occur in the rabbit brain preparation. These results suggest the presence of specific binding sites in mammalian tissue for maxadilan whose endogenous ligand remains unknown. 

When an insect probes for a blood meal in skin, blood vessels are injured and the basic hemostatic processes of vasoconstriction, coagulation, and platelet aggregation are initiated (1). To overcome these processes and ensure success at obtaining blood, insects have developed a number of potent anti-hemostatic compounds in their saliva. The ultimate goal of blood-feeding by arthropods is to allow completion of the life cycle in which the female needs the stimulus of a blood meal in order to lay eggs. Males typically do not blood feed. The small size of many arthropods and rapidity with which they can feed suggests that these compounds are potent substances that can be exploited in the development of pharmacological agents (1).

During the last several years a number of antihemostatic compounds have been found in salivary glands of arthropods and it would appear that these substances aid in bloodfeeding. Some of these substances inhibit platelet aggregation and others effect the coagulation pathway (2). Two vasodilators have been described - a nitrosovasodilator from *Rhodnius prolixus* (3) and maxadilan, a peptide from the sand fly *Lutzomyia longipalpis.* which acts directly on smooth muscle cells (4, 5).

Maxadilan is a vasodilator peptide isolated from salivary gland extracts of the sand fly, the vector of the protozoan disease leishmaniasis. Leishmaniasis occurs in many parts of the

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world including Central and South America, the Middle East and Indian subcontinent. The disease is typically manifest as ulcers on the skin or infection of the liver and spleen. It has been shown that sand fly salivary gland extracts exacerbate the infectious process by decreasing or turning off the local immune response of the host. Besides its vascular effects, maxadilan has immunomodulatory properties and appears to be the substance responsible for the exacerbative effect (submitted). The ability of maxadilan to dilate blood vessels and modulate immune responses is similar to the effects of CGRP, which has the additional property of being a neuropeptide (6,7).

Maxadilan is produced as a 63 amino acid peptide which undergoes C-terminal cleavage and amidation to a 61 amino acid peptide. It contains four cysteine residues which participate in the formation of disulfide bonds between positions 1-5 and 14-51. The peptide acts to raise level of cAMP in rabbit aorta suggesting that it interacts with a cell-surface receptor to mediate its effects (5).

In previous studies, it was demonstrated that maxadilan was functionally active on rabbit vessels and human skin but not on rat aorta, dog mesentery or pig or cow coronary vessels (5). To obtain a picture of the tissue distribution of binding sites for this peptide, maxdilan was stoichiometrically labeled with $^{125}I$ to high specific radioactivities (2000-4000 Ci/mmol). The iodinated peptide was incubated with membrane fractions prepared from a variety of rabbit tissues. These results, which revealed binding to membrane fractions obtained from rabbit brain, prompted an investigation of binding of maxadilan in the brain of various species.

## Materials and Methods

*Preparation of peptide* - Recombinant maxadilan produced in *E. coli* contained the four additional amino acid residues glycine, serine, isoleucine and leucine at the N-terminus as a result of construction in the pGEX vector designed for cleavage with thrombin (8). Termed "GSIL-maxadilan", it was purified to homogeneity using reverse phase HPLC.

Maxadilan was labeled with $^{125}I$ using Iodo-Beads (Pierce, Rockford, IL). Free iodine was removed by passing the peptide over a Sep-Pak C18 cartridge (Waters, Marlborough, MA) and unlabeled peptide was separated from radiolabeled products by reverse phase HPLC (Figure 1) (9). Specific activity was 2000-4000 Ci/mmole in different preparations. As maxadilan does not contain tyrosine residues, the reaction was carried out at pH 8.2 to promote iodination on either or both of the histidine residues in the peptide (10). Biological activity was assessed by stoichiometrically labeling maxadilan with cold $^{127}I$ followed by injection into rabbit skin. This material maintained complete functional activity relative to the unlabeled peptide. It was inferred from this result that the [$^{125}I$] labeled material was also biologically active.

