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VASORELAXATION INDUCED BY AMODIAQUINE IN RAT SUPERIOR MESENTERIC ARTERIES: *IN VIVO* AND *IN VITRO* STUDIES

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Abstract: The aim of this study was to investigate the mechanisms underlying vasorelaxation induced by amodiaquine (AMQ) in rat superior mesenteric arteries. In normotensive, conscious rats, AMQ at 1–20 mg/kg *i.v.* produced hypotension and dose-dependent bradycardia. In mesenteric rings pre-contracted with phenylephrine (PHE) (10^{-5} M), AMQ caused concentration-dependent relaxation [$IC_{50} = (1.34 \pm 0.04) \times 10^{-5}$ M, E_{max} = 67.5 ± 0.8%]. Vasorelaxation induced by AMQ was unaffected after removal of the endothelium (E_{max} = 66.9 ± 0.3%, $p > 0.05$), and in the presence of ouabain (10^{-4} M) (E_{max} = 65.4 ± 1.9%, $p > 0.05$). In contrast, vasorelaxation evoked by AMQ was significantly inhibited after pre-treatments with 4-aminopyridine (10^{-3} M), tetraethylammonium (10^{-3} M) and glibenclamide (10^{-5} M), blockers of voltage-dependent K^+ (Kv), large and intermediate conductance Ca^{2+}-activated K^+ (BK_{Ca}) and K_{ATP} channels, respectively. Additionally, AMQ reduced $CaCl_2$-induced contractions in Ca^{2+}-free solution containing KCl, probably due to its non-selective opening of K^+ channels or may be acting as Ca^{2+}-antagonist. Furthermore, AMQ did not interfere with Ca^{2+} release from intracellular stores mediated by either phenylephrine (10^{-5} M) or caffeine (0.02 M). Collectively, these results provide functional evidence that AMQ-induced hypotensive and bradycardic effects may involve the opening of K^+ channels sensitive to 4-aminopyridine, tetraethylammonium and glibenclamide or the blockade of extracellular Ca^{2+} influx.

Keywords: Ca^{2+} influx, K^+ channels, amodiaquine, mesenteric arteries, vasorelaxation

Between 300 to 500 million cases of malaria are reported yearly, which has resulted in over one million deaths annually (1). Also, about two billion people (approximately 40% of the world's population) in more than 100 countries are at risk of the disease (2). Ninety percent of the cases of malaria occur in children in Africa under 5 years of age (2). The incidence of malaria is made worse in most sub-Saharan Africa because of the emergence of drug-resistant strains of *plasmodium falciparum* which rendered some traditional antimalarial drugs especially the first-line drugs like chloroquine (CQ) and sulfadoxine-pyrimethamine (SP) ineffective. The spread of CQ-resistance has led to an increasing use of amodiaquine (AMQ).

AMQ, chemically known as 4-[(7-chloroquinolin-4-yl)amino]-2-[(diethylamino)methyl]phenol (Figure 1), is an alternative first-line drug for uncomplicated malaria (3). AMQ has been reported to remain remarkably effective in many malaria endemic countries and in particular in West Africa despite emerging CQ resistance (4–9). AMQ either as monotherapy or Artemisinin-based combination therapy (ACT) has proved highly effective in a number of field trials (10, 11). AMQ is a Mannich base derivative, an analog of CQ and has been in use since 1940s (12). Despite the long use of AMQ, some of its pharmacodynamic properties, especially electrocardiographic effects have not been exten-

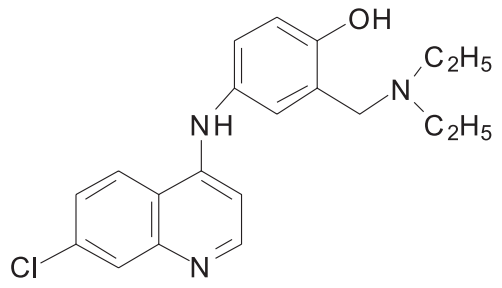

Figure 1. Structure of amodiaquine – 4-[(7-chloroquinolin-4-yl)amino]-2-[(diethylamino)methyl]phenol

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sively studied. Like other quinoline containing antimalarials, such as CQ, quinine, quinidine and halofantrine, AMQ has been reported to prolong ventricular repolarization, which is evidenced by increased QTc interval in electrocardiogram, and bradycardia and hypotensive effects in patients with acute uncomplicated *plasmodium falciparum* at usual therapeutic concentrations (12–16). Though symptomatic cardiac effects have not been reported after administration of AMQ, the changes in electrocardiographic and hemodynamic parameters of patients caused by AMQ warrants thorough scientific investigation. Against this background, this study was designed to establish and provide pharmacological basis for the vasorelaxation induced by AMQ.

