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ORIGINAL ARTICLE

Vascular activity of two silicon compounds, ALIS 409 and ALIS 421, novel multidrug-resistance reverting agents in cancer cells

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Abstract
Purpose The aim of this study was to investigate the effects of two novel multidrug-resistance reverting agents, ALIS 409 [1,3-dimethyl-1,3-*p*-fluorophenyl-1,3(3-morfolinopropyl)-1,3-disiloxan dihydrochloride] and ALIS 421 {1,3-dimethyl-1,3-(4-fluorophenyl)-1,3[3(4-buthyl)-(1-piperazinyl)-propyl]-1,3-disiloxan tetrahydrochloride}, on vascular functions in vitro.
Experimental design A comparison of their mechanical and electrophysiological actions in rat aorta rings and single rat tail artery myocytes, respectively, was performed.
Results In endothelium-denuded rat aorta rings, ALIS 409 and ALIS 421 antagonized 60 mM K^+-induced contraction in a concentration-dependent manner with IC_{50} values of 52.2 and 15.5 μM, respectively. ALIS 409 and ALIS 421 inhibited L-type Ca^{2+} current recorded in artery myocytes in a concentration-dependent manner with IC_{50} values of 6.4 and 5.6 μM, respectively. In rat aorta, ALIS 409 and ALIS 421 antagonized the sustained tonic contraction induced by phenylephrine with IC_{50} values of 58.0 and 13.7 μM (endothelium-denuded rings) and of 73.9 and 31.9 μM (endothelium-intact rings), respectively. In endothelium-denuded rings, ryanodine reduced significantly the response to phenylephrine in the absence of extracellular Ca^{2+} whereas nifedipine, ALIS 409 or ALIS 421 did not affect it. Phenylephrine-stimulated influx of extracellular Ca^{2+} was markedly reduced when tissues were pretreated with ALIS 409, ALIS 421 or nifedipine, and stimulated when they were pretreated with ryanodine. Application of ALIS 409 (up to 100 μM) to intact rat aorta rings failed to induce mechanical responses.
Conclusions Our results provide functional evidence that the myorelaxing effect elicited either by ALIS 409 or by ALIS 421 involved mainly the direct blockade of extracellular Ca^{2+} influx. This effect, however, took place at concentrations much higher than those effective as modifiers of multidrug resistance in cancer cells.

Keywords ALIS 409 · ALIS 421 · Rat aorta · Rat tail artery myocytes · Ca^{2+} influx · Whole-cell patch-clamp

Abbreviations

$I_{Ca(L)}$	L-type Ca^{2+} current
K60	60 mM K^+ PSS
MDR	Multidrug-resistance
PSS	Physiological salt solution
ROCCs/SOCCs	Receptor/store-operated Ca^{2+} channels
V_h	Holding potential
VOCCs	Voltage-operated Ca^{2+} channels

Introduction

The effectiveness of chemotherapy is often impaired by the development of either intrinsic or acquired (during therapy) resistance of cancer cells to cytotoxic drugs. The failure of

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chemotherapy can be due to several different mechanisms, such as increased drug efflux, inactivation of the drugs, alterations in drug target(s), avoidance of apoptosis, etc. [18]. One of the major (but not the only one) causes of failure is the over-expression of several ATP-dependent efflux pumps or transporters, known as the ATP-binding cassette (ABC) family, that confers a multidrug resistance (MDR) phenotype on cells in various diseases, including many forms of cancer [10]. The best known and most classical example in man of these efflux pumps is that of the plasma membrane glycoprotein, called *P*-glycoprotein (P-gp) and encoded by the multidrug resistance MDR1 gene [21]. Endeavouring to find MDR-reverters is a crucial task for anti-cancer therapeutic interventions. In the past few years, extensive studies have been performed with the aim of developing effective chemosensitizers to overcome MDR of human cancer cells. The process of chemosensitization involves co-administration of a MDR modulator along with an anticancer drug in order to enhance intracellular anticancer drug accumulation by impairing P-gp function [9]. The function of drug pumps can be effectively inhibited by substrate analogues, inhibitors of ATP binding or utilization, specific monoclonal antibodies and by various agents displaying yet unknown mechanisms of action. In the search for novel resistance modifiers, we have synthesised various groups of compounds (from phenothiazines to thioxanthenes and selenoxanthenes with diverse substituents, including fluorine and silicium derivatives) to reverse different bacterial and tumour resistance types. Among them, ALIS 409 [1,3-dimethyl-1,3-*p*-fluorophenyl-1,3(3-morfolinopropyl)-1,3-disiloxan dihydrochloride] and ALIS 421 {1,3-dimethyl-1,3-(4-fluorophenyl)-1,3[3(4-buthyl)-(1piperazinyl)-propyl]-1,3-disiloxan tetrahydrochloride} (Fig. 1), two water-soluble organosilicon compounds, have been shown to be effective in vitro MDR reverting agents [20]. The ALIS compounds inhibited specifically P-gp activity without affecting MDR1 gene expression. In particular, ALIS 409, in the concentration range 0.48–1.28 μM caused a 100% inhibition of the efflux pump activity in MDR1-transfected mouse lymphoma and colo-320 cell lines. Moreover, it enhanced the antiproliferative action of daunorubicin and displayed a synergistic interaction with epirubicin.

