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Effects of Methylene Blue on the Uptake, Release and Metabolism of Noradrenaline in Mesenteric Arterial Vessels

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Abstract—Methylene blue (3, 10 and 30 μM) increased the spontaneous outflow of endogenous dopamine and noradrenaline from sympathetic nerves supplying the dog mesenteric artery and drastically reduced the formation of endogenous dihydroxyphenylglycol (DOPEG). In addition, it decreased the accumulation of [^{3}H]noradrenaline in the tissue, reduced the formation of [^{3}H]DOPEG and [^{3}H]normetanephrine, without altering the formation of [^{3}H]dihydroxymandelic acid. In tissue homogenates of the same blood vessel, methylene blue 30 and 100 μM produced a significant reduction in the deamination of 5-hydroxytryptamine (5-HT), β-phenylethylamine (β-PEA) and tyramine. Methylene blue increased the accumulation of [^{3}H]isoprenaline in the tissue, and markedly reduced the formation of [^{3}H]*O*-methylisoprenaline ([^{3}H]OMI). These results show that methylene blue alters the storage and disposition of the adrenergic transmitter.

Over the last few years, methylene blue has been widely used as a guanylate cyclase inhibitor in studies of mechanisms involving vascular smooth muscle relaxation and cyclic guanosine monophosphate (cGMP) formation as caused by various agents (Ignarro et al 1981; Kukovetz et al 1982; Ignarro & Kadowitz 1985). Furthermore, the dye also abolishes vascular smooth muscle relaxation and cGMP formation elicited by vasodilator/relaxant agents which are known to stimulate the formation and/or release of endothelium-derived relaxing factors (EDRF) (Murad et al 1979; Kukovetz et al 1981; Gruetter et al 1981a, b; Furchgott 1984; Griffith et al 1985; Bowman et al 1985, 1986; Pascual et al 1987). In previous reports (Bowman et al 1985, 1986), methylene blue has been used to analyse the relaxant effect on the rabbit aorta of EDRF and of the smooth muscle inhibitory factor (IF) which can be extracted from the bovine retractor penis muscle. In these experiments the dye alone consistently caused a rise of the tone in the aortic strip. Though this could be due to inhibition of a background production of cGMP achieving levels capable of supressing spontaneous tone, as suggested by Griffith et al (1985), further investigations have shown that the contraction of rabbit aortic strips elicited by the dye was abolished by α-adrenoceptor blockade and denervation by 6-hydroxydopamine. Also, responses to both tyramine and field stimulation of adrenergic motor nerves of rabbit perfused ear artery preparations were significantly reduced by previous exposure to methylene blue (Soares-da-Silva & Gillespie 1987).

Although the results previously reported strongly support the view that the dye's contractile effect is due to the release of noradrenaline from adrenergic nerves, it has not been investigated whether other mechanisms, which influence the availability of the released transmitter in the biophase, are affected by the dye; these could as well be responsible for its indirect sympathomimetic effects. In the present work we have quantified the amount of noradrenaline released by methylene blue from sympathetic neurons supplying the proximal branches of the mesenteric artery and studied the effects of the dye on the neuronal uptake of the adrenergic transmitter and on monoamine oxidase (MAO) and catechol-*O*-methyl transferase (COMT) activities. A preliminary account of some of these findings has been previously presented (Caramona et al 1987).

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Materials and Methods

Assay of endogenous catecholamines and dihydroxyphenylglycol

Mongrel dogs of either sex, 15–21 kg, were anaesthetized with sodium pentobarbitone (30 mg kg^{-1} i.v. injected in the forelimb) and the proximal branches of the mesenteric artery removed, stripped of their mesentery, rinsed free from blood and longitudinally cut. Each segment, weighing about 40 mg and of 4 cm length, was preincubated for 30 min in 5 mL Krebs solution at 37°C, gassed with 95% O_2 and 5% CO_2, in the presence of 55 μM hydrocortisone to block extraneuronal uptake. The Krebs solution had the following composition (mM): NaCl 118, KCl 4·7, $CaCl_2$ 2·4, $MgSO_4$ 1·2, $NaHCO_3$ 25, KH_2PO_4 1·2 and glucose 11. EDTA 0·04 mM was added to the Krebs solution to prevent oxidation of catecholamines.

