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Effects of commonly used protein kinase inhibitors on vascular contraction and L-type Ca^{2+} current

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ABSTRACT

Regulation of smooth muscle contraction is driven by a number of protein kinases: the evidence for this often originates from studies that investigate the effects of extracellularly added specific protein kinase inhibitors. Six compounds, thought to be selective inhibitors of various kinases, were analysed for their effects on vascular L-type Ca^{2+} channels because this potential subsidiary activity could strongly influence our understanding of the pathways involved in smooth muscle contraction. Whole-cell L-type Ba^{2+} currents [$I_{Ba(L)}$] were recorded in single myocytes, and contractile responses were measured from endothelium-denuded rings taken from the rat tail artery. Although ML-7, ML-9, and wortmannin (MLCK inhibitors), HA-1077 and Y-27632 (Rho-associated kinase inhibitors), and GF-109203X (PKC inhibitor) relaxed rings pre-contracted with high KCl in a concentration-dependent manner, their effect on $I_{Ba(L)}$ intensity was surprisingly variable. Wortmannin showed negligible effects while HA-1077 and Y-27632 were ineffective. $I_{Ba(L)}$ was partly inhibited by GF-109203X and blocked by ML-7 and ML-9 in a concentration-dependent manner, with the blockade by ML-7 being voltage-dependent. Whilst ML-7, ML-9, and GF-109203X sped up the inactivation kinetics of $I_{Ba(L)}$, GF-109203X did not modify ML-7- or ML-9-induced effects, with both intensity and kinetics of the current remaining unchanged. In contrast, application of Bay K 8644 on myocytes pre-treated with ML-7 or ML-9 raised $I_{Ba(L)}$ beyond control values. In conclusion, ML-7 and ML-9 inhibit L-type Ca^{2+} channels via a mechanism independent of MLCK, PKC or Rho kinase activities, and as such caution should be used in employing these agents to elucidate the role of kinases in smooth muscle contraction.

1. Introduction

Vascular smooth muscle contraction is a highly specialized and complex phenomenon regulated by a variety of contractile proteins, ion channels, and signalling molecules. Understanding the precise subcellular mechanisms controlling contraction is relevant in gaining a deeper insight not only into vascular disorders based on hypercontractility, but also into how vessels respond to contractile, relaxant, and therapeutic agents. A rise of the concentration of cytosolic free Ca^{2+} is the major trigger for the contraction of smooth muscle [1]. Elevation of intracellular Ca^{2+} leads to calmodulin-dependent activation of myosin light-chain kinase (MLCK), and hence phosphorylation of the 20 kDa light chains of myosin II at Ser^{19} [2]. This simple phosphorylation reaction triggers the cycling of myosin cross-bridges along the actin filaments, with force development leading to shortening of the muscle. Under tonic force maintenance, the relationship between force and the extent of myosin light chain-phosphorylation can be tight, loose or even dissociated, as defined by the so-called Ca^{2+}-sensitization theory [3]. Dephosphorylation of the myosin light chain which is catalysed by myosin light-chain phosphatase is normally selectively down-regulated in this process [4]. This down-regulation is thought to be mediated through inhibition of the myosin light-chain phosphatase to which several different mechanisms have been proposed, involving: (i) Rho-associated kinase phosphorylation of myosin light-chain

Abbreviations: Bay K 8644, (S)-(−)-methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine-5-carboxylate; GF-109203X, 3-(N-[dimethylamino]propyl-3-indolyl)-4-(3-indolyl)maleimide; HA-1077, hexahydro-1-(5-isoquinolinylsulfonyl)-1H-1,4-diazepine; $I_{Ba(L)}$, L-type Ba^{2+} current; ML-7, 1-(5-iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine; ML-9, 1-(5-chloronaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine; MLCK, myosin light chain kinase; PKC, protein kinase C; V_h, holding potential; Y-27632, (R)-(+)-*trans*-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide.

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phosphatase-targeting subunit; (ii) protein kinase C (PKC) phosphorylation of the endogenous myosin light-chain phosphatase inhibitor CPI-17; and (iii) arachidonic acid. Both Rho and Rho kinase activation occur as a consequence of either agonist binding or high K^+ depolarization [5,6].

To fully understand the organization and function of the smooth muscle contractile machinery, specific and effective antagonists are needed to tease out the critical steps along the transduction pathway. The established opinion that protein kinases play a key role in smooth muscle contraction is based on studies using small, cell-permeable agents to effectively and specifically inhibit kinase activity and observing the concomitant inhibition of muscle contraction. Recently, however, some concern has arisen on the use of various protein kinase inhibitors in this role, as they have been shown to display physiological actions other than those on Ca^{2+} metabolism, which are particularly evident when employed at high concentrations. For instance, the naphthalene sulphonamide derivative ML-9 [1-(5-chloronaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine], and its analogue ML-7 [1-(5-iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine] when applied at high concentrations (30–100 μM) have been reported to not only reduce intracellular Ca^{2+} concentration, but also reduce K^+-induced contraction concomitantly, resulting in guinea pig tracheal smooth muscle [7], coronary artery [8], and pulmonary artery relaxation [9]. It has been hypothesized that fall of intracellular Ca^{2+} level may also be somewhat dependent on the influx of extracellular Ca^{2+}, and that these compounds restrict extracellular Ca^{2+} from entering the cell [7,9]. If this hypothesis is correct, the interpretation that such smooth muscle contraction is attributed solely to kinase inhibition could be misleading and may require re-evaluation.