*Preparation of membranes* - Rat, rabbit, mouse and bovine tissue was obtained from Pel-Freez (Rogers, AR). Rat brain material was also obtained from animals purchased from Charles River Laboratories. Human brain material was kindly provided by Tessa Hedley-Whyte of the neuropathology service at MGH. Membrane fractions from these tissues were prepared as described previously (11). Briefly, tissue was placed in 10 vol of ice-cold 50 mM Tris-HCl buffer (pH 7.6) containing 0.32M sucrose, 5 mM EDTA, 1 mg/ml leupeptin, 1 mg/ml pepstatin A, 2 mg/ml bacitracin and 10 mg/ml phenylmethylsulfonyl fluoride. Tissue was homogenized with a polytron PT 3000 (Brinkmann Instruments, Westbury, NY) for 30 s at power level 8 at 4°C. The homogenate was centrifuged for 10 min at 1000 x g at 4°C. The supernatant was removed, and the pellet resuspended in 15 ml of homogenizing buffer, homogenized again using the Polytron at the same setting as the first homogenization, and the homogenate was centrifuged at 1000 x g for 10 min at 4°C. The combined supernatant was centrifuged at 30,000 x g for 20 min at 4°C. The pellet was washed two times by successive suspension in 50 mM Tris-HCl buffer containing 1 mM $MgCl_2$, 0.3 % BSA, 1 mg/ml leupeptin, 1 mg/ml pepstatin A, 2 mg/ml bacitracin and 10 mg/ml phenylmethylsulfonyl floride.

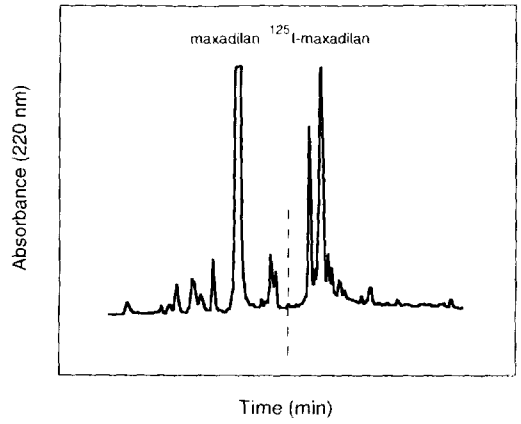


Figure 1. Maxadilan (100µg) was iodinated with 20mCi carrier-free Na$^{125}$I and two Iodo-Beads in 0.1M sodium phosphate, pH 8.2. After removal of unreacted iodide, the peptide was loaded on a reverse phase C4 column to separate labeled and unlabeled maxadilan. Since unlabeled peptide is essentially removed by this method, specific radioactivity of the labeled peptide may be calculated for that of carrier-free $^{125}$I.

*Binding of [$^{125}$I] maxadilan* - Crude membranes (250-400 mg) were incubated for 2 hr at 4°C in a final volume of 0.5 ml consisting of 50 mM Tris-HCl buffer (pH 7.6) containing 0.3 % BSA, 1mM $MgCl_2$, 1 mg/ml leupeptin, 1 mg/ml pepstatin A, 2 mg/ml bacitracin, 10 mg/ml phenylmethylsulfonyl fluoride and 70 pM maxadilan in the absence or presence of 1 µM maxadilan. At the end of incubation, samples were assayed for protein-bound radioactivity by vacuum filtration through GF/C Whatman glass microfiber filters pretreated with 0.5% polyethylenimine. Filters were then washed with 3 x 3 ml of incubation buffer at 4°C. The radioactivity trapped on the filters was measured using a gamma counter. Non-specific [$^{125}$I] maxadilan binding was determined by the addition of 1µM unlabeled maxadilan and represented between 10-20% of total binding depending upon the tissue preparation. In the Results, specific binding (total cpm minus non-specific cpm) is shown. Proteins were estimated by the method of Bradford using bovine serum albumin as standard.

*Chemicals* - All peptides were obtained from Peninsula Laboratories (Belmont, CA). All other reagents were of analytical grade from Sigma (St. Louis, MO).

## Results

*Binding of maxadilan to rabbit tissues*

Crude membrane homogenates were prepared from various rabbit tissues and assayed for specific maxadilan binding (Figure 2). Specific binding was noted in aorta, spleen and brain. Although binding to brain and spleen were seen consistently, binding to aorta was reproducible for some but not all batches of iodinated maxadilan.

