Abstract  Mibefradil is a tetralol derivative that inhibits cloned and native T-type voltage-operated calcium channels (VOCCs) at an IC50 of 1 µM and with an apparent 15-fold selectivity for T- over L-type VOCCs. Recent electrophysiological studies in Xenopus oocytes and inhibition of noradrenaline release studies in human isolated right atria concluded that mibefradil (0.3–3 µM) can block N-type VOCCs like ω-conotoxin GVIA. We tested this hypothesis in rat and guinea pig isolated driven left atria and rat vas deferens in response to sympathetic nerve stimulation. Mibefradil (3 µM) did not inhibit the inotropic responses to sympathetic electrical field stimulation in atria. In contrast, these responses were blocked by GVIA (10 nM) and tetrodotoxin (TTX 0.1 µM). In rat vas deferens, pretreated with benextramine (BNX 10 µM), contractions considered to be due to transmitter ATP evoked by electrical field stimulation were blocked by: (a) TTX 0.1 µM, (b) P2x receptor desensitisation (αβ-MeATP 100 µM), and (c) GVIA (pIC50 9.04±0.15, n=5). In contrast, mibefradil (0.3–30 µM) had no effect on these N-type VOCC-sensitive sympathetic nerve responses. Potassium (62 mM K+) and αβ-MeATP-induced contractions were unaffected by GVIA (10 nM), but mibefradil inhibited the potassium-induced contractions with a pIC50 6.02±0.08 (n=5), consistent with a T- and L-type VOCC blocking action on the postjunctional smooth muscle. We conclude that mibefradil up to 30 µM does not block N-type VOCCs in these isolated intact tissue assays of sympathetic nerve-muscle transmission. Therefore, at least in these tissues, mibefradil can be used to define T- and L-type VOCC activities at submicromolar and micromolar concentrations in sympathetic nerve-mediated responses.

Keywords  Mibefradil · ω-Conotoxin GVIA · N-type calcium channels · Rat vas deferens · Left atrium

Introduction

Mibefradil is a relatively selective T- over L-type voltage-operated calcium channel (VOCC) blocking drug. This 15-fold or so selectivity is exhibited by its potency (IC50) to inhibit the T-type calcium currents in cloned (α1G, 0.27 µM and α1H, 0.14 µM with 2.0 mM Ca2+ as charge carrier; Martin et al. 2000) and native T-type Ca2+ currents (rat vascular smooth muscle 0.2 µM; Mishra and Hermsmeyer 1994) at lower concentrations than required to inhibit L-type calcium currents (α1C from 1.7 µM to 21 µM, rat vascular smooth muscle 2.6 µM; Mehrke et al. 1994; Mishra and Hermsmeyer 1994; Bezprozvanny and Tsien 1995; Seisenberger et al. 1995; Welling et al. 1995; Martin et al. 2000). Some effects of mibefradil that may be due to T-type VOCC inhibition include: the selectivity for vascular relaxation over negative cardiac inotropism (Osterrieder and Holck 1989; Brixius et al. 1998; Sarsero et al. 1998), reduction in heart rate (Ertel et al. 1997), reduction in subendothelial cell proliferation (Schmitt et al. 1995, 1996), and protective benefits in hypertension and animals with heart failure (Bermink et al. 1996; Davies et al. 1997; Li and Schiffrin 1997; Massie et al. 1997; Oparil et al. 1997; Hermsmeyer 1998; Clozel et al. 1999; Sandmann et al. 2001). Although mibefradil has been withdrawn from therapy by the manufacturer because of interactions with other drugs (Griffin 1998; Krahenbuhl et al. 1998; Mullins et al. 1998; Spoendlin et al. 1998), the therapeutic benefits of mibefradil were considered to be mainly due to the blockade of T-type VOCCs.