After the preincubation period, tissues were incubated for 30 min in 5 mL warm (37°C) and gassed (95%O_2 and 5%CO_2) Krebs solution (containing hydrocortisone, as above). Methylene blue was added to the Krebs solution to obtain a concentration of 3, 10 or 30 μM, respectively. In control experiments no drug was present. At the end of the incubation period, tissues were rapidly removed from the incubation vials, blotted with filter paper, weighed, minced with fine scissors and placed in 2·0 mL 0·1 M perchloric acid. Tissue catecholamines were extracted during 48 h at −20°C. After removal of tissues from incubation vials 0·5 mL 1·0 M perchloric acid was added to the incubation fluid and samples were stored frozen until analysed, usually within 48 h.

Assay of dopamine (DA), noradrenaline (NA) and dihydroxyphenylglycol (DOPEG) was performed by HPLC with electrochemical detection, as previously described (Soares-

da-Silva 1987). Under our conditions, the lower limits of detection for dopamine, noradrenaline and DOPEG were 30, 10 and 20 pg/50 μL, respectively.

Removal, accumulation and metabolism of [^{3}H]noradrenaline ([^{3}H]NA)

Proximal branches of the canine mesenteric artery strips of about 50 mg were incubated with [^{3}H]NA 0·2 μM (specific activity 23·1 Ci mM^{-1}, batch No. 2113–113 NEN; 70% of total tritium in the 7 position, 30% in the 8 position). The incubation was carried out at 37°C in small beakers containing 3 mL of Krebs solution. To avoid autoxidation of the amine, 27 μM EDTA and 114 μM ascorbic acid were added to the incubation fluid, which was bubbled with 95% O_2 and 5% CO_2. The beakers were continuously shaken throughout the incubation period.

In control experiments, the strips were kept in a beaker with oxygenated (95% O_2 and 5% CO_2) and warmed (37°C) Krebs solution for 30 min before incubation with [^{3}H]NA. To study the influence of methylene blue on the accumulation, removal and metabolism of [^{3}H]NA tissues were preincubated with the dye for 30 min and thereafter incubated for another 30 min with [^{3}H]NA. At the end of the incubation, tissues were rapidly immersed in cold Krebs solution, blotted, weighed and homogenized in 5 mL cold 0·4 M perchloric acid containing 1 mg mL^{-1} EDTA and 1·25 mg mL^{-1} Na_2SO_3. Aliquots of both acid tissue extract and the incubation medium, which was also acidified, were analysed for the labelled amine and its metabolites.

In each experiment, one sample of medium was carried through the incubation procedure without being exposed to any tissue. This sample was analysed as described below. The values for the "metabolites" recovered from this control sample represent contamination plus spontaneous degradation of labelled amine. These values were subtracted from the value for the corresponding experimental sample. On the other hand, no corrections were made of the experimental values in relation with the presence of 30% of the ^{3}H in the 8-position.

Chromatography on columns of alumina and Dowex 50W × 4 was performed with aliquots of tissue homogenates and incubation fluid to separate [^{3}H] NA and its metabolites as described by Graefe et al (1973). The recovery of labelled NA was 88·4 ± 5·2 (n = 4). The recovery of radioactivity in the chromatographic procedure (sum of radioactivity in 5 fractions/total radioactivity in the samples) was 91·6 ± 2·8 (n = 4). Radioactivity was measured by liquid scintillation counting (Berthold BF 5000/300) using a mixture of 330 mL Triton X-100, 5·5 mL Permablend III and 1 mL toluene as scintillation fluid. For determination of extracellular space, the strips were incubated for 30 min in 3 mL Krebs solution containing 1 μCi of [^{14}C]sorbitol and radioactivity was measured by liquid scintillation counting as described above. The values found for [^{3}H]NA in the tissue were corrected for extracellular space.