EXPERIMENTAL

Animals

Male Wistar rats (280–300 g) were used for all experiments. The animals were kept in well-ventilated cages at controlled temperature (21 ± 1°C) and under controlled light cycles (12 h light/12 h dark). Animals were maintained on normal laboratory chow (PURINA, João Pessoa, Brazil) and tap water *ad libitum*. The study was approved by the Committee of Ethics for Animal Research (Comitê De Êtica Em Pesquisa Animal) CEPA No. 0805/07, Laboratório de Tecnologia Farmacêutica, Universidade Federal da Paraíba, João Pessoa, Brazil.

Drugs

The drugs used were: L-phenylephrine hydrochloride, acetylcholine hydrochloride, Tween-80, tetraethylammonium, glibenclamide, ouabain, 4-aminopyridine, caffeine, ethylene glycol-bis(β-aminoethylether)-N,N,N´,N´-tetraacetic acid EGTA) and amodiaquine (Sigma Chemical Co., St. Louis, MO, USA). Chemicals used for preparing Tyrode´s solutions were: calcium chloride, glucose, magnesium sulfate, potassium dihydrogen phosphate, sodium bicarbonate and sodium chloride (E. Merck, Darmstadt, Germany). The stock solutions were dissolved in distilled water, except glibenclamide that was dissolved in ethanol. The solutions were prepared fresh on the day of experiments.

Direct blood pressure measurements in non-anesthetized rats

Intra-aortic blood pressure was recorded using the technique described by Oliveira et al. (17). Briefly, under sodium thiopental anaesthesia (45 mg/kg; *i.v.*), the lower abdominal aorta and inferior vena cava were canulated *via* left femoral artery and vein using polyethylene catheters. Thereafter, catheters were filled with heparinized saline solution and led under the skin to emerge between the scapulae. Arterial pressure was measured after 24 h by connecting the arterial catheter to a pre-calibrated pressure transducer (Statham P23 ID; Gound, Cleveland, OH, USA) coupled to an amplifier-recorder (Model TBM-4M, WPI, Sarasota, FL, USA) and then connected to a computer equipped with an analog-digital converter board (CIO-DAS16/JR, Computer Boards, Inc., Mansfield, MA, USA) and CVMS software (WPI, Sarasota, FL, USA). The data were sampled at a frequency of 500 Hz. For each cardiac cycle, the mean arterial pressure (MAP) and heart rate (HR) (pulse interval) were calculated by the computer. The venous catheter was used for drug administration. Sodium nitoprusside (10 mg/kg; *i.v.*) was injected to check the efficacy of the venous catheter insertion. After cardiovascular parameters had been stabilized, the MAP and HR values were recorded before (baseline) and after administration of randomized doses of AMQ (1, 5, 10, 15 and 20 mg/kg; *i.v.*). For the construction of a dose-response curve, the difference between the baseline and after administration values for each dose was expressed as percentage of baseline value. Successive injections of AMQ were well separated by a time interval sufficient to allow full recovery of hemodynamic parameters (40–50 minutes) (18–20).

Preparation of rat superior mesenteric arteries rings

The superior mesenteric arteries from the second order branches were quickly removed and cleaned of adherent connective tissues and fat. Mesenteric rings (2–4 mm length) were obtained and suspended by cotton threads in organ bath containing 10 mL of Tyrode´s solution, maintained at 37°C and gassed with a 95% O_2 + 5% CO_2 mixture (pH 7.4). The composition of the Tyrode´s solution (in 10^{-3} M): NaCl 158.3; KCl 4.0; $CaCl_2.2H_2O$ 2.0; $MgCl_2.6H_2O$ 1.05; $NaHCO_3$ 10.0; $NaH_2PO_4.H_2O$ 0.42 and glucose 5.6 (21). Rings were stabilized with a resting tension of 0.75 g, for at least 60 min, with constant changing of Tyrode´s solution (every 15 min) to prevent the accumulation of metabolites that could otherwise lead to misinterpretation of results (22). The isometric tension was recorded by a force-displacement transducer (Miobath-4, WPI, Sarasota, FL, USA) coupled to an amplifier-recorder (Transbridge-4; WPI, Sarasota, FL, USA) and a computer equipped with an analog–to–digital con-

