Interactions between calcium antagonists and the sympathetic nervous system in various pharmacological models
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Chapter 6

EVIDENCE FOR A SYMPATHOLYTIC EFFECT OF MIBEFRADIL IN THE PITHEDED RAT PREPARATION

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1. Introduction

Mibefradil, a non-dihydropyridine calcium antagonist displays an unique hemodynamic profile when used in therapeutic dosages. It reduces blood pressure and heart rate without inducing a negative inotropy. This clinically advantageous characteristics were claimed to be due to T-type calcium channel blockade. Indeed. MIB has a high affinity to this particular type of calcium channel [1] which might explain the observed phenomena. However, MIB blocks at least three types of calcium channels in the micromolar range: the T-, L- and the N-type calcium channels, respectively [2]. This combined calcium channel blockade makes MIB an interesting pharmacological tool, despite the withdrawal of the drug in 1998 from the market because of several interactions with other drugs. Several of its clinically relevant effects, e.g. the antihypertensive and anti-ischaemic activities, can be readily explained by L-type calcium channel blockade [3]. Whether or not the T-type channel blockade contributes to the observed hemodynamic profile of this drug continues to be subject to debate. Beside its inhibitory activity on T- and L-type calcium channels, the interaction with the N-type channels might be considered to contribute to the overall clinical effect of this drug. The N-type calcium channel is crucially involved in the release of neurotransmitters from the varicosities of sympathetic nerves [4]. An inhibition of these channels might therefore exert a sympatholytic effect. In fact, it was shown [5] that MIB inhibits α-conotoxin GVIA-sensitive sympathetic noradrenaline-release in human right atrial appendages, which is indicative for a sympatholytic effect induced by N-type calcium channel blockade. It is the aim of the present study to investigate whether MIB can exert this particular effect under in vivo conditions and at dosages that might be considered to be in the therapeutic range. The pithed rat preparation was used to study the effect of MIB on the functional consequences of an electrical stimulation of the sympathetic cardioaccelerator nerves. To isolate the effect of MIB on the neurotransmitter release, its effect on externally applied noradrenaline was investigated. Furthermore, electrical stimulation of the preganglionic cardioaccelerator nerves as well as the noradrenaline dose-response curves were performed with the classic L-type calcium channel blocker verapamil (VER), an L-type calcium channel blocker with no known affinity for the N-type calcium channel.
2. Methods

During anesthesia (hexobarbitone, 150 mg/kg, i.p.) male normotensive Wistar rats of 250-300g (Broekman, The Netherlands) received a tracheal cannula and one group of animals were pithed and immediately thereafter subjected to artificial respiration with room air (60 strokes/mm, 10 ml/kg) using a positive pressure pump. The body temperature of 37°C was maintained by means of a thermostatically controlled heating table. The right jugular vein was catheterized and heparin (1 000 IU/kg) was injected via this route. A cannula was placed in the ipsilateral common carotid artery and arterial blood pressure was measured via a pressure transducer connected to a MacLab data acquisition system (ADInstruments, Castle Hill, Australia). The heart rate was derived on-line from the blood pressure recording. The animals were pretreated with d-tubocurarine (1 mg/kg, i.v.) and atropine (1 mg/kg s.c.) and bilateral vagotomy was performed in the cervical region. The pithing rod was coated with enamel, except for a segment of 1 cm length, 7cm distally from the tip for a maximal stimulation of the cardiac sympathetic nerves (C7-T1). After an equilibration period of 5 min, the animals received saline (1 ml/kg) (controls), MIB or VER either 3 or 10 μmol/kg intra-arterially. After an incubation period of 15 min, in the pithed animals the preganglionic cardioaccelerator nerves were electrically stimulated between the pithing rod and the dorsally-located indifferent electrode using monophasic rectangular pulses of 2 ms duration and supramaximal voltage (50V). Trains of pulses of 25s duration were applied and the increase in cardiac frequency (beats/min) was measured. Between the periods of stimulation, sufficient time was allowed to ascertain complete return of the cardiac frequency to pre-stimulation values. The second group received cumulatively noradrenaline 0.1-1000 nmol/kg in a total volume of 0.5ml/kg intravenously. For both stimuli, electrical stimulation and noradrenaline, one control group was compared against the four treatment groups with 3 and 10 μmol/kg of both calcium antagonists.

2.1. Statistical evaluation
All values in the text are given as means ± standard error of the mean (SEM). Statistical significance was evaluated by one-way ANOVA, followed by Newman-Keuls' test for multiple comparisons. The level of significance was set at p<0.05.