Assay of MAO activity

Homogenates of the proximal branches of canine mesenteric artery were prepared in 0·06 M phosphate buffer, pH 7·2, using a Duall-Kontes homogenizer. MAO activity was determined using [^{3}H]5-hydroxytryptamine ([^{3}H]5HT) as preferential substrate for MAO type A, [^{14}C]β-phenylethylamine ([^{14}C]PEA) as preferential substrate for MAO type B and [^{3}H]tyramine ([^{3}H]TYR) as a substrate for both types of MAO. The reaction mixture contained 25 μL of tissue homogenate, 50 μL 0·06 M phosphate buffer (pH 7·2) with 2 mM [^{3}H]5HT, 100 μM [^{14}C]PEA or 100 μM [^{3}H]TYR and 25 μL bidistilled water. The concentration of substrates was approximately 3 times the K_m of the enzyme (Caramona 1982). After 30 min of incubation at 37°C with continuous shaking, the tubes were transferred to ice-water and the reaction stopped by adding 10 μL of 3 M HCl. The deamination product was then extracted and measured by liquid scintillation counting, as described by Jarrott (1971). When different concentrations of methylene blue (3, 10, 30 or 100 μM) were used, 25 μL of tissue homogenate were preincubated for 15 min with 25 μL of the inhibitor solution, before addition of the substrate.

Influence on O-methylating activity

Segments of the proximal branches of the mesenteric artery were preincubated in beakers containing 3 mL oxygenated (95% O_2 5% CO_2) and warm (37°C) Krebs solution as controls, with added U-0521 (100 μM) or with methylene blue in different concentrations (3, 10 and 30 μM) for 30 min, under constant agitation at 37°C. After preincubation, tissues were placed in 3 mL Krebs solution with added [^{3}H]isoprenaline (2 μM) for another 30 min. At the end of this period, tissues were immersed for a few seconds in Krebs solution, blotted with filter paper, weighed and processed for scintillometric determination of [^{3}H]isoprenaline and its methylated metabolite [^{3}H]*O*-methylisoprenaline (OMI); in the fluid only OMI was determined. The details of the method were reported by Azevedo & Osswald (1976).

Drugs

Drugs used were: [^{3}H]5-hydroxytryptamine creatinine sulphate (23·6 Ci mM^{-1}) (NEN Chemical, RFA), isoprenaline (isoproterenol)-(±)-[7-^{3}H(N)] (8·9 Ci mM^{-1}) (NEN), methylene blue (T & H Smith, Ltd), [^{3}H](−)-noradrenaline (23·1 Ci mM^{-1}) (NEN), [^{14}C]β-phenylethylamine hydrochloride (50 Ci mM^{-1}) (NEN), [^{3}H]tyramine (11·3 Ci mM^{-1}) (NEN).

Statistics

Differences of two means were estimated by Student's *t*-test for unpaired data; a probability of less than 0·05 was assumed to denote a significant difference.

Results

Effect of methylene blue on amine spontaneous outflow and DOPEG formation

As shown in Table 1, methylene blue (3, 10 or 30 μM) did not significantly change tissue catecholamine content, though a

Table 1. Noradrenaline (NA) and dopamine (DA) tissue content (in μg g^{-1}) after 30 min incubation in control experiments and in the presence of 3, 10 and 30 μM methylene blue. Results are the means ± s.e. of five experiments.

		Methylene blue		
	Control	3 μM	10 μM	30 μM
NA	3·3 ± 0·3	3·4 ± 0·1	3·2 ± 0·2	2·7 ± 0·3
DA	0·17 ± 0·02	0·15 ± 0·02	0·18 ± 0·02	0·14 ± 0·02

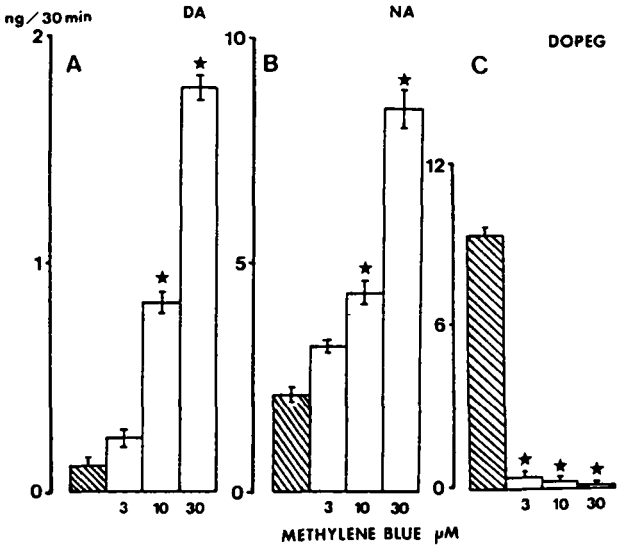

FIG. 1. The effect of methylene blue (3, 10 and 30 μM) on the spontaneous outflow of (A) noradrenaline (NA), (B) dopamine (DA) and (C) DOPEG from the proximal branches of the dog mesenteric artery. Each column represents the mean of five experiments per group; vertical lines show s.e. Hatched columns, control; open columns, effect of methylene blue. *Significantly different from control values ($P<0.01$).

slight decrease in DA an NA could be observed with the highest concentration used.