More recently, the multidrug resistance reversal effect of ALIS 409 was demonstrated in vivo, in human pancreatic cancer xenografts, without appreciable toxicity [29]. In addition, ALIS 409 increased the apoptotic activity and delayed tumour growth, without affecting the mitotic rate in inbred, artificially immunosuppressed CBA mice, bearing subcutaneously-growing PZX-40/19G human pancreatic cancer xenografts.

Down-regulation of the caspase cascade is another possible MDR mechanism. Earlier studies have reported that resistance to apoptosis is correlated with reduced caspase-3 activity in some tumor cell lines [5]. Interestingly, both ALIS 409 and ALIS 421 are capable of activating caspases, as recently shown by Kars et al. [16]. In addition, these authors demonstrated that both organosilicon compounds are effective P-gp modulators in novel models of drug resistance using MCF-7 cell lines and also show synergism with the chemotherapeutic effects of paclitaxel and docetaxel, drugs of the taxoid group used in the treatment of breast cancer. Taken together, these observations make the two disiloxanes, and in particular ALIS 409, good candidates for advanced experimental as well as clinical investigations, since they may represent useful adjuvant drugs in cancer therapy owing to their apparently mild side effects.

Many MDR reverters—in particular those belonging to the first generation—have been shown to possess inherent pharmacological activity. For instance, the doses of verapamil required to overcome MDR were found to be associated

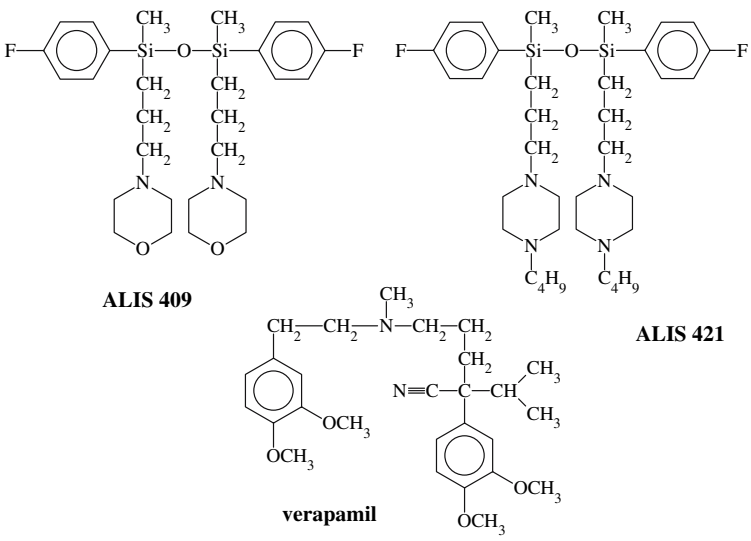

Fig. 1 Chemical structures of the silicon compounds ALIS 409 and ALIS 421, and of the phenylalkylamine verapamil

with severe and sometimes life-threatening cardiovascular toxicity such as atria-ventricular block and hypotension, thus rendering its clinical application impossible. Furthermore, some MDR modifiers possess a dihydropyridine-like molecular structure [26] that makes them potentially effective Ca^{2+} channel blockers. Therefore, it is desirable to have compounds that can be used at much lower doses than those shown to be toxic or are devoid of major pharmacodynamic "side"-effects at the doses effective for reverting MDR. As a consequence, in the last few years, much attention has been focused on the development of safer MDR inhibitors characterized by proper selectivity. This task should regard any novel drug designed to be administered with the traditional antineoplastic agents. When inspecting the structure of both ALIS 409 and ALIS 421 (see Fig. 1) some analogies with phenylalkylamines—verapamil-like derivatives—are evident, though the former compounds exhibit two chiral Si atoms while verapamil has only one chiral C atom. Aim of this study, therefore, was to investigate the effects of these two silicon compounds on large blood vessels function in vitro, by comparing their mechanical and electrophysiological actions. ALIS 409 turned out to be the least effective vasorelaxant of the two, thus representing a lead compound for the development of potent MDR chemosensitizers characterised by negligible vascular effects.

Materials and methods

Aorta rings preparation and equilibration period

The investigation conforms 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). Aorta rings (1.5-mm wide), either endothelium-intact or -denuded, were prepared from male Sprague-Dawley rats (358.8 ± 18.1 g, $n = 17$, Charles River Italia, Calco, Italy) anaesthetised with a mixture of Ketavet® (0.3 mg kg^{-1}; Gellini, Italy) and Rompum® (0.08 mg kg^{-1}; Bayer, Germany), decapitated and bled, as described elsewhere [7].

The rings were allowed to equilibrate for 1 h at a resting tension of 1 g. During this period, a modified Krebs-Henseleit physiological salt solution (PSS) (see below for composition) was changed every 15 min and the passive tension was re-adjusted to 1 g. Following equilibration, in order to test their contractile activity, rings were stimulated with 60 mM K^+ PSS (K60) until a sustained response was obtained (~15 min). Upon conclusion of a 45-min wash out period, the presence or absence of functional endothelium was assessed with 10 μM ACh in rings precontracted with 0.3 μM phenylephrine. The latter evocked maximal plateau values for active tension of 483.3 ± 29.0 mg ($n = 62$). Rings were then washed and equilibrated for another 45 to 60-min period before testing the various experimental settings (see below). Control preparations were treated with drug vehicle only.