Recently, a series of papers, using functional and electrophysiological approaches, have concluded that mibefradil may also block N-type VOCCs (Bezprozvanny and Tsien 1995; Göthert and Molderings 1997; Molderings et al. 2000; van der Lee et al. 2000). In addition, in some human and rat CNS and rat spinal motoneurone preparations, mibefradil may inhibit other VOCCs including N, P
or Q channels-mediated mediated calcium influx (Meder et al. 1997; Viana et al. 1997; Meder et al. 1999). N-type VOCCs, first defined by their electrophysiological properties, are sensitive to the highly selective N-type VOCC blocker ω-conotoxin GVIA (GVIA). If mibefradil displays N-type VOCC antagonism, then the mechanism for the clinical actions of mibefradil may be due to T-, N- and L-type VOCC blockade. Thus, our knowledge about the cardiovascular role of T- and L-type VOCCs as defined by mibefradil would need to be re-evaluated. In this work, we have explored the action of mibefradil in a series of functional, in vitro sympathetic nerve-muscle transmission assays that display N-type VOCC sensitivity to the now classical pharmacological tool, GVIA. We report here that mibefradil failed to show any inhibition in these assays. Therefore, our data do not support the proposition that mibefradil inhibits N-type VOCCs in our functional assays. Part of this work has been published in abstract form (Xi and Angus 2000).

Materials and methods

Tissue isolation

Sprague-Dawley rats (300–350 g) and guinea pigs (400–450 g) were killed with 80% CO2 in O2 and exsanguination. The left atrium was isolated from rat or guinea pig hearts and placed vertically on stainless steel S-shaped hooks attached to a Grass FT03C force transducer in an organ bath (20 ml) at 37°C in a physiological salt solution (PSS) of the following composition (in mM): NaCl 119, KCl 4.7, KH2PO4 1.18, MgSO4 1.17, NaHCO3 25, CaCl2 2.5 (for rat left atria with 1.25 mM CaCl2), ethylene-diamine-tetracetic acid (EDTA) 0.026, glucose 11 and saturated with 95% O2 and 5% CO2. Rat vasa deferentia were dissected with no EDTA 0.026, glucose 11 and saturated with 95% O2 and 5% CO2. Rat vasa deferentia were allowed to equilibrate for at least 30 min. Tissues were then contracted by a potassium depolarisation solution (KPSS, K+ 62 mM with Na+ 62 mM) replacing the normal PSS. After the tissues were washed thoroughly over 30 min, another KPSS exposure was made as the second control contraction. Drugs studied were GVIA (0.1–10 nM), mibefradil (0.1–10 µM) and nicardipine (0.1–1000 nM). Higher concentrations of the drugs were added every 30 min after the KPSS was washed out thoroughly.

αβ-MeATP contraction. Following the equilibration for at least 30 min, the tissues were contracted by KPSS replacing the normal PSS. After the tissues were washed thoroughly, GVIA (10 nM) or vehicle control (Mili Q water) was added into the organ baths and equilibrated for 30 min. The tissues were exposed to isoprenaline (10 nM). This protocol was repeated three times at an interval of 30 min where either GVIA (10 nM), mibefradil (3 µM), vehicle control (Mili Q water), or TTX (0.1 µM) was equilibrated between the second and the third stimulation.

Rat vasa deferentia

The upper end (epididymal) was attached to an isotonic force transducer (Grass FT03C; Quincy, Mass., USA) and the lower end (prostatic) threaded through two platinum electrodes (2 mm apart, 5 mm long) and tied to a fixed support. Output from the transducer amplifier was recorded on a flat-bed recorder (Linear recorder WR3300; Graphitec, Tokyo, Japan). Tissues were initially stretched to 2 g force.

