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Milrinone and theophylline act as lower oesophageal sphincter relaxing agents: a comparative pharmacodynamic study in the rabbit

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This study demonstrates that the inotropic agent milrinone and the bronchodilator drug theophylline exert a relaxing effect on the rabbit lower oesophageal sphincter *in vitro*. The relaxing effect of milrinone and theophylline, which is concentration-dependent, involves a second messenger 3′,5′-cyclic adenosine monophosphate pathway and most probably it is accomplished through inhibition of phosphodiesterase (PDE) type III, as according to the obtained results it is not significantly modified either by nicotinic acid, an inhibitor of adenylate cyclase, or by the inhibitor of nitric oxide-synthetase N_{ω}-nitro-L-arginine methylester and the purinergic antagonist suramin; moreover, it persists under non-adrenergic non-cholinergic conditions and it is both hexamethonium- and tetrodotoxin-insensitive. Both milrinone and theophylline display equal efficacy, comparable to that of the calcium blocker verapamil and the non-selective PDE inhibitor papaverine, but milrinone appears 50 times more potent than theophylline and three times less potent than verapamil, as, according to the pIC_{50} values the potency rank of order is found to be verapamil (5.56) > milrinone (5.12) > theophylline (3.42). The here obtained pharmacodynamic profiles of the drugs suggest that both milrinone and theophylline may be considered as potent relaxing agents of the lower oesophageal sphincter.

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INTRODUCTION

Oesophageal or lower oesophageal sphincter (LOS) achalasia associated with peripheral neuropathies, myasthenia gravis, oesophagitis or chronic gastric dilation which may lead to megaoesophagus have been reported to occur in several animal species including dogs (Boria *et al.*, 2003), cats (Martínez *et al.*, 2001), pigs (Moses *et al.*, 2003) and horses (Broekman & Kuiper, 2002). On the other hand, it is known that several groups of drugs including anticholinergics, calcium channel blockers, nitrates, beta-adrenergic agonists and phosphodiesterases (PDEs) inhibitors, besides their main indications, are also recommended for the treatment of oesophageal dismotility disorders or for disorders characterized by hypertensive LOS (Boeckxstaens, 2005; Pohl & Tutuian, 2007).

Lower oesophageal sphincter relaxation is accompanied by an increase in 3′,5′-cyclic adenosine monophosphate (cAMP) and 3′,5′-cyclic guanosine monophosphate (cGMP), which are considered as the key second messengers regulating the tone. Increase in the intracellular levels of the two cyclic nucleotides results either from the activation of adenylate and/or guanylate cyclase, or following inhibition of the cyclic nucleotides degradation; this latter process is highly dependent on the PDEs isoenzymes. There is an evidence that among the 11 up to now detected different families of PDEs (Boswell-Smith *et al.*, 2006), types III, IV and V are present in the LOS (Barnette *et al.*, 1991; Osinki *et al.*, 1998), and that their inhibition decreases the LOS tone (Park *et al.*, 2003). Consequently, drugs possessing inhibitory activity at these types of PDEs are expected to be effective LOS relaxants. On the basis of the available clinical evidence, the non-selective PDE inhibitor theophylline, a methylxanthine used in the treatment of asthma in human (Barnes, 2005) and veterinary medicine (Foreman, 1999; Johnson, 2000), is among the PDEs inhibitors proven to effectively lower the LOS pressure in human. It is noteworthy that although the relaxing activity of theophylline on the LOS is considered as a side effect when the drug is administered for its bronchodilatory and/or anti-inflammatory effects, it proves to be advantageous in patients with oesophageal dismotility disorders (Boeckxstaens, 2005). However, information for the *in vitro* relaxing effect of theophylline on the LOS is limited to human and opossum tissues (Fox & Daniel, 1979; Tottrup *et al.*, 1990; Park *et al.*, 2003).