Effect of ALIS 409 and ALIS 421 on aorta rings contracted with either K60 or phenylephrine

Steady tension was evoked either by K60 (in endothelium-denuded rings) or by 0.3 μM phenylephrine (in endothelium-intact or in endothelium-denuded rings) and then drugs under investigation were added cumulatively (0.01–100 μM). Relaxations were evaluated as a percentage of the initial response to either K60 or phenylephrine.

Effects of ALIS 409 and ALIS 421 on Ca^{2+} release from intracellular stores and Ca^{2+} influx triggered by phenylephrine

In order to investigate whether ALIS 409 and ALIS 421 could interfere with Ca^{2+} release from intracellular stores, PSS was replaced with a Ca^{2+}-free solution containing 1 mM EGTA. Endothelium-denuded rings were exposed to this solution for 5 min [4] and then stimulated with 10 μM phenylephrine (this contraction was used to identify the portion of phenylephrine-induced response that depends on intracellularly stored Ca^{2+}). External Ca^{2+} was then restored in the presence of phenylephrine and the ensuing contraction was taken as an index of the influx of Ca^{2+} from the extracellular space triggered, in part, by the emptied stores, and, in part, by α-receptor stimulation. The contractions were obtained under control conditions as well as after a 30-min incubation period with vehicle alone (DMSO or ethanol), ALIS 409 or ALIS 421 (100 μM), ryanodine (30 μM) or nifedipine (0.3 μM).

Effect of ALIS 409 on spontaneous tone of rat aorta rings

The cumulative concentration-response relationship for ALIS 409 (3–100 μM), the less effective vasorelaxant silicon compound, was determined in endothelium-intact tissues bathed with PSS.

Tail artery cells isolation procedure

Smooth muscle cells were freshly isolated from the tail main artery incubated at 37°C in 2 ml of 0.1 mM Ca^{2+} external solution (see below for composition) containing 1 mg/ml collagenase (type XI), 1 mg/ml soybean trypsin inhibitor and 1 mg/ml BSA, gently bubbled with a 95% O_2–5% CO_2 gas mixture, as previously described [8]. Cells exhibited an ellipsoid form (10–15 μm in width, 35–55 μm in length) and were continuously superfused with external

solution containing 0.1 mM Ca^{2+} using a peristaltic pump (LKB 2132, Bromma, Sweden), at a flow rate of 500 µl min^{-1}. Electrophysiological responses were tested at room temperature (22–24°C) only in those cells that were phase dense. Cell membrane capacitance and access resistance averaged 35.5 ± 2.4 pF and 8.58 ± 1.22 MΩ (n = 17).

Whole-cell patch clamp recording

The conventional whole-cell patch-clamp method [11] was employed to voltage-clamp smooth muscle cells. Recording electrodes were pulled from borosilicate glass capillaries (WPI, Berlin, Germany) and fire-polished to obtain a pipette resistance of 2–5 MΩ when filled with internal solution. A low-noise, high-performance Axopatch 200B (Axon Instruments, USA) patch-clamp amplifier driven by an IBM computer in conjunction with an A/D, D/A board (DigiData 1200 A/B series interface, Axon Instruments, USA) was used to generate and apply voltage pulses to the clamped cells and record the corresponding membrane currents. Current signals, after compensation for whole-cell capacitance, series resistance and liquid junction potential, were low-pass filtered at 1 kHz and digitized at 3 kHz prior to being stored on the computer hard disk. The liquid junction potential between the bath and pipette solutions was compensated electronically.

Cells used in this study expressed only L-type Ca^{2+} channels (see [22]). L-type Ca^{2+} current [$I_{Ca(L)}$] was always recorded in 5 mM Ca^{2+}-containing external solution.

$I_{Ca(L)}$ was elicited with 250-ms clamp pulses to 10 mV from a holding potential (V_h) of −90 mV. Data were collected once the current amplitude had been stabilized (usually 7–10 min after the whole-cell configuration had been obtained; maximal $I_{Ca(L)}$ values of 58.8 ± 6.0 pA, n = 17). $I_{Ca(L)}$ did not run down during the following 30–40 min under these conditions ([22]; see Fig. 3a).

K^+ currents were blocked with 30 mM tetraethylammonium in the external solution and Cs^+ in the internal solution (see below).

Current values were corrected for leakage using 300 µM Cd^{2+}, which was proved to block completely $I_{Ca(L)}$.

Solutions and chemicals

Solutions used for functional experiments

PSS contained (in mM): 118 NaCl, 4.75 KCl, 2.5 $CaCl_2$, 1.19 $MgSO_4 \cdot 7H_2O$, 1.19 KH_2PO_4, 25 $NaHCO_3$ and 11.5 glucose, bubbled with a 95% O_2–5% CO_2 gas mixture to create a pH of 7.4. PSS containing KCl at a concentration of more than 4.75 mM was prepared by replacing NaCl with equimolar KCl.

