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The interaction between methylene blue and the cholinergic system

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1 The inhibitory effects of methylene blue (MB) on different types of cholinesterases and [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) binding to muscarinic receptors were studied.

2 Human plasma from young healthy male volunteers, purified human pseudocholinesterase and purified bovine true acetylcholinesterase were incubated with acetylcholine and increasing concentrations of MB (0.1–100 μmol l^{-1}) in the presence of the pH-indicator m-nitrophenol for 30 min at 25°C. The amount of acetic acid produced by the enzymatic hydrolysis of acetylcholine was determined photometrically.

3 Rat cardiac left ventricle homogenate was incubated with [^{3}H]-NMS and with increasing concentrations of MB (0.1 nmol l^{-1}–100 μmol l^{-1}) at 37°C for 20 min. The binding of [^{3}H]-NMS to the homogenate was quantified by a standard liquid scintillation technique.

4 MB inhibited the esterase activity of human plasma, human pseudocholinesterase and bovine acetylcholinesterase concentration-dependently with IC_{50} values of 1.05 ± 0.05 μmol l^{-1}, 5.32 ± 0.36 μmol l^{-1} and 0.42 ± 0.09 μmol l^{-1}, respectively. MB induced complete inhibition of the esterase activity of human plasma and human pseudocholinesterase, whereas bovine acetylcholinesterase was maximally inhibited by 73 ± 3.3%.

5 MB was able to inhibit specific [^{3}H]-NMS binding to rat cardiac left ventricle homogenate completely with an IC_{50} value of 0.77 ± 0.03 μmol l^{-1}, which resulted in a K_i value for MB of 0.58 ± 0.02 μmol l^{-1}.

6 In conclusion, MB may be considered as a cholinesterase inhibitor with additional, relevant affinity for muscarinic binding sites at concentrations at which MB is used for investigations into the endothelial system. In our opinion these interactions between MB and the cholinergic system invalidate the use of MB as a tool for the investigation of the L-arginine-NO-pathway, in particular when muscarinic receptor stimulation is involved.

Keywords: Methylene blue; cholinesterase, muscarinic receptors

Introduction

Methylene blue (methylthionine chloride; MB) is a widely accepted pharmacological tool in the analysis of the nitric oxide (NO)-pathway. MB is known to be an inhibitor of soluble guanylate cyclase (Ignarro *et al*., 1984), although a few authors consider this effect to be rather weak (Marczin *et al*., 1992). Others have shown that MB is a direct inhibitor of NO-synthase and other iron-containing enzymes (Mayer *et al*., 1993). MB is known to inhibit directly the vascular smooth muscle relaxation induced by the endothelium-derived relaxing factor (EDRF, NO) itself or by nitrates, which are known to release NO *in vitro* and *in vivo* (Martin *et al*., 1985; McMahon & Kadowitz, 1992).

We recently used MB as a tool in a clinical pharmacological study, where the vasodilator effects of acetylcholine (ACh) and methacholine (MCh) in the human forearm vascular bed were compared by means of venous occlusion plethysmography (Bruning *et al*., 1995). Both ACh and MCh are considered to be non-selective muscarinic receptor agonists, which both activate the various M-receptor subtypes (M_1–M_4) (Eglen & Whiting, 1990). The endothelium-dependent vasodilatation caused by both MCh and ACh is triggered by stimulation of endothelial M_3-receptors and the subsequent release of EDRF/NO. MCh has proved to be a significantly more potent vasodilator than ACh (Kemme *et al*., 1995), and we explained this quantitative difference by the sensitivity of ACh for degradation by acetylcholinesterases (AChE), which does not affect MCh. When MB was used as a pharmacological tool, our attention was drawn to the finding that MB markedly potentiated the vasodilator response to ACh but not that to MCh (Bruning *et al*., 1994). MB may be speculated to activate the cholinergic system, possibly by inhibiting AChE and/or other esterases. Furthermore, intoxication with MB in man is known to provoke a series of symptoms (bronchial, gastrointestinal, haemodynamic) which would be in accordance with cholinergic activation (e.g. Martindale 30th edition, 1993). These considerations prompted us to study the possible interaction between MB and certain components of the cholinergic nervous system *in vitro*.