The effects of methylene blue on the spontaneous loss of catecholamines from the proximal branches of the mesenteric artery are shown in Fig. 1A, B. The effects were concentration-dependent; the dye caused a significant rise of DA and NA outflow.

Fig. 1C shows the effect of 3, 10 and 30 μM methylene blue on DOPEG spontaneous outflow during the 30 min incubation period; at all concentrations it drastically reduced DOPEG outflow.

Influence of methylene blue on the removal, accumulation and metabolism of [^{3}H]NA

The accumulation and metabolism of [^{3}H]NA 0·2 μM by isolated mesenteric arteries was studied during a 30 min incubation period in Krebs solution. The results are expressed in pmol g^{-1} of tissue/30 min (Table 2). Methylene blue 3 μM and higher concentrations reduced by about 90% the formation of the deaminated metabolite [^{3}H]DOPEG, without affecting the formation of other deaminated metabolites namely [^{3}H]dihydroxymandelic acid.

Methylene blue 10 and 30 μM reduced the accumulation of [^{3}H]NA by about 52 and 60%, respectively; 3 μM did not affect the accumulation of [^{3}H]NA in the tissue. At 10 an 30 μM it also decreased the formation of [^{3}H]normetanephrine by about 60%.

The effect of methylene blue on MAO A and MAO B activities

In view of the findings reported above and considering the difference between the inhibition of [^{3}H]DOPEG formation and blockade of [^{3}H]NA accumulation in the tissue, a preferential MAO inhibition by methylene blue appeared as possible.

Table 3 shows the influence of 3, 10, 30 or 100 μM methylene blue on the deamination of the three different MAO substrates, 5-HT, PEA and tyramine. Only 30 and 100 μM methylene blue significantly reduced the deamination of the substrates. The reduction of [^{3}H]5-HT (MAO A) and [^{14}C]PEA (MAO B) deamination by the dye (30, 100 μM) was of about 25%. When [^{3}H]TYR (MAO A and B) was used the percentage of inhibition of deamination by the dye was of about 50%.

Table 2. Accumulation and metabolism of [^{3}H]noradrenaline ([^{3}H]NA, 0·2 μM) by isolated mesenteric arteries pretreated with methylene blue (MB; 3, 10 and 30 μM) during 30 min. The results are expressed in pmol g^{-1} 30 min. Values are means ± s.e. of four experiments.

		Methylene blue		
	Control	3 μM	10 μM	30 μM
[^{3}H]NA accumulated	494 ± 17	487 ± 109	234 ± 15*	195 ± 17*
[^{3}H]DOPEG	328 ± 71	46 ± 4**	38 ± 3**	27 ± 12**
[^{3}H]DOMA	296 ± 81	241 ± 24	212 ± 38	230 ± 39
[^{3}H]OMDA	1441 ± 321	1077 ± 236	1039 ± 207	1028 ± 87
[^{3}H]NMN	277 ± 61	198 ± 52	112 ± 17*	101 ± 13*

Significantly different from corresponding control values, *$P<0.05$, **$P<0.01$.

NA, noradrenaline; DOPEG, dihydroxyphenylglycol; DOMA, dihydroxymandelic acid; OMDA, *O*-methylated and deaminated metabolites (methoxy-hydroxyphenylglycol + methoxyhydroxymandelic acid); NMN, normetanephrine.

Table 3. Influence of methylene blue on the deamination of the substrates of MAO. Results are expressed in nmol g^{-1} 60 min. Values are means ± s.e. of four experiments.