Solutions used for electrophysiological experiments

The Ca^{2+}-free external solution contained (in mM): 130 NaCl, 5.6 KCl, 10 HEPES, 20 glucose, 1.2 $MgCl_2$ and 5 Na-pyruvate (pH 7.4).

The internal solution (pCa 8.4) consisted of (in mM): 105 CsCl, 10 HEPES, 11 EGTA, 2 $MgCl_2$, 1 $CaCl_2$, 5 Na-pyruvate, 5 succinic acid, 5 oxalacetic acid, 3 Na_2-ATP and 5 phosphocreatine; pH was adjusted to 7.4 with CsOH.

The osmolarity of external solution (320 mosmol) and that of the internal solution (290 mosmol) [27] was checked with an osmometer (Osmostat OM 6020, Menarini Diagnostics, Italy).

ALIS 409 and ALIS 421 were originally synthesized as compounds to reverse multidrug resistance in cancer cells, as described in the European Patent No. 1432717B1 [12].

The chemicals used included: collagenase (type XI), trypsin inhibitor, BSA, tetraethylammonium chloride, phenylephrine, and nifedipine (Sigma Chimica, Milan, Italy). Calbiochem (La Jolla, CA, USA) supplied ryanodine. ALIS 409 and ALIS 421 were dissolved in water. Ryanodine, dissolved directly in DMSO and nifedipine, dissolved in ethanol, were diluted at least 1,000 times prior to use. The resulting concentrations of DMSO and ethanol (below 0.1%, *v/v*) failed to alter the response of the preparations (data not shown). Final drug concentrations are stated in the text.

Curve fitting and statistics

Acquisition and analysis of data were accomplished by using GraphPad Prism version 4.03 (GraphPad Software, USA). Data are reported as mean ± SEM.; n is the number of rings or cells analysed (indicated in parentheses), isolated from at least three animals. Statistical analyses and significance as measured by either ANOVA (followed by Dunnett's post test) or Student's t test for either paired or unpaired samples (two-tail) were obtained using GraphPad InStat version 3.06 (GraphPad Software, USA). In all comparisons, $P < 0.05$ was considered significant.

The pharmacological response to each substance was described in terms of IC_{50}.

Results

Effect of ALIS 409 and ALIS 421 on aortic rings precontracted with K60

The compounds were assayed on rat aorta rings, precontracted with K60. As shown in Fig. 2, ALIS 409 and ALIS 421 inhibited, in a concentration-dependent manner, the sustained tonic contraction induced by K60 with IC_{50}

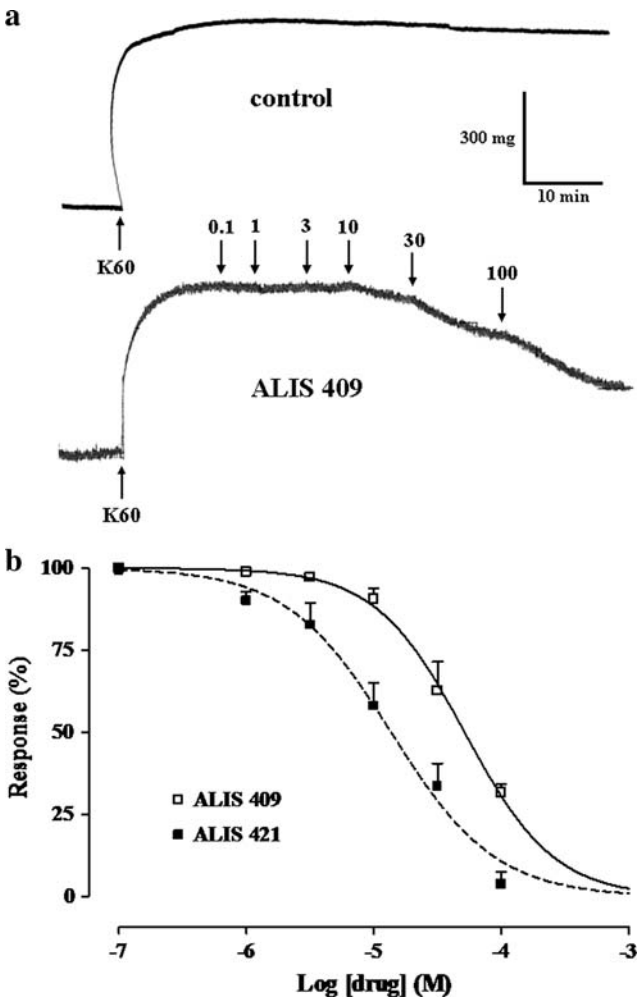

Fig. 2 Effect of ALIS 409 and ALIS 421 on endothelium-denuded rat aorta rings depolarised with K60. **a** Trace (representative of five experiments) of relaxation developed in response to cumulative concentrations (μM) of ALIS 409 added on the plateau of 60 mM K^+-elicited contraction in endothelium-denuded rings. The time-matched control is also reported for a comparison. The reason for the backward in time curving of the upper recording is due to the kymograph-like characteristics of the Basile pen recorder (Basile, Varese, Italy). **b** Concentration-response curves for ALIS 409 and ALIS 421. In the ordinate scale, relaxation is reported as a percentage of the initial tension induced by K60. Data points are mean ± SEM (n = 4–5)