On the other hand, milrinone, a potent cardiac bipyridine with positive inotropic and vasodilator properties, referred also as selective cardiac or PDE III inhibitor, exhibits pronounced effects on both heart contractility and vasorelaxation, and it is recommended in the treatment of congestive heart failure in both human and animals (Muir, 1995; Uechi *et al.*, 2006). Apart from relaxing the arteriolar and venous vascular smooth muscle in several animal species (Tobata *et al.*, 2004; Wu *et al.*, 2005) milrinone has been shown to relax the guinea pig trachea (Wu *et al.*, 2004), the mouse jejunum (Sato *et al.*, 2006), the guinea pig taenia coli (Kaneda *et al.*, 2004), the guinea pig ileum (Kaneda *et al.*, 1997), the guinea pig gall bladder (Kaneda *et al.*, 2005) and the rat urinary bladder (Qiu *et al.*, 2001). However, information about the effect of milrinone on the LOS is missing.

The positive inotropic and vasodilatory effects of milrinone have been attributed to the selective inhibitory effect of the drug on PDE type III, leading to elevation of only cAMP. On the other hand, either only cAMP or both cyclic nucleotides are involved in theophylline's effects, following the non-selective inhibitory effect of the drug on PDEs (Wu *et al.*, 2004; Barnes, 2005). Adenosine antagonism has also been suggested as an additional mechanism for the effects of milrinone and theophylline, as both drugs are recognized as potent competitive inhibitors at adenosine receptors (Floreani *et al.*, 2003).

This study attempts to explore the use of milrinone and theophylline as LOS relaxing agents and compares their effects with the effect of two drugs with well-known pronounced smooth muscle relaxing properties, but different mechanism of action, the calcium entry blocker verapamil (Pohl & Tutuian, 2007) and the non-selective PDE inhibitor papaverine (Inatomi *et al.*, 1975). It also attempts to explore the underlying mechanism of their action.

MATERIALS AND METHODS

LOS preparations

In this study, 20 rabbits (New Zealand) of either sex weighing approx. 2.5 kg were used. The *in vitro* experiments were carried out with the permission of the local bureau of the Hellenic Ministry of Agriculture (Veterinary Service), conforming the Ethical Committee of the Aristotle University of Thessaloniki, and in accordance to EU directives (86/609/European Council) and state law (1197/81 and 2015/92).

Following the killing of the rabbits according to the experimental principles of laboratory animals, the chest and abdomen were opened and the oesophagus was transected and excised along with a cuff of gastric tissue out of the animals and transferred immediately into aerated with carbogen (95% O_2–5% CO_2) Krebs' solution (millimolar concentrations: NaCl 117, $NaHCO_3$ 25, NaH_2PO_4 1.2, KCl 4.7, $MgCl_2$ 1.2, $CaCl_2$ 2.5, glucose 11.1). The sphincter region was opened along the longitudinal axis and the LOS was excised by sharp cutting in a circular direction, making strips about 2 mm wide and 10 mm long. The mucosa was then removed by a sharp dissection. The smooth muscle strips were tied at both ends with silk sutures. Then the LOS preparations were suspended in 20 mL of organ baths (Hugo Sachs Electronic KG, Hugstetten, Germany), containing Krebs' solution, which was bubbled constantly with a mixture of 95% O_2–5% CO_2 gas and maintained at a temperature of 37 °C. The preparations were connected to isotonic myograph transducers (Narco Co., Houston, TX, USA) under a resting tension of 1 g and responses were recorded on a physiograph recorder (desk model type DMP-4A; Narco Co.). The preparations were allowed to equilibrate for 60 min (with intervening washings) before any compound addition.

The preparation of the strips, as well as the whole experimental procedure (equilibration period, resting tensions, aeration of the tissues), were based on *in vitro* methods previously described (Rattan *et al.*, 2002; Kohjitani *et al.*, 2005).

Reagents

The following compounds were used: milrinone, theophylline, verapamil, atropine, propranolol, phentolamine, hexamethonium (HEX), N_ω-nitro-L-arginine methylester (L-NAME), tetrodotoxin (TTX), nicotinic acid, suramin and papaverine (Sigma Chemical Co., St Louis, MO, USA). Milrinone and theophylline were freshly dissolved, before each experiment, in pre-warmed and pre-aerated bathing solution. These two substances were treated this way to get crystal-clear and steady solutions throughout the experiment without adding some other solvent except bathing solution. All other substances were freshly dissolved in distilled water. All solutions were gently added directly to the organ bath fluid by a micropipette.