	5-HT	β-PEA	TYR
Control	12·8 ± 0·8	11·4 ± 0·6	19·0 ± 0·6
Methylene blue			
3 μM	11·6 ± 0·9	11·0 ± 0·9	15·9 ± 1·5
10 μM	11·0 ± 1·1	10·4 ± 0·7	13·5 ± 1·3
30 μM	9·7 ± 0·9*	8·5 ± 0·7*	11·4 ± 1·5*
100 μM	9·6 ± 0·6*	8·1 ± 0·8*	9·1 ± 0·7*

*Significantly different from corresponding control values ($P<0.02$).

5-HT, 5-hydroxytryptamine; β-PEA, β-phenylethylamine; TYR, tyramine.

Table 4. Accumulation and metabolism of [^{3}H]isoprenaline (2 μM) in mesenteric artery strips during 30 min of incubation. Results are expressed in nmol g^{-1} 30 min. Values are means ± s.e. of four experiments.

	[^{3}H]ISO accumulated	[^{3}H]OMI	
		in tissue	in fluid
Control	121·9 ± 5·2	380·1 ± 0·8	99·7 ± 9·8
U-0521 (100 μM)	108·7 ± 6·1	15·2 ± 0·4*	32·0 ± 8·9*
Methylene blue (μM)			
3	164·7 ± 8·1*	27·5 ± 0·1*	61·9 ± 6·9*
10	151·4 ± 2·7*	25·8 ± 1·1*	51·1 ± 4·9*
30	113·6 ± 1·8	17·4 ± 0·3*	39·9 ± 2·7*

*Significantly different from corresponding control values ($P<0.05$).

ISO, isoprenaline; OMI, *O*-methylisoprenaline.

Metabolism of [^{3}H]isoprenaline

Table 4 shows the effect of U-0521 (100 μM) and methylene

blue (3, 10 and 30 μM) on the accumulation and methylation of [^{3}H]isoprenaline. OMI accumulated in the tissue was significantly reduced by U-0521, as was the amount of OMI found in the incubation fluid. The effects of methylene blue on the methylation of [^{3}H]isoprenaline were dose-dependent and characterized by a reduction in the amount of OMI in the tissue and incubation fluid.

Discussion

Classical textbooks of pharmacology mention methylene blue for its antimalarial, disinfectant, atropine like activity, basic dye properties and therapeutic effects in methaemoglobinaemia. More recently, however, the dye has been shown to be useful as a guanylate cyclase inhibitor, and has been used for this purpose in the study of the processes of vascular smooth muscle relaxation which occur through cGMP generation. The discovery that methylene blue has also some indirect sympathomimetic properties (Soares-da-Silva & Gillespie 1987), which to some extent could conflict with other mechanisms involved in the process of vascular smooth muscle relaxation, led us to pursue the work on the effects of the dye upon sympathetic neurotransmission. The present study shows that the dye affects the storage and disposition of the adrenergic transmitter, and we conclude that this should be kept in mind in future studies involving sympathetically innervated preparations.

Methylene blue in a concentration-related manner increased spontaneous DA and NA outflow from sympathetic nerves supplying the proximal branches of the dog mesenteric artery. The results presented do not give any information concerning the mechanism through which the dye does this. However, the observation that it produced a simultaneous phentolamine-sensitive rise of tone and reduced by 30% the response to field stimulation of adrenergic motor nerves in the rabbit perfused ear artery, suggest that the NA releasing properties of the dye are not due to a facilitation of exocytosis but probably to a tyramine like effect (Soares-da-Silva & Gillespie 1987). Though the NA releasing properties of methylene blue are consistent with the previously demonstrated indirect sympathomimetic action of the dye, other mechanisms appear to be involved, which also could account for such a sympathomimetic effect. Evidence in favour of this view is supported by the fact that the dye blocks neuronal uptake of NA and inhibits the enzymes which are responsible for the metabolism of the adrenergic transmitter, MAO and COMT. In fact, both mechanisms would make possible an increased availability of NA at the biophase, and potentiate the effects of the NA released by the dye.