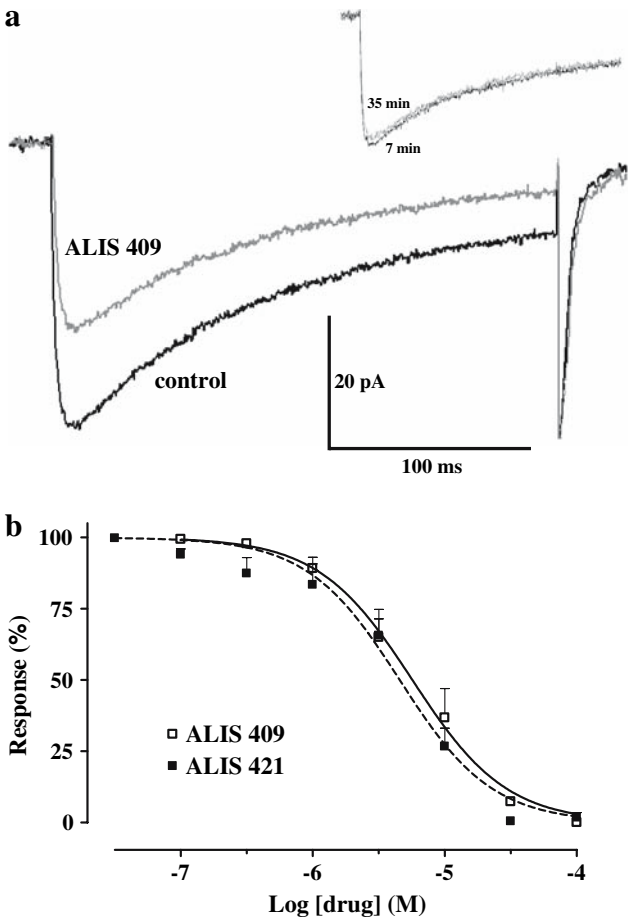

Fig. 3 ALIS 409 and ALIS 421 inhibition of $I_{Ca(L)}$ in rat tail artery myocytes. **a** Average traces (recorded from five cells) of conventional whole-cell $I_{Ca(L)}$ elicited in rat tail artery myocytes with 250-ms clamp pulses to 10 mV from a V_h of −90 mV, measured in the absence (control, *black*) or presence of 3 μM ALIS 409 (*gray*). Inset: control traces recorded in the same cell 7 min (*black*) and 35 min after going whole-cell (*gray*), respectively. **b** Concentration-effect relationship for ALIS 409 and ALIS 421. On the ordinate scale, response is reported as percent of control. Data points are mean ± SEM (n = 3–6)

values of 52.2 ± 6.6 μM (n = 5) and 15.5 ± 3.6 μM (n = 4; $P < 0.01$, Student's t test for unpaired samples), respectively.

Effect of ALIS 409 and ALIS 421 on $I_{Ca(L)}$

Figure 3a shows average recordings of $I_{Ca(L)}$ elicited with a clamp pulse to 10 mV from V_h of −90 mV under control conditions and after the addition of 3 μM ALIS 409. Both ALIS 409 and ALIS 421 inhibited $I_{Ca(L)}$ in a concentration-dependent manner (Fig. 3b) with IC_{50} values of 6.4 ± 2.0 μM (n = 5) and 5.6 ± 1.7 μM (n = 6), respectively.

The current evoked at 10 mV from a V_h of −90 mV activated and then declined with a time course that could be fitted by a mono-exponential equation (2.31 ± 0.15 ms and 95.33 ± 4.27 ms, respectively; n = 10). At the highest concentrations allowing for a reliable measurement of current kinetics (i.e. 3 μM), neither ALIS 409 (2.65 ± 0.29 and 97.52 ± 12.96 ms, respectively; n = 5) nor ALIS 421 (2.08 ± 0.29 and 86.14 ± 6.48 ms, respectively; n = 5) modified both τ of activation and τ of inactivation.

Effect of ALIS 409 and ALIS 421 on aortic rings precontracted with phenylephrine

ALIS 409 and ALIS 421 were assayed on rat aorta rings, precontracted with 0.3 μM phenylephrine. As shown in Fig. 4, both compounds inhibited significantly, in a