2.2. Data analysis
Using a computer program (GraphPad Prism, GraphPad. San Diego, USA) all curves of noradrenaline in the absence and the presence of the low concentration of the calcium antagonists were fitted to log concentration-effect data for 4-6 individual
experiments. The underlying equation is \( E = E_{\text{max}} \cdot A^p \cdot (A^p + IC_{50}^p)^{-1} \). In this equation \( E \) is the response obtained at a given concentration or stimulation frequency \( A \), \( E_{\text{max}} \) is the maximally attainable response, \( IC_{50} \) the concentration for the half maximal effect, and the exponent \( p \) describes the slope of the relationship (Hill-coefficient). The averaged half-maximal concentrations and the maximal responses to noradrenaline were compared by a one-way ANOVA test followed by Newman-Keuls' test for multiple comparisons.

2.3. Drugs used
Atropine sulfate, d-tubocurarine, verapamil (Sigma Chemical Co., St. Louis, MO, USA), indomethacin (Merck Sharp and Dohme, Haarlem, The Netherlands), noradrenaline bitartrate (Hoechst, Amsterdam, The Netherlands), guanethidine, (Pfizer, Brussels, Belgium), heparin (Leo Pharmaceuticals, Weesp, The Netherlands, hexobarbitone (OPG, Utrecht, The Netherlands). Mibefradil was kindly donated by Hoffman-LaRoche, Basel, Switzerland.

3. Results

3.1. Basal parameters
The mean blood pressure and the heart rate 15 minutes after the pithing procedure were \( 39.6 \pm 4.2 \text{ mmHg} \) (n=12) and \( 318 \pm 7 \text{ (n=12) bpm} \), respectively. Both calcium antagonists reduced the heart rate significantly and dose-dependently to \( 263.5 \pm 7.6 \) (MIB \( 3 \mu\text{mol/kg} \), n=13), \( 229.2 \pm 7.8 \) (MIB \( 10 \mu\text{mol/kg} \), n=12), \( 278.5 \pm 5.5 \) (VER \( 3 \mu\text{mol/kg} \), n=13) and \( 218.0 \pm 10.6 \) (VER \( 10 \mu\text{mol/kg} \), n=11). No difference could be observed between VER and MIB when applied at the same dosages.

3.2. Electrical stimulation
The maximum increase in heart rate (bpm) in response to electrical nerve stimulation was \( 96\pm7 \) (control, n=6) which was reached at 1 Hz. Guanethidine (5 mg/kg, i.v.) completely blocked the chronotropic response to electrical nerve stimulation (Fig. 1).

At doses of 3 and 10 \( \mu\text{mol/kg} \) both calcium antagonists shifted the frequency response curve to the right. The maximally obtainable increase in heart rate was \( 71\pm8 \) (MIB \( 3\mu\text{mol/kg} \), n=6), \( 57\pm6 \) (MIB \( 10\mu\text{mol/kg} \), n=5), \( 94\pm8 \) (VER \( 3\mu\text{mol/kg} \), n=4) and \( 46\pm7 \) (VER \( 10\mu\text{mol/kg} \), n=5). At a dose of 3 \( \mu\text{mol/kg} \) only MIB was able to depress the maximal chronotropic response to electrical stimulation. At the higher
concentration both calcium antagonists reduced the maximum to an identical extent (Fig. 1).

![Graph](image.png)

Fig.1. Frequency-dependent increase of heart rate as response of a pithed rat preparation to electrical stimulation of the spinal cord (C7-Th1) in the absence and presence of mibebradil, verapamil and 5mg/kg guanethidine. (a) Results with 3 μmol/kg mibebradil or verapamil; (b) results with 10 μmol/kg mibebradil or verapamil. Data given as mean ± SEM, n=4-6. $fP<0.05, ffP<0.01$ verapamil versus control; *$P<0.05$, **$P<0.001$ mibebradil versus control; $SP<0.05$ mibebradil versus verapamil. In graph (b) all values in the presence of mibebradil and verapamil show a $P<0.01$ versus control.

3.3. Noradrenaline
The maximum increase in heart rate (bpm) in response to noradrenaline was 96±4 bpm with a pEC$_{50}$ of 8.5 ± 0.031 (control, n=6). At doses of 3 and 10 μmol/kg of both calcium antagonists there was no significant shift of the noradrenaline dose-
response curve to the right. The pEC$_{50}$ and the maximally obtainable increase in heart rate were 7.62 ± 0.04 and 87±20 (MIB 3 µmol/kg, n=6), 42±9 (MIB 10 µmol/kg, n=5), 7.49 ± 0.05 and 73±5 (VER 3µmol/kg, n=5) and 40±7 (VER 10 µmol/kg, n=6). Under control conditions and in the presence of 3 µmol/kg MIB and VER the maximal effect of noradrenaline was reached at 0.1 µmol/kg whereas in the presence of 10 µmol MIB and VER it was reached at a dose of 1 µmol/kg.

![Diagram](image)

**Fig. 2.** Concentration-dependent increase of heart rate as response of a pithed rat preparation to intravenously applied noradrenaline in the absence and presence of mibefradil and verapamil. (a) Results with 3 µmol/kg mibefradil or verapamil; (b) results with 10 µmol/kg mibefradil or verapamil. Data given as mean ± SEM, n=4-6. *P<0.05 verapamil versus control; ** P<0.005 mibefradil and verapamil versus control; $P<0.05 $$$P<0.01$ mibefradil versus verapamil.