The ultimate mechanism responsible for the drastic reduction of endogenous DOPEG and [^{3}H]DOPEG formation could be that of MAO inhibition or the reduced offer of NA to intraneuronal MAO through an inhibition of neuronal uptake. Our data suggest that both inhibition of MAO and blockade of the NA uptake system could be responsible for the inhibition of DOPEG formation caused by methylene blue; 10 and 30 μM blocked by 50 and 60%, respectively, the accumulation of [^{3}H]NA and inhibited by 30 and 40%, respectively, MAO (A and B) activities. One inconsistency appears to be that 3 μM of the dye drastically reduced the formation of endogenous DOPEG and [^{3}H]DOPEG without any effect either on MAO (A and B) activities or on [^{3}H]NA accumulation. The possibility that either endogenous DOPEG or [^{3}H]DOPEG were chemically inactivated by methylene blue during the incubation period was investigated by studying the recoveries of exogenous DOPEG added to incubation vials containing different concentrations of dye and subsequently treating as described for tissue incubation experiments; the dye did not interfere with recoveries of endogenous and tritiated DOPEG (data not shown). It is possible, however, that the effect of methylene blue on DOPEG formation is not only related to its ability to interfere with MAO and neuronal uptake of the adrenergic transmitter, but to a decreased offer of NA to the intraneuronal MAO, as has been found for tyramine (Brandão et al 1978). In fact, it has been shown that tyramine produces an increase in noradrenaline outflow accompanied with a decrease in DOPEG outflow, and the relative importance of DOPEG in the efflux declines with increasing concentrations of tyramine (Brandão et al 1980). The finding that methylene blue did not affect the formation of [^{3}H]dihydroxymandelic acid which is a deaminated metabolite of noradrenaline of extraneuronal origin (Osswald & Guimarães 1983; Osswald & Caramona 1984), also agrees with this view.

Although the effects of low concentrations of methylene blue on the formation of endogenous DOPEG and [^{3}H]DOPEG do not clearly suggest that MAO has been inhibited, the finding that 30 and 100 μM dye has inhibited the deamination of [^{3}H]5-HT, [^{14}C]PEA and [^{3}H]TYR in tissue homogenates shows that the dye, to some extent, inhibits MAO. It appears, however, that it is an unspecific MAO inhibitor, since the degree of inhibition of deamination of [^{3}H]5-HT (preferential substrate for MAO A) was of about the same magnitude as that for [^{14}C]PEA (preferential substrate for MAO B).

Another enzymic step in the metabolic degradation of the adrenergic transmitter we have found to be affected by methylene blue was that of NA methylation by COMT; 10 and 30 μM dye significantly reduced the formation of [^{3}H]normetanephrine. In view of the variety of the effects of methylene blue upon nerves, it could be also suspected that the reduction of [^{3}H]normetanephrine formation could be the result of the interference of the dye upon other mechanisms which occur at the extraneuronal level, namely the inhibition of the enzyme COMT, the blockade of the extraneuronal uptake system for NA or both. The finding that the dye did not reduce, but actually increased, the accumulation of [^{3}H]isoprenaline and drastically reduced the formation of [^{3}H]OMI suggests that only COMT has been inhibited.

Though the results presented in this study demonstrate that methylene blue releases NA from sympathetic nerves and alters the disposition of the adrenergic transmitter, several other questions remain unsolved, namely those concerning the intrinsic mechanisms through which the dye exerts its action. Methylene blue is a well-known oxidizing agent, and it is possible that some of its releasing, neuronal uptake blocking and inhibitory enzyme properties are due to changes of redox potentials in the tissues (Lehninger 1975) which to some extent would alter the outcome of enzymic reactions. On the other hand, the inhibition of COMT by methylene blue could be attributed to a reduced availability

of the enzyme cofactor adenosylmethionine, as methionine reacts with the dye to form a sulphoxide (Bowman & Rand 1980).

In conclusion, the results presented show that methylene blue releases NA and DA from sympathetic nerves, decreases the overall accumulation of NA in vascular-innervated tissues and inhibits MAO A and B and COMT. These results are in good agreement with those reported previously on the indirect sympathomimetic effects of methylene blue (Soares-da-Silva & Gillespie 1987). In view of these findings care should be taken in using methylene blue as a guanylate cyclase inhibitor, at least when sympathetically innervated preparations are used. Finally, the finding that haemoglobin inhibits cGMP formation and abolishes some of the vascular responses which are dependent upon activation of guanylate cyclase (Bowman & Gillespie 1981; Bowman et al 1982; Bowman & Drummond 1984; Furchgott 1984; Martin et al 1986) without affecting sympathetic transmission (Pascual et al 1987), suggests that it could be a better experimental tool than methylene blue for future studies.

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