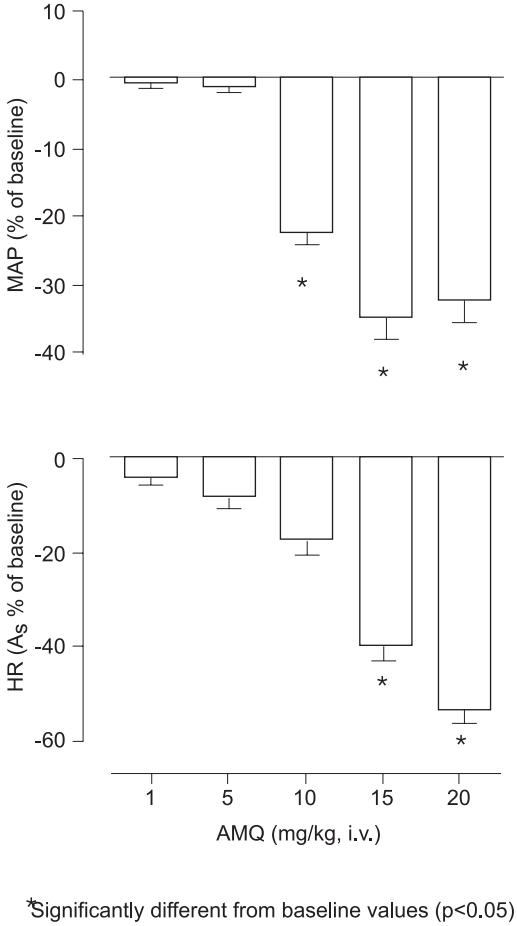

Figure 2. The effect of amodiaquine (1–20 mg/kg *i.v.*) on mean arterial pressure (MAP) and heart rate (HR) in non-anesthetized rats

verter board (AD16JR; WPI, Sarasota, FL, USA). In some experiments, the endothelium layer of the rings was removed by gently rubbing the external surface with a finger moistened with Tyrode´s solution. Endothelial integrity was assessed qualitatively by the degree of relaxation caused by acetylcholine (10^{-5} M) in the presence of contractile tone induced by phenylephrine (PHE) (10^{-5} M). Rings were considered to be endothelium-denuded when acetylcholine-induced relaxant effects were less than 10% and, endothelium-intact when the relaxant effects were more than 90%.

Effect of AMQ on contractions induced by phenylephrine (10^{-5} M) or KCl (0.08 M) in isolated rat superior mesenteric arteries

After equilibration, steady tension was evoked by phenylephrine (10^{-5} M) for endothelium-intact and -denuded rings to induce contraction of similar magnitude and AMQ was added cumulatively (10^{-8} – 10^{-3} M). The ability of AMQ to attenuate KCl (0.08 M)-induced sustained contraction in the rings was also examined for both endothelium-intact and -denuded rings. The relaxations were measured by comparing the developed tension before and after addition of AMQ.

Investigation of the role of K^+ channels in AMQ-induced vasorelaxation in isolated superior mesenteric arteries

In another set of experiments, the rings without endothelium were pre-contracted with phenylephrine (10^{-5} M) for 30 min after being pre-incubated with one of the following inhibitors: glibenclamide (10^{-5} M), selective blocker of K_{ATP} channels, tetraethylammonium (TEA) (10^{-3} M), non-selective blockers of BK_{ca} channels, 4-aminopyridine (4-AP) (10^{-3} M), a selective blocker of K_v channels and ouabain (10^{-4} M), a selective inhibitor of Na^+- K^+ ATPase. The concentration used for each inhibitor of K^+ channels is sufficient to antagonize selectively those channels in arterial smooth muscle (19, 23–25). After stabilization of the tonic contraction induced by phenylephrine (10^{-5} M), increasing cumulative concentrations of AMQ (10^{-8} – 10^{-3} M) were added to the organ bath.