MIB at a dose of 3 µmol/kg was significantly more effective in reducing the chronotropic response to electrical stimulation when compared to externally applied
noradrenaline. For verapamil the opposite holds true. These differences were not observed with doses of 10 μmol/kg MIB and VER (Fig. 2).

4. Discussion

Electrical stimulation of the cardioaccelerator nerves in the pithed rat preparation at C7-Th1 induces a stable and reproducible chronotropic response [6] which has been proven to be sensitive to guanethidine, a blocker of noradrenaline release from sympathetic varicosities. Since all central regulatory mechanisms are eliminated in this particular preparation, this model can be assumed to be suitable for the investigation of drug actions at the level of the peripheral sympathetic nerve transmission [6]. The release of neurotransmitters from sympathetic varicosities is calcium dependent [7]. On depolarization calcium enters the cell via voltage-dependent calcium channels. Although L- and T-type calcium channels have been found in the membrane of prejunctional sympathetic nerves, the N-type plays the major role in the supply of trigger calcium for the noradrenaline release [8]. This has been shown by Pruneau and Angus [9] who used ω-conotoxin GVIA, a 27-amino acid peptide venom from the fish-hunting snail Conus geographus, to block the chronotropic responses to elective electrical spinal cord stimulation in the pithed rat preparation.

Mibefradil is a new non-dihydropyridine calcium antagonist which has a high affinity towards T-type calcium channels [1]. However, it also interacts with L-type calcium channels and, as has been demonstrated recently, N-type calcium channels in therapeutically relevant concentrations [2]. Göthert and Molderings [5] have shown that mibefradil is able to inhibit the ω-conotoxin GVIA-sensitive noradrenaline release from human right atrial appendages.

It was the aim of the present study to demonstrate a contribution of the N-type calcium channel blocking properties of mibefradil to the overall pharmacological profile of this drug.

At the low concentration of 3 μmol/kg of both calcium antagonists induced a comparable rightward shift of the noradrenaline dose-response curve with no depression of the maximum which might indicate that there are no differences in the potency or efficacy of both drugs in inhibiting the neurotransmitter mediated effects at the postjunctional level.

At the higher concentration of 10 μmol/kg both calcium antagonists produced an identical rightward shift and a marked depression of the maximal response to noradrenaline. Since there was no difference between mibefradil and verapamil in the
Experiments with externally applied noradrenaline, both at 3 and 10 μmol/kg, it can be assumed that the postjunctional effects of noradrenaline are influenced in a qualitatively and quantitatively identical manner by both calcium antagonists. This clearly points to the L-type being the predominant calcium channel in the mediation of the response to adrenoceptor stimulation at the postjunctional level.

The same behavior, a rightward shift without a depression of the maximal response, was seen with verapamil 3 μmol/kg in the electrically stimulated pithed rat which might indicate that verapamil, i.e. pure L-type calcium channel blockade, interacts merely on the postjunctional level and is therefore identical with the results obtained with externally applied noradrenaline.

Mibefradil at a dose of 3 μmol/kg, however, induced a significant depression of the maximal response to electrical spinal cord stimulation which indeed might point to a reduction of the pre junctional neurotransmitter release.

At the higher dose of 10 μmol/kg the effects of mibefradil and verapamil on the electrically-induced chronotropic response were undistinguishable and similar to the effects seen in the experiments with externally applied noradrenaline. This might indicate that at this concentration the L-type calcium channel blockade, which is a common feature of both drugs, is getting prominent and thereby overruling the effect on the neurotransmitter release. This notion is supported by the finding that at this concentration both calcium antagonists are equipotent in inhibiting the noradrenaline as well as the electrically provoked response.

N-type calcium channel blockade by calcium antagonists is not a new concept. An affinity for this particular type of calcium channel has been demonstrated for the 1,4-dihydropyridine amlodipine in a concentration range that can be assumed to be clinically relevant [10]. Indeed, amlodipine has been shown to influence noradrenaline release in essential hypertensive patients [11].

Additional N-type calcium channel blockade and thereby a reduction of the sympathetic activity, might explain at least some of the differences observed in the hemodynamic profile of various L-type calcium channel blockers. Since there is a strong L-type calcium channel independent component in the response of the cardiovascular system to adrenergic stimulation [12], a combined N- and L-type calcium channel blockade might be a still mainly unexplored therapeutic option.

It is concluded that mibefradil, beside its direct effect on cardiac T- and L-type calcium channels reduces the N-type calcium channel-dependent release of noradrenaline from sympathetic nerve endings. In the model used this effect is only observable at relatively low concentrations, most probably due to the direct cardiodepressant action of mibefradil provoked by cardiac L-type channel blockade.
References