For this purpose we investigated the influence of MB on two types of cholinesterases and on human serum.

Since the stimulation of muscarinic receptors is often used to provoke the release of EDRF/NO from the endothelium we extended our study to test the possibility of a direct interaction of MB with muscarinic receptors. For this purpose the influence of MB on [^{3}H]-N-methylscopolamine binding in rat ventricular homogenate preparations was investigated as well.

Methods

Esterase activity

Esterase activity was quantified by the method of Rappaport *et al*. (1959), by use of a commercially available, colorimetric kit (cholinesterase endpoint, Sigma diagnostics). To establish the enzymatic activity we used the serum of 6 healthy male volunteers (mean age 24 years, range 19–31) as well as commercially available, purified human pseudocholinesterase and bovine erythrocyte true acetylcholinesterase. The protocol for the use of human material was approved by the Medical Ethics

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Committee of the University Hospital and informed consent was obtained from all subjects. The serum 0.2 ml was diluted with 0.2 ml isotonic saline. The purified human pseudocholinesterase and bovine true acetylcholinesterase were dissolved in isotonic saline to a concentration of 8 u 0.4 ml^{-1} and 2 u 0.4 ml^{-1}, respectively. One unit pseudocholinesterase is defined as the activity that will hydrolyse 2.5 μmol ACh min^{-1} at 37°C at pH 8.0. One unit true acetylcholinesterase is defined as the activity that will hydrolyse 1 μmol ACh min^{-1} at 37°C, at pH 8.0. Blanks were prepared by esterase inactivation at 60°C for 10 min to compensate for background absorbance induced by the sample. The active and inactivated enzymes were incubated with ACh, m-nitrophenol and various concentrations of MB (0.1–100 μmol l^{-1}) in a final volume of 5.6 ml at 25°C at pH 7.8 for 30 min. The decreasing pH, induced by the formation of acetic acid from the enzymatic hydrolysis of acetylcholine, causes a change of colour of the acid-base indicator m-nitrophenol. This results in an increased absorption of light at a wavelength of 420 nm which is proportional to the amount of acetic acid. The measurements were performed in 1 cm cuvettes with a Zeiss Specord S10 spectrophotometer, with distilled water as reference. The actual results were obtained by subtracting the absorption values of the test tubes from those of the blanks. The calibration was performed by adding increasing amounts of acetic acid to the assay with inactivated esterases and measuring the absorption. The results are expressed as Rappaport units which are defined as the amount of cholinesterase which will liberate 1 μmol of acetic acid from ACh in 30 min at 25°C, at pH 7.8, under the conditions of this test. A spectrogram of MB (100 μmol l^{-1}) and a calibration curve in the presence MB were recorded to rule out any interference with the colorimetric assay.

[^{3}H]-N-methylscopolamine binding

Male Wistar rats (approximately 12 weeks of age, weight 200–250 g) were obtained from IFFA Credo (Les Oncins, France). After cervical dislocation, the carotid arteries were opened and the hearts were rapidly excised. The binding experiments were performed according to Doods *et al*. (1987). The left ventricles were immersed in ice-cold HEPES buffer (20 mmol l^{-1} HEPES, 100 mmol l^{-1} NaCl, 10 mmol l^{-1} $MgCl_2$, pH 7.5) and homogenization was performed with a Polytron PT homogenizer. The homogenate was filtered through four layers of cloth gauze and centrifuged at 50,000 × *g* for 20 min. The pellet was rehomogenized and diluted to a final membrane concentration of 0.5–1.0 mg ml^{-1}. The protein content of the final membrane preparation was assessed by using the method of Bradford (1976). All procedures were carried out at 4°C. Samples, (500 μl), of the membrane suspension were incubated at 37°C for 20 min with 0.4 nmol l^{-1} [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) in the presence of various concentrations of MB (0.1 nmol l^{-1}–100 μmol l^{-1}) in a final volume of 1 ml. Non-specific binding was defined as binding in the presence of 10 μmol l^{-1} dexetimide. The incubation was terminated by dilution with 3 ml ice-cold HEPES standard assay buffer. Separation of bound and free [^{3}H]-NMS was achieved by rapid vacuum filtration across Whatman GF/B filters (Whatman International Ltd, Maidstone, Kent, U.K.), followed by three washes with 3 ml ice-cold HEPES buffer. The radioactivity on the filters was measured by standard scintillation counting techniques.