Concentration–response curves

After the 60-min equilibration period the LOS preparations were exposed to cumulatively increasing concentrations of milrinone, theophylline or verapamil, respectively, (from 10^{-9} to 10^{-3} M) to obtain full concentration–response curves. The contact time of each concentration of the above substances with the preparations was dependent upon the time needed for the previous concentration to reach the maximal level, and after a series of preliminary experiments it was determined to be 5 min for verapamil and 10 min for milrinone and theophylline (data not shown).

The construction of the concentration–response curves to milrinone and to theophylline was repeated after pre-incubation of the preparations with either (i) the ganglion blocker HEX (10^{-4} M) for 15 min; (ii) the Na^+ blocker TTX (3×10^{-6} M) for 30 min; (iii) the inhibitor of nitric oxide (NO)-synthetase L-NAME (10^{-4} M) for 30 min; (iv) the adenylate cyclase-inhibitor nicotinic acid (10^{-4} M) for 30 min; (v) the purinergic receptor antagonist suramin (10^{-4} M) for 30 min; or (vi) under non-adrenergic non-cholinergic (NANC) conditions (phentolamine at 3×10^{-6} M plus propranolol at 3×10^{-6} M plus atropine at 3×10^{-6} M) for 15 min.

Maximal relaxation was obtained by application of a supramaximal dose (10^{-4} M) of the non-selective PDE inhibitor

papaverine, a drug related to morphine with strong smooth muscle relaxant properties (data not shown).

Statistical analysis of the results

The responses obtained were expressed as a percentage of the maximum response obtained by the control substance (papaverine, milrinone or theophylline). Statistical evaluation of the data was performed using SPSS 15.0 software (SPCC Inc., Chicago, IL, USA). Student's *t*-test for paired or unpaired data was used when appropriate. The data were expressed as mean ± SEM and P values of <0.05 were considered to be significant.

RESULTS

Effect of milrinone

Milrinone, at concentrations higher than 10^{-8} produced a sustained decrease in the basal tone of the rabbit LOS preparations; the pIC_{50} value (the negative logarithm of the concentration necessary to induce 50% of the maximal relaxation) was 5.12. The maximum relaxation was produced by milrinone at the concentration of 10^{-3} M (Fig. 1).

Effect of theophylline

Theophylline, at concentrations up to 10^{-5} M did not affect, while at concentrations higher than 3×10^{-5} M it produced a sustained decrease in the basal tone of the rabbit LOS preparations; the pIC_{50} value was 3.42. The maximum relaxation was produced by theophylline at the concentration of 10^{-3} M (Fig. 1).

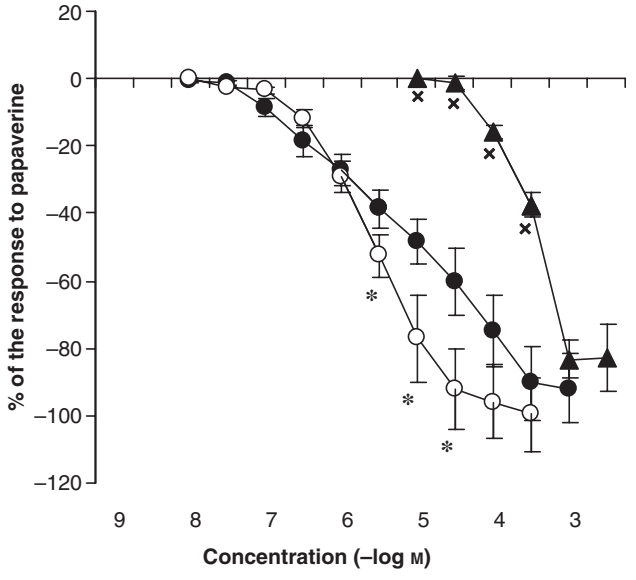

Fig. 1. Concentration–response curves of the milrinone- (●, $n = 15$), theophylline- (▲, $n = 14$) and verapamil- (○, $n = 6$) induced changes in the basal tone of the isolated rabbit lower oesophageal sphincter preparations. The ordinate is expressed as a percentage of the response of the preparations to papaverine at the concentration of 10–4 M. Each point represents mean ± SEM. * and × indicate the significant differences in response between milrinone and verapamil, or milrinone and theophylline respectively ($P < 0.05$–0.0001).

Effect of verapamil

Verapamil, at concentrations higher than 3.2×10^{-8} M produced a sustained decrease in the basal tone of the rabbit LOS preparations; the pIC_{50} value was 5.56. The maximum relaxation was produced by verapamil at the concentration of 3.2×10^{-4} M (Fig. 1).