Effect of AMQ on contractions induced by $CaCl_2$, and Ca^{2+} release from intracellular stores

To further investigate the mechanism of vasorelaxation induced by AMQ, concentration-response curves to $CaCl_2$ were constructed using endothelium-denuded rings (24). Briefly, the rings were pre-contracted with KCl (0.06 M) to confirm tissue viability. The Tyrode´s solution was replaced with depolarizing Tyrode´s solution (KCl 0.06 M) nominally without Ca^{2+} (15 min). Thereafter, concentration-response curves to $CaCl_2$ (10^{-6} M – 10^{-2} M) were constructed in the absence or presence of AMQ (10^{-6}, 10^{-5}, 10^{-4}, 10^{-3} M). To determine the effect of AMQ on the release of Ca^{2+} from intracellular stores, the endothelium-denuded rings were pre-contracted with KCl (0.06 M), washed and exposed to Ca^{2+}-free Tyrode´s solution containing EGTA (10^{-3} M). The rings were then stimulated with phenylephrine (10^{-5} M) or caffeine (0.02 M) (26). The contractions of agonists were obtained in absence (control) or after incubation with AMQ (10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} M).

Statistical analysis

Two pharmacological parameters were ana-

lyzed in this study; E_{max} (maximal effect generated by agonist) and pD_2 (–log IC_{50}). Values are expressed as the means ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA) or Student's *t*-test using GraphPad Prism TM 5.0 version software, San Diego, CA, USA. *Post hoc* comparisons were performed using Dunnett's test. The level of significance considered in all the tests was 0.05.

RESULTS

Effect of AMQ on mean arterial pressure (MAP) and heart rate (HR) in non-anesthetized rats

In this study, the baseline values of MAP and HR were 118 ± 2 mmHg and 334 ± 6 bpm, respectively. AMQ at doses 10, 15 and 20 mg/kg (*i.v.*) induced hypotension by 22.3 ± 1.2%, 34.5 ± 3.1% and 32.1 ± 3.4%, respectively (expressed as percentage of baseline values) (Fig. 2). The hypotensive response was found to be short-live (4 ± 1 min). The hypotensive response was accompanied by prolonged and dose-dependent bradycardia (4.1 ± 1.6%, 8.4 ± 2.3%, 17.0 ± 3.3%, 39.3 ± 3.5% and 53.2 ± 2.9% at doses of 1, 5, 10, 15 and 20 mg/kg, respectively). The bradycardic effect lasted for 10 ± 3 min at each dose.

Effect of AMQ on mesenteric rings pre-contracted with phenylephrine or KCl

AMQ at 10^{-6} – 10^{-3} M significantly ($p < 0.05$) relaxed the sustained contractions induced by phenylephrine (PHE) and KCl in a concentration-dependent manner in both endothelium-intact and -denuded rings (Fig. 3). The E_{max} and pD_2 values of AMQ for intact and denuded rings in both agonists are statistically similar. Thus, endothelium removal has no effect on the vasorelaxation induced by AMQ. AMQ was active in inhibiting both the PHE- and KCl-induced contractions of the arterial rings. The IC_{50} values of AMQ were 1.34 ± 0.04 × 10^{-5} M and 5.88 ± 0.05 × 10^{-5} M for the inhibition of PHE- and KCl-induced contractions of the rings, respectively.

Role of K^+ channels in AMQ-induced vasorelaxation in superior mesenteric arteries

In denuded rings, the incubation with KCl (0.02 M) before constriction with PHE significantly decreased the pD_2 values of AMQ when compared to controls (Table 1), indicating the involvement of K^+ channels in the vasorelaxation induced by AMQ. Similarly, incubation of denuded rings with glibenclamide (10^{-5} M), tetraethylammonium (10^{-3} M) and 4-aminopyridine (10^{-3} M) shifted the concentration-response curve for AMQ to right with reduction of the pD_2 values (Table 1 and Fig. 4). Interestingly, in endothelium-denuded rings, the concentration-response curve for AMQ was insignificantly changed after incubation with ouabain (10^{-4} M), (Fig. 4B).