*Binding of maxadilan to brain and spleen tissues*

Because of the specific binding of maxadilan to rabbit brain and spleen, such tissues from mouse, rat, bovine and human sources were examined. In all cases, specific binding was noted in the brain (Figure 3). In the case of spleen, specific binding was only seen in rabbit. The equilibrium binding and Scatchard analysis of rabbit brain membrane revealed a Kd of 136 pM (Figure 4). The distribution of [$^{125}$I] maxadilan binding sites was examined in rat brain, where binding appeared to be essentially equal across the areas examined including cortex, hippocampus,

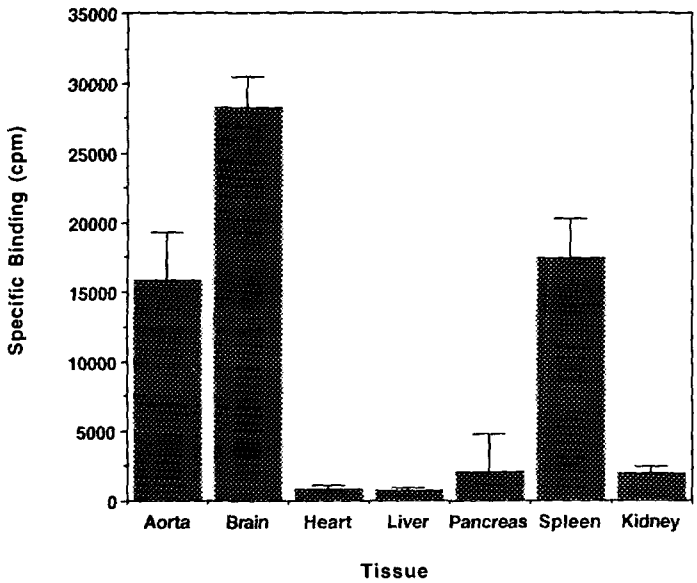


Figure 2. Specific binding of maxadilan to membrane fractions of rabbit tissues. Crude membrane fractions were prepared from the indicated tissues and incubated with [$^{125}$I] maxadilan and excess unlabeled peptide in a competition assay. Specific binding was noted in brain and spleen.

brain stem, striatum and cerebellum (Figure 5). Specific binding was noted to all areas of human brain examined including substantia nigra, pons, vermis (anterior portion), medulla/olive, thalamus, parietal, hippocampus and cerebellar gray and white matters (data not shown). The least binding

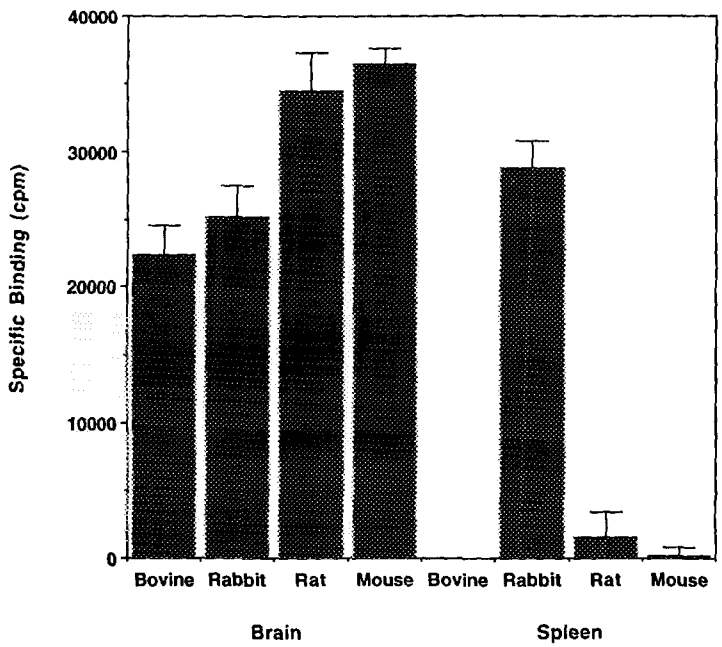


Figure 3. Specific binding of maxadilan to membrane fractions of brain and spleen of various species. Maxadilan binds to all brains examined, including mouse, rat, rabbit and bovine. Binding to human brain occurred (not shown). Binding to spleen was only noted in rabbit tissue.