Responses of the rabbit LOS preparations to either milrinone or theophylline after pretreatment with HEX, TTX, L-NAME, nicotinic acid, suramin or under NANC conditions

The relaxing effect of milrinone was not modified by pretreatment of the preparations with HEX, TTX, L-NAME, nicotinic acid or suramin, and it persisted under NANC conditions (Fig. 2).

Similar were the results regarding the effects of all above antagonists on the relaxing effect of theophylline (Fig. 2).

DISCUSSION

This study shows that milrinone, a cardiac bipyridine with positive inotropic and vasodilatory properties, as well as the bronchodilator methylxanthine theophylline, produce a concentration-dependent, NANC, HEX- and TTX-insensitive relaxation on the rabbit LOS. Milrinone displays higher potency than theophylline, but equal efficacy, which is comparable to that of the calcium channel blocker verapamil and the PDE inhibitor papaverine.

The relaxing effect of milrinone on the LOS is reported for the first time; interestingly, the here obtained milrinone molar concentrations that relax the LOS are found to be 10–100 times lower the ones reported to produce relaxation on tissues of the lower gastrointestinal tract (Kaneda *et al.*, 1997, 2004; Sato *et al.*, 2006), but comparable to those needed to produce vasodilatation (Tobata *et al.*, 2004; Wu *et al.*, 2005).

The relaxation produced by milrinone and theophylline remains under NANC conditions, thus excluding the involvement of either adrenergic or cholinergic pathways. According to existing knowledge, the smooth muscle relaxing effects of milrinone are ascribed to the increase of cellular cAMP (Qiu *et al.*, 2001; Kaneda *et al.*, 2005), while the ones of theophylline to increase of either cAMP or of both cAMP and cGMP (Wu *et al.*, 2004; Barnes, 2005). The elevated concentrations of the cyclic nucleotides initiate a cascade of events including inhibition of calcium influx and/or stimulation of calcium uptake into the intracellular stores, finally resulting in a decrease in intracellular calcium concentration, which in turn leads to smooth muscle relaxation (Nishimura, 2006).

It is generally accepted that milrinone and theophylline affect the intracellular levels of cyclic nucleotides by inhibiting the PDEs. Considering that in LOS the degradation of cAMP depends

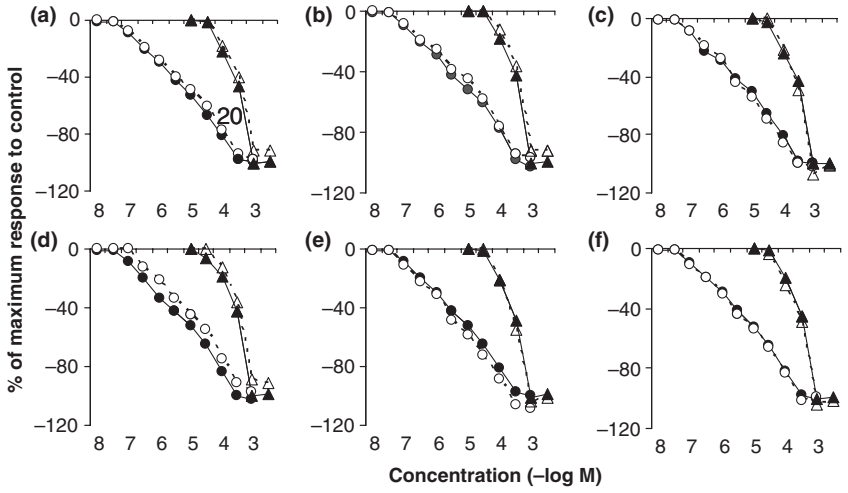

Fig. 2. Responses of the isolated rabbit lower oesophageal sphincter preparations to milrinone (●, ○) and to theophylline (▲, △), before (●, ▲ = control) and after (○, △) preincubation of the preparations with either (a) hexamethonium, (b) tetrodotoxin, (c) N_{ω}-nitro-L-arginine methylester, (d) nicotinic acid, (e) suramin, (f) or under non-adrenergic non-cholinergic conditions. The ordinate is expressed as a percentage of the maximum response of the lower oesophageal sphincter to milrinone alone. Each point represents the mean ± SEM obtained from five preparations.