Nerve stimulation. The rat tissues were equilibrated with benexamine (BNX, 10 µM) for 30 min to cause irreversible blockade of α1- and α2-adrenoceptors leaving ATP as the functional transmitter from nerve stimulation. The tissues were contracted to a nerve stimulation by applying electrical field stimulation of 0.05 Hz, 150 V, 0.5 ms duration (Grass S88 stimulator). Following these nerve stimulations and the contraction (R s A), a concentration of calcium channel antagonist (GVIA, mibefradil, or nicardipine) or vehicle (DMSO) was added to the bath to equilibrate for 30 min prior to the next field stimulation. Each drug concentration was added cumulatively and equilibrated for 30 min before the responses to field stimulation were reassessed. In order to pharmacologically calibrate the assay, tissue responses were recorded in the presence of TTX (0.1 µM), GVIA (10 nM), or αβ-MeATP (100 µM).

Potassium depolarisation. Rat vasa deferentia were allowed to equilibrate for at least 30 min. Tissues were then contracted by a potassium depolarisation solution (KPSS, K+ 62 mM with Na+ 62 mM) replacing the normal PSS. After the tissues were washed thoroughly over 30 min, another KPSS exposure was made as the second control contraction. Drugs studied were GVIA (0.1–10 nM), mibefradil (0.1–10 µM) and nicardipine (0.1–1000 nM). Higher concentrations of the drugs were added every 30 min after the KPSS was washed out thoroughly.

Drugs

Drugs used and suppliers were: atropine sulphate (Sigma, St. Louis, Mo., USA), αβ-MeATP (Sigma), benexatrine tetrachloride (BNX; Sigma), ω-conotoxin GVIA (GVIA; synthesised by J.P. Flinn and R. Murphy, Department of Pharmacology, University of Melbourne, Melbourne, Australia), desipramine HCl (DMI; Sigma), phentolamine HCl (Aldrich, Milwaukee, Wis., USA), (+)-isoprenaline bitartrate (Sigma), mibefradil (Roche, Australia), tetrodotoxin (TTX; Calbiochem, La Jolla, Calif., USA). Aliquots of αβ-MeATP (1.0 mM), GVIA (0.1 mM) and TTX (0.1 mM) in Mili Q water were lyophilised and stored at −20°C. A solution of phentolamine was prepared daily in Mili Q water. An aliquot was used for each experiment. The other chemicals were stored as stock solutions at 4°C.

For rat left atria, in addition to the field stimulation protocol, the tissues were exposed to isoprenaline (10 nM). This protocol was repeated three times at an interval of 30 min where either GVIA (10 nM), mibefradil (3 µM), vehicle control (Mili Q water), or TTX (0.1 µM) was equilibrated between the second and the third stimulation.

Rat and guinea pig left atria

A partially (0.5 g) stretched left atrium rested against two punctuate platinum electrodes protruding from the tissue holder 3 mm apart. These punctuate electrodes were used to drive the tissue at 1 Hz (0.05 ms duration with just supra-threshold voltage) using Grass SD9/S88 stimulators (Quincy, Mass., USA). The force signal was amplified by an amplifier Model 108 (Baker Medical Research Institute, Prahran, Australia). A pair of platinum wire electrodes were arranged along side and parallel to the atrium. When required, field stimulation of eight trains of three pulses per train (8×3/1, 200 Hz, 100 V, 2 ms duration and 1 ms delay following the punctuate twitch) were delivered across the tissue (Angus and Harvey 1981). Each three pulse train was delivered in one atrial refractory period to allow depolarisation of the autonomic varicosities and release of acetylcholine (ACh) and noradrenaline (NA) without affecting the atrial rhythm. To examine the sympathetic response, the atria were incubated with phentolamine (1 µM), desmethylimipramine (DMI 0.1 µM) and atropine (1 µM) in the PSS to block prejunctional α-adrenoceptors, neural uptake, and muscarinic receptors respectively but leaving postjunctional β-adrenoceptors intact.

In guinea pig left atria, two sets of control field stimulation (8×3/1) were applied before adding GVIA (0.1–10 nM), mibefradil (0.1–30 µM), or vehicle (Mili Q water). Each drug was added after two washes with fresh PSS and equilibrated for 30 min before the responses to field stimulation were reassessed. The effects of tetrodotoxin (TTX, 0.1 µM) were tested following the vehicle control.