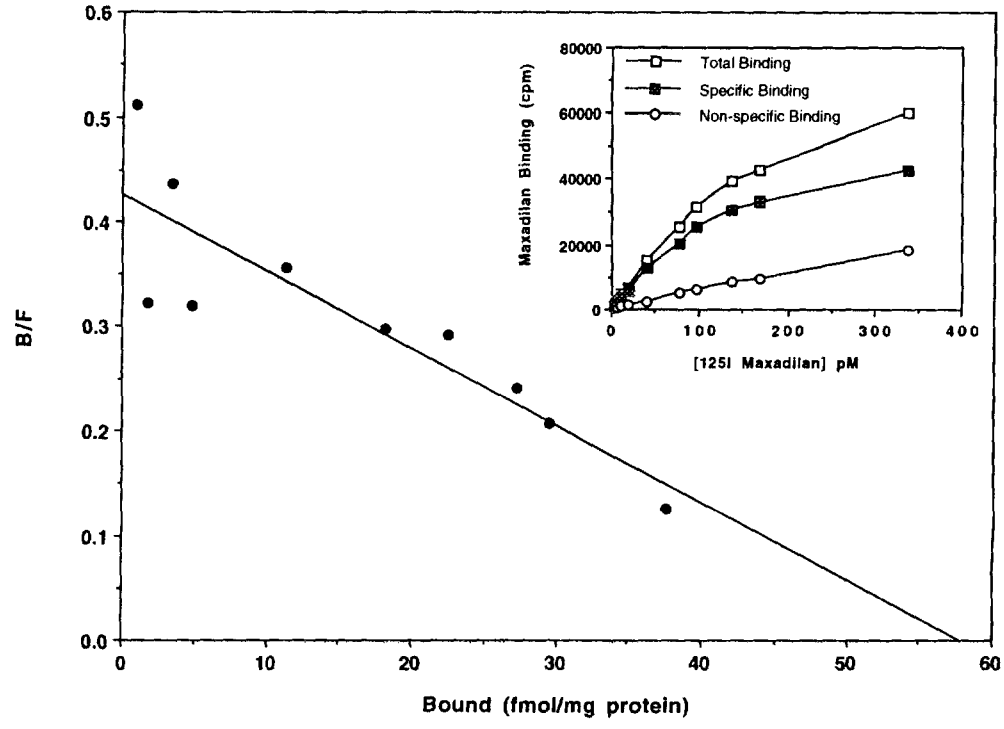


Figure 4. Equilibrium binding and Scatchard analysis of binding of maxadilan to rabbit brain. Saturable binding occurs and it appears that a single class of high affinity receptors is present.

was noted in cerebellar white matter. This finding correlates well with preliminary autoradiographic data in which preferential binding occurs in gray matter of rat brain sections (not shown). The dissociation constants and Bmax values for binding to brain membranes from the

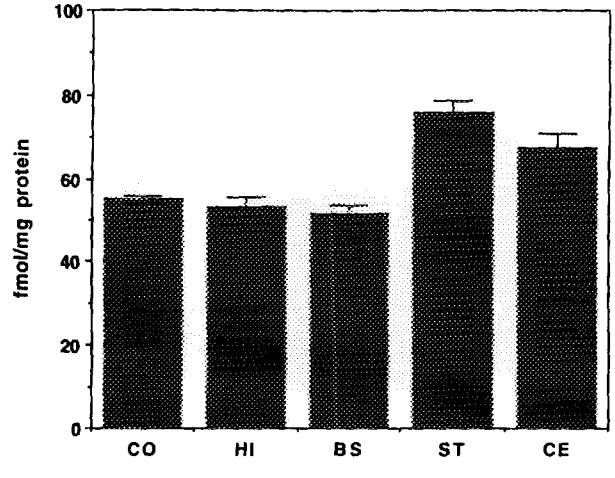


Figure 5. Specific binding of maxadilan to areas of rat brain. Crude membrane fractions were prepared from the indicated tissues and incubated with [$^{125}$I] maxadilan. CO - cortex, HI- hippocampus, BS - brain stem, ST- striatum, CE - cerebellum.

various species and rabbit spleen are noted in Table 1. In all cases, it appears that maxadilan binds with high affinity to a single class of receptors.