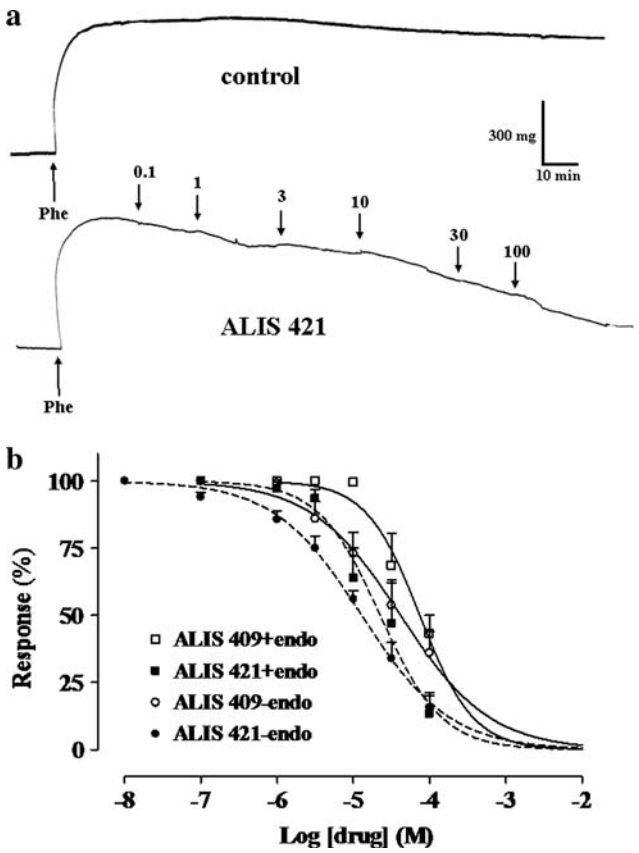

Fig. 4 Effect of ALIS 409 and ALIS 421 on rat aorta rings precontracted with phenylephrine. Steady tension was evoked by 0.3 μM phenylephrine and then each compound was added cumulatively. **a** Trace (representative of four experiments) of relaxation developed in response to cumulative concentrations (μM) of ALIS 421 added on the plateau of phenylephrine-elicited contraction in endothelium-denuded rings. The time-matched control is also reported for a comparison. The reason for the backward in time curving of the recordings is due to the kymograph-like characteristics of the Basile pen recorder (Basile, Varese, Italy). **b** Concentration-response curves for ALIS 409 and ALIS 421, either in endothelium-denuded (−endo) or endothelium-intact (+endo) aorta rings. In the ordinate scale, relaxation is reported as a percentage of the initial tension induced by phenylephrine. Data points are mean ± SEM (n = 3–6)

concentration-dependent manner, the sustained tonic contraction induced by phenylephrine. IC_{50} values of the relaxant effect of both ALIS 409 and ALIS 421 were not affected significantly by the presence of endothelium (73.9 ± 19.7 μM, n = 5, with endothelium, and 58.0 ± 23.0 μM, n = 5, without endothelium, ALIS 409; 31.9 ± 11.4 μM, n = 5, with endothelium, and 13.7 ± 1.7 μM, n = 4, without endothelium, ALIS 421) and did not differ significantly.

Effects of ALIS 409 and ALIS 421, ryanodine, and nifedipine on Ca^{2+} release from the intracellular stores and on extracellular Ca^{2+} influx induced by phenylephrine

When rings were incubated in a Ca^{2+}-free medium containing 1 mM EGTA, the addition of phenylephrine (10 μM) elicited release of intracellularly stored Ca^{2+}, which caused a small contraction (this response was taken as an index of the internal stored Ca^{2+}) (Fig. 5a). Restoring external Ca^{2+}, while phenylephrine was still present, caused an additional contraction, which was ascribed to the influx of extracellular Ca^{2+} subsequent to the opening of voltage-operated Ca^{2+} channels (VOCCs), receptor-operated Ca^{2+} channels (ROCCs) and/or store-operated Ca^{2+} channels (SOCCs). As shown in Fig. 5b, ryanodine (30 μM), but not nifedipine (0.3 μM), ALIS 409 or ALIS 421 (both at 100 μM) antagonized phenylephrine-induced contraction in the absence of extracellular Ca^{2+}. The latter, however, was reduced significantly by both ALIS compounds in rings preincubated with nifedipine. When external Ca^{2+} was restored, ALIS 409, ALIS 421, and nifedipine inhibited significantly phenylephrine-triggered Ca^{2+} influx, whereas ryanodine stimulated it. The inhibitory effect induced by ALIS 409 or ALIS 421 was not altered in rings pretreated with nifedipine.

Effect of ALIS 409 on spontaneous tone of rat aorta rings

Since ALIS 409 was the less potent vasodilator among the two silicon compounds, this experiment was conducted on endothelium-intact rings to investigate other mechanism(s) possibly responsible for its vascular activity. In the presence of extracellular Ca^{2+}, application of ALIS 409 (3–100 μM) to aorta rings failed to induce any mechanical responses.

Discussion

The present findings demonstrate that both ALIS 409 and ALIS 421 exhibited vasodilatory effects on rat aorta rings mainly *via* a negative modulation of plasmalemmal Ca^{2+} influx responsible for the contraction of vascular smooth muscle. In particular, ALIS 409 was the least active between the two silicon derivatives, displaying significant antispasmodic and spasmolytic activity at concentrations >10 μM.