Drugs used

The colorimetric cholinesterase kit (Cholinesterase endpoint No. 420, Sigma Diagnostics), the bovine true cholinesterase (from erythrocytes), the human pseudocholinesterase (from serum) and methylene blue were obtained from Sigma Chemical Co (St Louis, MO, U.S.A.). The radioligand used to analyse muscarinic receptor binding sites was [^{3}H]-N-methylscopolamine (NEN Dupont de Nemours, Dreieich, Germany). The specific activity was 78.9 Ci $mmol^{-1}$. Dexetimide was purchased from Merck & Co. (Rathway, NJ, U.S.A.). All other chemicals were of analytical or best commercial grade available.

Statistical evaluation

The results concerning the esterase activity are presented as percentage of the absorbance in the absence of MB at a wavelength of 420 nm. Means ± s.e.mean of 4–6 individual experiments, each performed twice, are depicted in Figure 1. The concentrations which produced a half-maximal inhibition (IC_{50}) were obtained by subjecting the results of the individual experiments to a non-linear regression algorithm, by use of the equation $E = E_{max}\ I^n(IC_{50}{}^n + I^n)^{-1}$. In the equation E is the actual effect at an inhibitor concentration I, E_{max} is the maximal attainable effect and n is the steepness of the relationship. The IC_{50} values are expressed as mean ± s.e.mean of 4–6 individual experiments, each performed twice.

The results of the binding experiments are presented as pecentage of specific [^{3}H]-NMS binding in the absence of MB. Means ± s.e.mean of 4 individual experiments, each performed twice, are depicted in Figure 2. The concentrations MB which displaced 50% of the radioligand [^{3}H]-NMS (IC_{50}) were obtained by subjecting the results of the individual experiments to a non-linear regression algorithm, by use of the equation $B = B_{max}\ (1 + 10^{A\text{-}\log IC_{50}})^{-1}$. In the equation B represents the

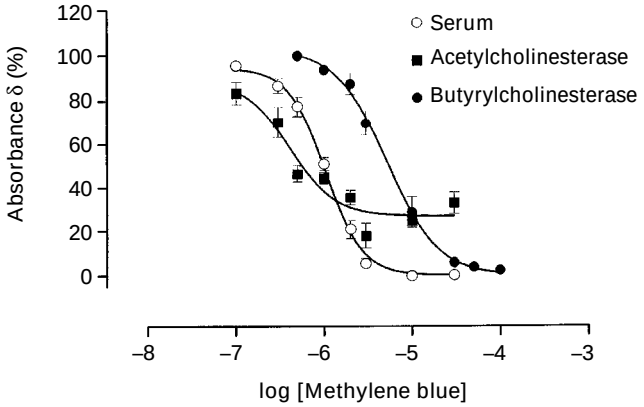

Figure 1 Effects of methylene blue on the activity of various cholinesterases. Results are expressed as percentage of the absorbance δ of the samples at a wavelength of 420 nm; 100% equals the absorbance in the absence of methylene blue. Data shown are means and vertical lines indicate s.e.mean ($n = 4–6$).

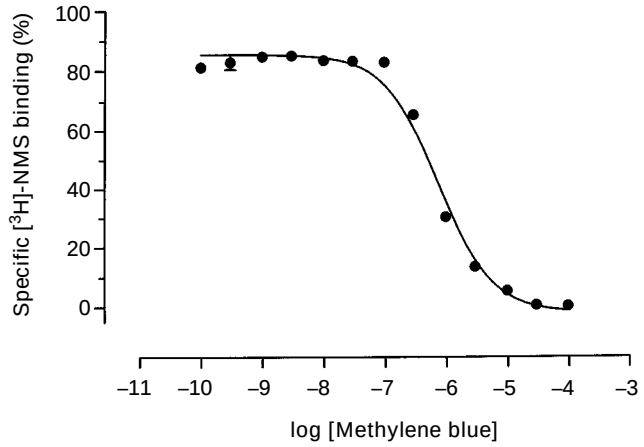

Figure 2 Effect of increasing concentrations of methylene blue on specific [^{3}H]-N-methylscopolamine (0.4 nmol l^{-1}) binding to rat cardiac left ventricle homogenate; 100% equals the specific binding in the absence of methylene blue. Data shown are means ± s.e.mean ($n = 4$).

actual binding at an inhibitor concentration A, B_{max} is the maximal binding and IC_{50} the concentration of the competitor that competes for half of the specific binding. The IC_{50} value is expressed as mean ± s.e.mean of 4 individual experiments, each performed twice. The K_i value of MB was calculated according to Cheng and Prusoff (1973).