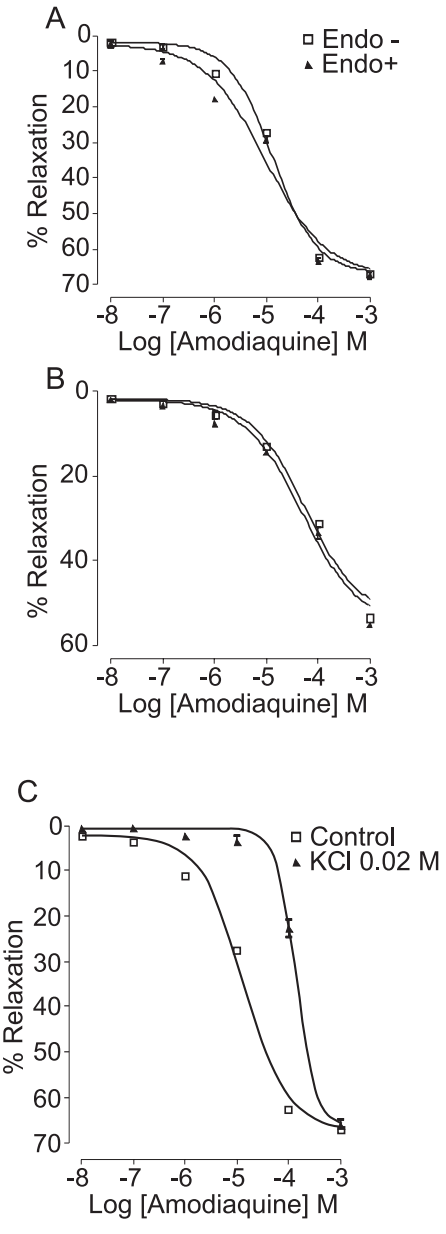

Figure 3. Relaxation responses induced by amodiaquine when endothelium-intact and -denuded rings were pre-contracted with phenylephrine (10^{-5} M) (A), KCl (0.08 M) (B), and when denuded rings were pre-incubated with KCl (0.02 M) before contracted with phenylephrine (C)

Table 1. Effect of KCl (0.02 M), TEA (10^{-3} M), glibenclamide (10^{-5} M), 4-AP (10^{-3} M) and ouabain (10^{-4} M) on E_{max} (%) and pD_2 values of amodiaquine (AMQ)-induced relaxant responses in endothelium-denuded mesenteric rings pre-contracted with phenylephrine (10^{-5} M).

Groups	E_{max} (%)	pD_2
Control	66.9 ± 0.30	4.87 ± 0.03
KCl (0.02 M)	65.8 ± 1.33	3.83 ± 0.04*
TEA (10^{-3} M)	65.0 ± 1.60	3.71 ± 0.06*
Glibenclamide (10^{-5} M)	65.6 ± 1.13	3.82 ± 0.05*
4-AP (10^{-3} M)	65.4 ± 1.64	3.92 ± 0.03*
Ouabain (10^{-4} M)	66.4 ± 1.92	4.84 ± 0.05

Values are the mean ± SEM, n = 7–8 experiments. * Significantly different from control ($p < 0.05$) (ANOVA followed by Dunnett´s multiple comparison test).

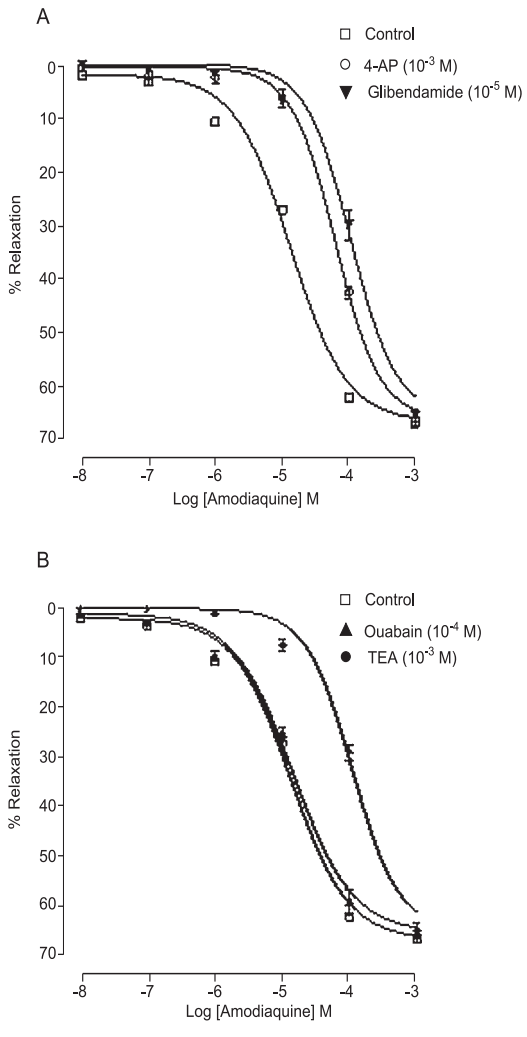

Figure 4. Relaxation responses induced by amodiaquine on endothelium-denuded rat mesenteric rings pre-contracted with phenylephrine in the presence of 4-aminopyridine (4-AP) (10^{-3} M), glibenclamide (10^{-5} M) (A) and ouabain (10^{-4} M), tetraethylammonium (TEA) (10^{-3} M) (B). The rings were pre-incubated with the inhibitors for 30 min. Steady tension was evoked by phenylephrine (10^{-5} M) and amodiaquine (10^{-8} – 10^{-3} M) was added cumulatively