It is well established that contraction of smooth muscle is initiated, and to a lesser extent maintained, by a rise in the concentration of free Ca^{2+} in the cell cytoplasm [15]. This activator Ca^{2+} can originate from two intimately linked sources, the extracellular space (Ca^{2+} entry) [14] and intracellular stores (Ca^{2+} release), most notably the sarcoplasmic reticulum [28]. The former event occurs through a variety of Ca^{2+} permeable ion channels in the sarcolemma membrane [17]. The best-characterized Ca^{2+} entry pathway utilizes VOCCs. The effects of the two silicon compounds on vascular smooth muscle have been presently investigated under experimental conditions that allow for the identification of blockers of these channels. High K^+-induced contraction of

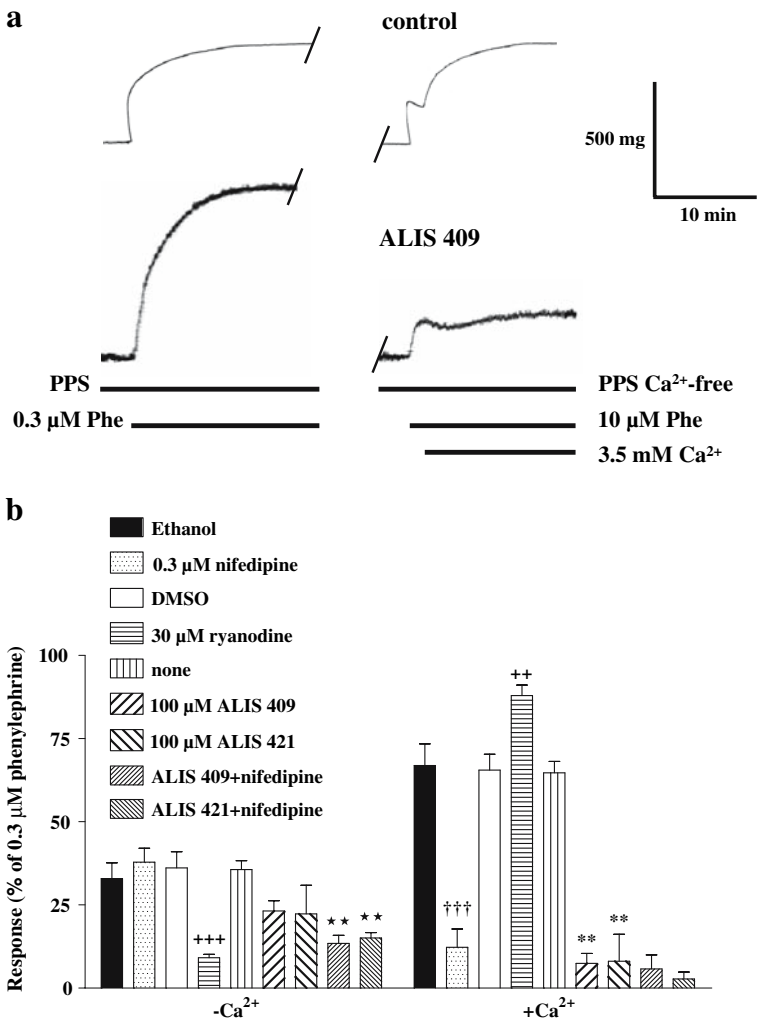

Fig. 5 Effects of ALIS 409, ALIS 421, ryanodine, and nifedipine on Ca^{2+} release from the intracellular stores and on extracellular Ca^{2+} influx induced by phenylephrine. **a** Recordings from two different preparations [representative of 9 (control) and 7 (ALIS 409) experiments, respectively]. *Right*: stimulation with 10 µM phenylephrine (Phe) in a Ca^{2+}-free medium, containing 1 mM EGTA, to empty Ca^{2+} internal stores produced a contraction. At its plateau, external Ca^{2+} was added to trigger the Ca^{2+} influx component. *Left*: the response to 0.3 µM phenylephrine in PSS is also reported for a comparison. Time between breaks (approximately 80 min) corresponds to wash out of 0.3 µM phenylephrine, pre-incubation with drugs or vehicle and exposure to Ca^{2+}-free solution. The reason for the backward in time curving of the upper recordings is due to the kymograph-like characteristics of the Basile pen recorder (Basile, Varese, Italy). **b** Columns represent phenylephrine-induced contractions either in the absence ($-Ca^{2+}$) or in the presence ($+Ca^{2+}$) of extracellular Ca^{2+}, recorded under control conditions (vehicle only, ethanol or DMSO, or none) or in the presence of drug. These contractions were measured independently, the response to phenylephrine in the absence of extracellular Ca^{2+} representing the baseline for that obtained after the addition of extracellular Ca^{2+}. Columns are mean ± S.E.M. (n = 3–13) and represent the percentage of the response to 0.3 µM phenylephrine, taken as 100%. ††† $P < 0.001$ versus ethanol, ++ $P < 0.01$, +++ $P < 0.001$ versus DMSO, Student's t test for unpaired samples; ★★ $P < 0.01$ versus nifedipine, **$P < 0.01$ versus none, Dunnett's post test

aorta rings, in fact, is the result of an increased Ca^{2+} influx through VOCCs and is specifically inhibited by Ca^{2+}-antagonists. ALIS 409 and ALIS 421 were shown to antagonize high K^+-induced contraction in a concentration-dependent manner. Therefore, this inhibition might be interpreted as a consequence of the blockade of VOCCs. The electrophysiological data presented here directly confirm this hypothesis, since both drugs inhibited $I_{Ca(L)}$ in rat tail artery myocytes with a complete block at the highest concentration assayed; their potency, however, was one order of magnitude lower than that exhibited in inhibiting high K^+-induced contractions. This observation suggests that the vasorelaxing activity, lower as one should expect from the VOCCs-blocking effect, is likely limited either by the diffusion of the drugs into the whole tissue or by the $t_{0.5}$ of the agents. Furthermore, slight differences in L-type Ca^{2+} channels expressed in the two tissues examined might explain the incomplete matching of data from the two experimental settings.