Results

Cholinesterase activity

After 30 min of incubation at 25°C the chosen amounts of serum, true acetylcholinesterase and pseudocholinesterase hydrolysed about 100 to 120 μmol ACh which equals 100 to 120 Rappaport units. The colorimetric measurement of ACh hydrolysis by m-nitrophenol at a wavelength of 420 nm was not disturbed by MB, which shows significant absorption below 350 and above 450 nm. The calibration curve, obtained with increasing concentrations of acetic acid to validate the indicator reaction with m-nitrophenol, was not affected by MB. The measurements of the samples at the endpoint (30 min) showed that MB inhibited the activity of the bovine true acetylcholinesterase, the human pseudoesterase and the human serum in a concentration-dependent manner. The cholinesterase activity in the human serum and the purified human pseudocholinesterase were blocked completely by the highest concentrations of MB, whereas maximal inhibition of the bovine true acetylcholinesterase was found to be 73 ± 3.3%. The half maximal inhibitory concentrations of MB and the steepness of the concentration-inhibition curves are presented in Table 1.

[^{3}H]-NMS binding

[^{3}H]-NMS binds to muscarinic receptors in rat cardiac left ventricle homogenate with a B_{max} of 213 fmol mg^{-1} protein and a K_d of 1.1 nmol l^{-1} (data not shown). The unspecific binding, determined by co-incubation with an excess of unlabelled dexetimide (10 μmol l^{-1}) amounted to 4.74 ± 0.39% of the total binding. Co-incubation of increasing concentrations of MB (0.1 nmol l^{-1} – 100 μmol l^{-1}) with [^{3}H]-NMS (0.4 nmol l^{-1}) resulted in a concentration-dependent decrease of specific [^{3}H]-NMS binding, indicating competition at the level of the muscarinic binding site. The IC_{50} value was found to be 0.77 ± 0.03 μmol l^{-1}, which resulted in a K_i value for MB of 0.58 ± 0.02 μmol l^{-1}. At high concentrations MB was able to displace the radioligand completely from its specific binding site. However, a non-concentration-dependent reduction of the specific binding by 13 ± 2% was visible at MB concentrations in the range of 0.1 nmol l^{-1} to 0.1 μmol l^{-1}.

Discussion

MB was and is widely used as a highly specific inhibitory tool for the analysis of the L-arginine-NO pathway and the endothelium. It can inhibit EDRF/NO-induced vasodilatation by scavenging and inactivating nitric oxide (Marshall *et al*., 1988; Wolin *et al*., 1990). Beside this oxyhaemoglobin-like effect (Doyle & Hoekstra, 1981) MB interacts with the active haeme centre (Martin *et al*., 1985) and the haeme-deficient apoenzyme (Tsai *et al*., 1983) of soluble guanylate cyclase, thereby inhibiting the formation of the relaxant second messenger guanosine-3′,5′-cyclic monophosphate (cyclic GMP). So far the inhibitory effect of methylene blue on induced vasodilatation was taken as indicative of an involvement of nitric oxide and/or the soluble guanylate cyclase (Ignarro *et al*., 1984).

Table 1 Maximal inhibition, half maximal concentration and the steepness of the relationship of methylene blue inhibitory effects on various cholinesterases

Cholinesterase	*Maximal inhibition* (%)	*EC_{50}* (μmol l^{-1})	*Steepness of the relationship*
Bovine true cholinesterase	73 ± 33.3	0.42 ± 0.09	−1.70 ± 0.65
Human psuedo-cholinesterase	100 ± 0	5.32 ± 0.36	−1.54 ± 0.11
Human serum	100 ± 0	1.05 ± 0.05	−2.10 ± 0.14

Data are presented as means ± s.e.mean, $n = 4-6$.