*Competition between maxadilan and selected receptor ligands*

Because maxadilan receptors were present both in the vasculature and the brain, a number of known vasoconstrictor or vasodilator peptides, also found in brain, and small molecules were examined for their ability to compete with binding of [$^{125}$I] maxadilan to rabbit brain. In a preliminary experiment, a competitive binding assay between a fixed quantity of [$^{125}$I] maxadilan and increasing concentrations of unlabeled maxadilan was performed (Figure 6) yielding an IC50 of maxadilan of 2.0+/-.3nM. None of the peptides tested, including, CGRP, amylin, endothelins and VIP competed with maxadilan (Figure 7). Results of an analogous experiment performed with small molecule agonists, including receptor ligands (carbachol, dopamine, histamine, isoproterenol, norepinephrine, serotonin, glycine, GABA and glutamate) and ion channel blockers (TEA, verapamil, and nifedipine) revealed that none of these small molecules had the ability to compete with labeled maxadilan binding to brain extracts (not shown).

**Discussion**

Maxadilan is a vasodilator peptide present in sand fly salivary glands and the fly probably uses this peptide as an aid in obtaining a blood meal. When sand flies transmit leishmaniasis, their salivary gland extracts exacerbate this process and recent data indicates that maxadilan participates in this process (submitted). To examine these cutaneous effects in more detail, an iodinated probe would be useful. The data presented here reveal that the peptide has been labeled to very high specific radioactivities (equimolar or greater ratio of $^{125}$I to peptide) with maintenance of biological activity despite a lack of tyrosines residues.

Because vasoactive molecules might be expected to interact with receptors in a variety of tissues, a survey was undertaken to examine the binding of labeled maxadilan to membrane fractions obtained from various rabbit tissues, instead of using this probe to assess binding sites in skin by autoradiography. Maxadilan was found to bind with high affinity to a single class of receptors in rabbit aorta and spleen and in brain from a variety of species. The binding in spleen is consistent with the interaction of maxadilan with cells of the immune system. The studies in rat and human brain reveal wide distibution of binding and the preferential binding to white

Table 1
Kd and Bmax of maxadilan binding to membrane homogenates

| species/tissue | Kd (pM) | Bmax (fmol/mg protein) |
|---|---|---|
| mouse brain | 90 | 80 |
| rat brain | 201 | 95 |
| rabbit brain | 136 | 58 |
| rabbit spleen | 85 | 14 |
| bovine brain | 103 | 27 |

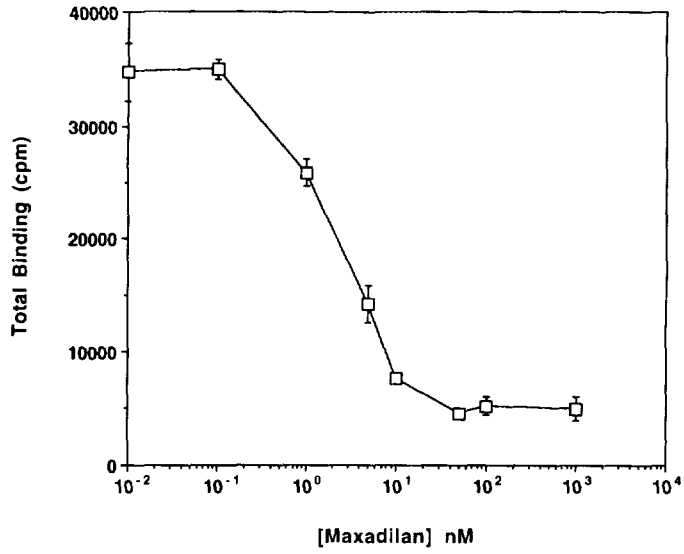


Figure 6. Competitive binding assay between labeled and unlabeled maxadilan in rabbit brain. Crude rabbit brain membranes were incubated with [$^{125}$I] maxadilan (70pM) and the indicated concentrations of cold maxadilan. The $IC_{50}$ for maxadilan was approximately 2nM.