The whole-cell patch-clamp data were obtained from cells whose cytoplasm underwent extensive dialysis with the conventional patch-clamp method [24]. It is conceivable, therefore, that ALIS 409- and ALIS 421-induced inhibition of $I_{Ca(L)}$ is not mediated by diffusible intracellular factors but is rather the consequence of its direct interaction with the channel protein. Furthermore, neither ALIS 409 nor ALIS 421 modified Ca^{2+} channel activation and inactivation rates, thus excluding a possible interaction of these drugs with the transition from the closed to the open as well as from the open to the inactivated state of the channel (see [1]).

ALIS compounds relaxed preparations precontracted with phenylephrine in both endothelium-intact and -denuded rings. Therefore, the participation of endothelium-derived vasodilators (e.g. NO) in the vasorelaxant effects of the silicon compounds was excluded and not further investigated.

Smooth muscle contraction activated by α_1 adrenergic agonists originates from the combination of Ca^{2+} release and Ca^{2+} entry. The latter occurs through ROCCs, activated by G-protein, SOCCs, activated by depletion of the Ca^{2+} stores (i.e. the sarcoplasmic reticulum), in addition to VOCCs activated by membrane depolarization brought about by ROCCs and SOCCs opening [3, 13, 25], as well as by G-protein acting on other membrane conductances (such as inhibition of K^+, and activation of Cl^- and cation non-specific channels). The observation that nifedipine inhibited phenylephrine-induced Ca^{2+} entry is consistent with this hypothesis [19, this paper]. Therefore, the antagonism exerted by ALIS 409 and ALIS 421 on phenylephrine-induced contraction might be the result of a specific action on one of these targets (namely ROCCs/SOCCs, VOCCs, and sarcoplasmic reticulum) or, more likely, of a concerted effect on two or more of these targets. The latter hypothesis is supported by the fact that both ALIS 409 and ALIS 421 relaxed preparations precontracted with either KCl or phenylephrine with similar efficacy and IC_{50} values. Furthermore, high concentrations of ALIS 409 and ALIS 421 antagonized phenylephrine-triggered Ca^{2+} influx as well as the release of Ca^{2+} from the sarcoplasmic reticulum, although the latter reached a level of statistical significance only in rings pretreated with nifedipine. These results suggest that both ALIS 409 and ALIS 421, present during the incubation period, trigger the depletion of phenylephrine-sensitive Ca^{2+} stores [6], which is, at least in part, balanced by Ca^{2+} influx through nifedipine-sensitive Ca^{2+} channels [19].

When extracellular Ca^{2+} was restored, still in the presence of phenylephrine, Ca^{2+} influx occurred through ROCCs/SOCCs, and VOCCs (see above), thus causing an additional tonic response. This was significanctly reduced in the presence of ALIS compounds, thus suggesting that ROCCs/SOCCs, beyond VOCCs, likely represent additional targets responsible for ALIS 409 and ALIS 421 antispasmodic activity. This may not be particularly surprising in light of what appears to be a high degree of structural homology between the pore-forming α_1 subunits of the VOCCs and the proteins thought to form their store-operated counterparts [2, 23]. However, preincubation of rings with nifedipine did not modify significantly the antagonistic efficacy of both silicon compounds towards Ca^{2+} influx, thus indicating that this action is exerted selectively on VOCCs. In contrast to what observed with ALIS compounds and nifedipine, ryanodine increased Ca^{2+} entry, possibly by stimulating indirectly SOCCs.

Both ALIS 409 and ALIS 421 relaxed endothelium-intact preparations precontracted with phenylephrine with higher, although not significantly, IC_{50} values as compared to endothelium-denuded rings. This might be due either to an inhibitory effect on the release of endothelium-derived relaxing factors (e.g. NO) or to the release of endothelium-derived contracturing agents (e.g. thromboxane A2). However, since ALIS 409 was not capable to elicit any contraction in endothelium-intact aorta rings bathed in PSS, we can hypothesized that its action is not exerted at the endothelium, although further experiments are needed to clarify this point.

In conclusion, the present results demonstrate that ALIS 409 and ALIS 421 exert myorelaxing effect on in vitro rat aorta rings both by blocking Ca^{2+} influx through VOCCs and by emptying Ca^{2+} stores. It must be underlined, however, that, at least in the case of ALIS 409, the vasodilatatory activity is negligible at concentrations one order of magnitude higher than those effective as a P-gp inhibitor (see [20]), thus outlining the clear divergence of its P-gp inhibition and vascular activity. The absence of in vitro vascular activity per se, however, is not sufficient to guarantee for ALIS 409 safety and further experiments in combination with conventional chemotherapy drugs as well as on other physiological systems are needed before it can be addressed to the clinical investigation. Nevertheless, since reversion of MDR is a field of research of overwhelming importance, ALIS 409 may represent a lead compound for the design of novel and potent MDR chemosensitizers needed for the chemotherapy of cancer and other diseases.

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