mainly on the activity of PDEs III and IV, and the one of cGMP on PDE V (Park *et al.*, 2003), milrinone, as a specific PDE III inhibitor, is expected to increase only cellular cAMP. On the other hand theophylline, which has no selectivity for any particular isoenzyme (Barnes, 2005), is expected to increase the concentrations of cAMP via its inhibitory effect on PDEs III and IV, as well as of cGMP, by inhibiting PDE V. The intracellular accumulation of cGMP may also result via activation of guanylate cyclase by the inhibitory neurotransmitter NO, which besides its well-known relaxant effect in the vasculature (Berhane *et al.*, 2006; Giles, 2006), it plays a major role in oesophageal peristalsis and LOS relaxation (Hirsch *et al.*, 2000). However, according to the present results, the inhibitor of NO-synthetase L-NAME fails to affect either the milrinone- or the theophylline-induced relaxation; moreover, the relaxing effect of the drugs was found to be both HEX- and TTX- insensitive. These results imply for a post-synaptic, muscle-mediated effect, which does not involve the cGMP/NO pathway, and they highly suggest that the milrinone- and theophylline-induced LOS relaxations depend merely on cAMP. Moreover, taking into account that theophylline and the selective PDE III inhibitor milrinone exhibit equal efficacy, and that the decrease in the LOS tone is known to depend more on the inhibition of PDEs III and V, and less on PDE IV (Park *et al.*, 2003), we could suggest that the theophylline-induced relaxation in the rabbit LOS results mainly from its inhibitory effect on PDE III.

On the other hand, results of this study rule out the possibility that an antagonistic effect of milrinone and theophylline at inhibitory adenosine receptors mediating inhibition of adenylate cyclase activity may account for the increase in cellular cAMP levels and lead to LOS relaxation, as nicotinic acid, an inhibitor of adenylate cyclase, failed to modify the concentration–response curve of either milrinone or theophylline. Similarly, despite the existing evidence for the inhibitory activity of both milrinone (Zhang & Colman, 2007) and theophylline (Inbe *et al.*, 2004) on purinergic P2Y receptors, the involvement of such receptors is also excluded, as the purinergic antagonist suramin was without effect on the drugs-induced relaxations.

The above data suggest that the relaxing effect of milrinone and theophylline on LOS is mediated merely via the inhibitory effect of the drugs on PDEs. This is further supported by the fact that the maximum responses of the LOS to milrinone and theophylline do not significantly differ from the response of the tissue to a supramaximal dose of the non-selective PDE inhibitor papaverine, a morphine-related drug with strong smooth muscle relaxant properties (Inatomi *et al.*, 1975). This observation is also indicative of the high efficacy of the drugs. In addition, results of the present study show that the maximum effects of theophylline and milrinone are also comparable to that of verapamil, a drug known to produce considerable relaxation in smooth muscle tissues, including LOS (Pohl & Tutuian, 2007), via a cAMP-independent mechanism, i.e. by direct inhibition of calcium entry through slow-calcium channels. Taking into account that LOS tone depends mainly on extracellular calcium (Fox & Daniel, 1979; Biancani *et al.*, 1987; Salapatek *et al.*, 1998), the above observation, apart from highlighting the high efficacy of the drugs, also implies that the relaxation produced by milrinone and theophylline, which act as cAMP-elevating agents, relies to a great extent on inhibition of calcium influx.

In conclusion, results of this study show that the inotropic drug milrinone and the bronchodilator theophylline produce a considerable relaxation on the isolated rabbit LOS. Considering that milrinone is found here to be 50 times more potent than theophylline, we could expect milrinone to be *in vivo* more potent than theophylline. Moreover, the fact that the milrinone molar concentrations which relax the LOS are comparable to those producing vasodilatation allows us to hypothesize that when milrinone is administered for its inotropic and/or vasodilator properties, it may also produce a more or less pronounced LOS relaxation. Clarification of the exact biological potency of milrinone and theophylline as LOS relaxant agents should await results of their *in vivo* examination; however, data of this study give evidence that in animal species where PDE III plays a major role in the regulation of the LOS tone, milrinone and theophylline may prove to be effective LOS relaxing drugs and may be recommended in disorders characterized by hypertensive LOS.

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