Many pathways involved in vascular smooth muscle contraction are Ca^{2+}-controlled and mostly involve Ca^{2+} influx from the extracellular space through L-type Ca^{2+} channels. These channels regulate excitation-contraction coupling in vascular smooth muscles, which is the primary process for voltage-gated Ca^{2+} influx. Subsidiary effects of kinase inhibitors on Ca^{2+} currents in vascular tissues could strongly revise our understanding of the transduction pathways involved in smooth muscle contraction and Ca^{2+}-sensitization phenomena. To clarify this point, data is presented on in vitro studies using artery rings and single myocytes derived from the rat tail main artery aimed at identifying whether the specific kinase inhibitors, used to investigate the role of kinases in muscle contraction, interact with L-type Ca^{2+} channels. The effects on L-type Ba^{2+} currents by ML-7, ML-9, wortmannin (MLCK inhibitors; [8,10]), GF-109203X (3-(N-[dimethylamino]propyl-3-indolyl)-4-(3-indolyl)maleimide, a PKC inhibitor; [11]), HA-1077 [hexahydro-1-(5-isoquinolinylsulfonyl)-1H-1,4-diazepine], and Y-27632 [(R)-(+)-*trans*-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide, Rho kinase inhibitors; [5]; Fig. 1], classified as highly selective kinase inhibitors, were examined. This is the first recorded study detailing the effects that these compounds have on the electrophysiological properties of vascular smooth muscle L-type Ca^{2+} channels. It was found that ML-7 and ML-9 block the L-type Ca^{2+} channel and this effect was independent of the inhibition of MLCK, PKC or Rho kinase activities. As such, caution should be used in utilizing these tools to elucidate the transduction pathway that leads to smooth muscle contraction.

2. Material and methods

2.1. Animals

All animal care and experimental procedures complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996), and were approved by the Animal Care and Ethics Committee of the Università degli Studi di Siena, Italy (08-02-2012) and Università degli Studi di Padova, Italy (14-04-2010). Male Sprague-Dawley rats (300–400 g, Charles River Italia, Calco, Italy) were anaesthetized (i.p.) with a mixture of Ketavet® (30 mg kg^{-1} ketamine; Intervet, Aprilia, Italy) and Xilor® (8 mg kg^{-1} xylazine; Bio 98, San Lazzaro, Italy), decapitated and exsanguinated. The tail was removed immediately, cleaned of skin and placed in physiological salt solution (containing, in mM: 125 NaCl, 5 KCl, 2.7 $CaCl_2$, 1 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, and 11 glucose; pH 7.35) or external solution (containing, in mM: 130 NaCl, 5.6 KCl, 10 HEPES, 20 glucose, 1.2 $MgCl_2 \cdot 6\ H_2O$, and 5 Na-pyruvate; pH 7.4). The tail main artery was dissected free of its connective tissue.

2.2. Contraction experiments

Drugs were tested on rat tail artery rings to assess their possible vasodilating effect. The endothelial layer was removed by inserting and gently rolling a stainless steel wire on the intimal surface of the vessel. Two mm wide rings were mounted, under a preload of 1.5 g using 40 μm tungsten wires inserted inside the

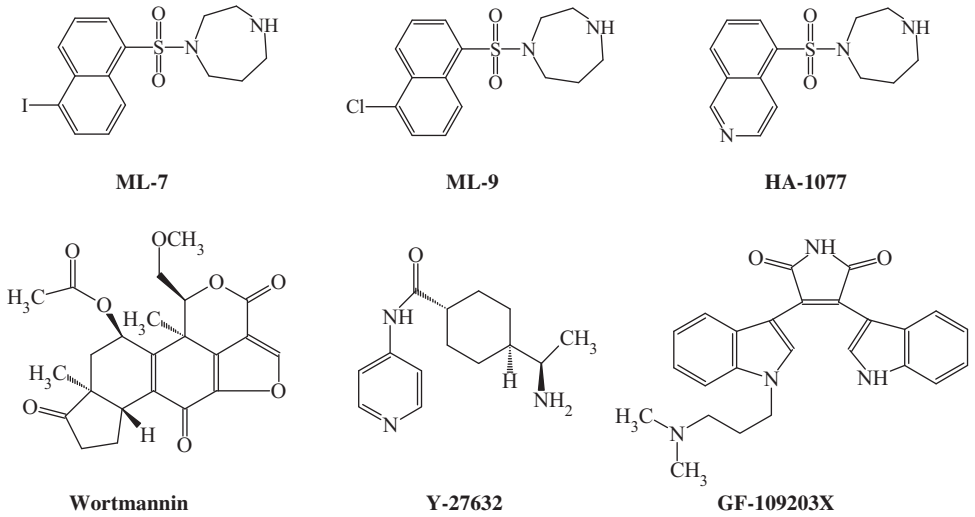

Fig. 1. Chemical structures of the compounds investigated.