However, recent investigations have revealed the MB exerts effects other than those aforementioned, like inhibition of the endothelial nitric oxide synthase (Mayer *et al*., 1993), inhibition of prostacyclin synthesis (Martin *et al*., 1989; Okamura *et al*., 1990), impairment of noradrenaline uptake, release and metabolism (Soares-Da-Silva & Caramona, 1988) and a possible interaction with G-proteins (Han *et al*., 1995).

Classical textbooks on pharmacology and pharmacopoeia mention effects of MB which might suggest an influence on certain elements of the cholinergic nervous system and/or its adjacent receptors (e.g. Martindale 30th edition, 1993). As discussed in the Introduction, we observed a clear potentiation by MB of the vasodilatation induced by acetylcholine in the human forearm vascular bed (Bruning *et al*., 1994).

In the present investigation we explored two mechanisms, one stimulating and one inhibitory, by which MB could possibly interact with the cholinergic system. The first was interference with the elimination of acetylcholine and, the second, an interaction with ACh muscarinic receptors.

When exposing different types of cholinesterases to MB, we indeed observed a significant inhibitory effect on the cholinesterase activity of human serum, as well as on purified bovine true acetylcholinesterase and human pseudocholinesterase. The esterase inhibition proved to be concentration-dependent in all three preparations studied. The MB concentrations required to bring about a significant degree of esterase activity inhibition are within the range of those frequently applied for the analysis of the L-arginine-NO-pathway, where MB is commonly used in concentrations of 10^{-5} mol l^{-1} or higher (Martin *et al*., 1985). In other words, MB may be considered as a cholinesterase inhibitor at the concentrations at which it is used for the investigation of the L-arginine-NO-pathway.

This finding also explains the potentiation of the vasodilator effects of ACh by MB in the human forearm vascular bed, established in clinical pharmacological studies (Bruning *et al*., 1994). Furthermore, it seems likely that the symptoms of cholinergic activation associated with MB poisoning are caused by inhibition of cholinesterase activity. It might even contribute to the ileal/jejunal stenosis seen in the newborn after intra-amniotic injection of MB, an adverse effect of MB still with an unknown mechanism of action (Nicolini & Monni, 1990; Dolk, 1991).

Muscarinic receptor stimulation by means of appropriate agonists, such as acetylcholine or methacholine, is a generally accepted procedure to investigate the functional role of the endothelium in blood vessels, where endothelial M_3-receptors are known to mediate the release of EDRF/nitric oxide, both *in vivo* (McMahon & Kadowitz, 1992) and *in vitro* (Martin *et al*., 1985). For this reason it seemed of interest to know whether MB may display affinity for muscarinic receptors. Our displacement experiments with [^{3}H]-NMS indeed indicate that MB possesses substantial affinity for muscarinic receptors, with a K_i value of 5.8×10^{-7} mol l^{-1}, which corresponds to or is even lower than that used for the analysis of the L-arginine-NO-pathway (Martin *et al*., 1985). This muscarinic receptor antagonism has also been demonstrated recently in electrophysiological experiments (Gerges *et al*., 1997).

In the present study, MB was shown to display opposing effects on the cholinergic system: an activating effect by inhi-

bition of the elimination of ACh, versus an inhibitory effect by the occupation of muscarinic receptors. It might be speculated that in those systems where the elimination of ACh by esterases effectively determines the concentration of the transmitter in the biophase, as in various *in vivo* conditions, MB exerts ACh potentiating properties. If a competitive type of antagonism is assumed the increasing concentrations of ACh under those conditions will displace MB from the muscarinic receptors. On the other hand, if the elimination of ACh does not play a decisive role, in particular under *in vitro* conditions, MB might effectively compete with the transmitter for its binding site, which would be manifest as mere ACh antagonism.

In conclusion, MB may be considered as a cholinesterase inhibitor with additional, relevant affinity for muscarinic binding sites at the concentrations in which it is used for investigations into the endothelial system. In our opinion these interactions between MB and the cholinergic system invalidate the use of MB as a tool for investigation of the L-arginine-NO-pathway, in particular when muscarinic receptor stimulation is involved.

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