matter suggests that maxadilan is interacting with a neuronal component in the central nervous system. The result that a high degree of specific binding occurred in brain may, in retrospect, not be surprising as a number of neuropeptides have vascular effects (12). However, the

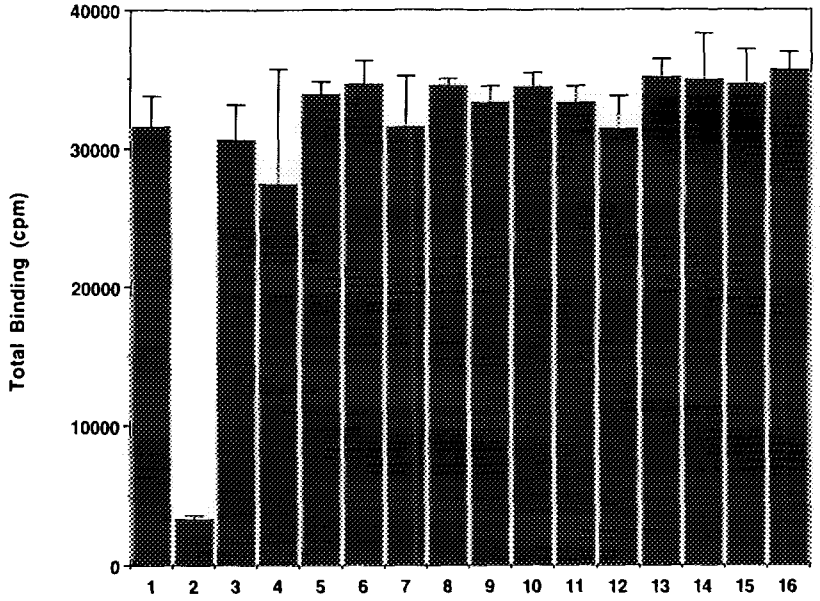


Figure 7. Competition between maxadilan and endogenous brain-derived peptides. The ability of a number of peptides to compete with labeled maxadilan was determined by competition. 1: no peptide, 2: maxadilan, 3: VIP, 4: CGRP, 5: amylin, 6: neurotensin, 7: bradykinin, 8: bombesin, 9: oxytocin, 10: somatostatin, 11: angiotensin II, 12: parathyroid hormone, 13: substance P, 14: endothelin I, 15: endothelin II, 16: endothelin III.

observations with maxadilan come from the opposite direction - it is an exogenous peptide whose primary effect is exerted on the vasculature to aid in blood-feeding, and it incidentally binds to brain membranes. A possible interpretation of the observation that some but not all batches of the labeled peptide bind to rabbit aorta while all preparations bind to rabbit brain is that subclasses of a "maxadilan receptor" exist and the iodination process alters the structure of the peptide enough to "distinguish" between such subclasses.

That maxadilan raises levels of cAMP and appears to bind to a membrane receptor suggests that it interacts with a member of the G-protein coupled receptor family (13). Coupled with earlier observations of slight primary sequence homology to CGRP, it is possible that maxadilan interacts with a subclass of CGRP or related receptors (4). It should be possible to identify a cell line, rather than whole tissue, which binds and signals upon incubation with maxadilan. Such a cell line would be useful for functional studies and for expression cloning of a maxadilan receptor. It is also possible that a maxadilan-like peptide is present in mammals, a scenario which could be examined in a number of ways including probing western blots of mammalian brain using antibody to maxadilan or Southern blots of mammalian genomic libraries using the gene encoding maxadilan as a probe. A positive result could lead to the identification of a previously unknown neuropeptide.

Identification of a maxadilan receptor or endogenous maxadilan-like peptide will advance our understanding of how this arthropod-derived molecule works. This result will shed light on how maxadilan exerts its long-lasting erythema effect and elucidate further the potential therapeutic role of this potent vasodilator.

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