Effect of AMQ on contractions induced by $CaCl_2$, and Ca^{2+} release from intracellular stores

Pre-incubation of endothelium denuded rings with AMQ attenuated $CaCl_2$-induced contraction in Ca^{2+}-free medium containing KCl (0.06 M). Administration of $CaCl_2$ induced a concentration-dependent contraction of mesenteric rings (E_{max} = 100%; pD_2 = 3.49 ± 0.06, Table 2). Pre-incubation of the rings with AMQ at 10^{-4} M and 10^{-3} M significantly ($p < 0.05$) inhibited the $CaCl_2$-induced contraction (Fig. 5). The effects of AMQ on the E_{max} and pD_2 values for $CaCl_2$-induced contractions are given in Table 2. In contrast, AMQ did not produce any remarkable effect on the transient contractions induced by phenylephrine (10^{-5} M) and caffeine (0.02 M) in endothelium denuded rings in Ca^{2+}-free media containing EGTA (10^{-3} M) (Fig. 6).

DISCUSSION AND CONCLUSION

The present study was designed to clarify the pharmacological basis for the observed vasorelaxation induced by AMQ in rat superior mesenteric arteries. The choice of this artery is due to its substantial contribution to the regulation of systemic circulation and, also reflects the variation of vascular resistance in circulation (27, 28). The major finding of this work was that AMQ induced concentration-dependent relaxation of rat mesenteric arteries that may be mediated by the blockade of Ca^{2+} influx and the opening of K^+ channels sensitive to 4-aminopyridine, tetraethylammonium and glibenclamide with a resultant membrane hyperpolarization/ repolarization. Furthermore, in this study AMQ induced hypotensive and bradycardic effects in non-anesthetized rats. The hypotensive and bradycardic effects of AMQ may be due, at least in part, to the blockade of Ca^{2+} influx by AMQ. The effects are common with cholinergic compounds, which are known to cause a fall in blood pressure by activation

Table 2. Effect of amodiaquine (AMQ) on E_{max} and pD_2 for $CaCl_2$-induced contraction in endothelium denuded mesenteric rings in Ca^{2+}-free medium.

AMQ (M)	pD_2	E_{max} (%)
Control	3.49 ± 0.06	100
10^{-6}	3.42 ± 0.08	95.4 ± 3.2
10^{-5}	3.45 ± 0.05	93.3 ± 2.7
10^{-4}	2.86 ± 0.07*	72.4 ± 1.6*
10^{-3}	2.23 ± 0.04*	61.3 ± 1.7*

Values are the mean ± SEM, n = 28 for control; n = 7 for others. * Significantly different from control ($p < 0.05$) (ANOVA followed by Dunnett´s multiple comparison test).

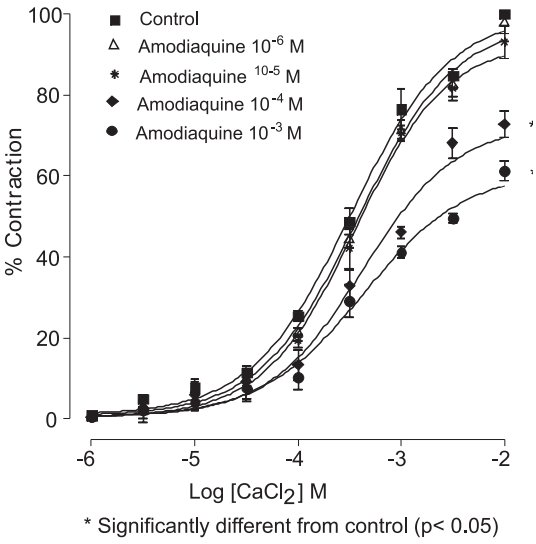

Figure 5. Effect of amodiaquine on $CaCl_2$-induced contractile response in endothelium-denuded mesenteric rings. Concentration-response curves for $CaCl_2$ were determined in Ca^{2+}-free solution containing KCl (0.06 M). The curves were determined in the absence (control) and after incubation with amodiaquine (10^{-6} – 10^{-3} M)

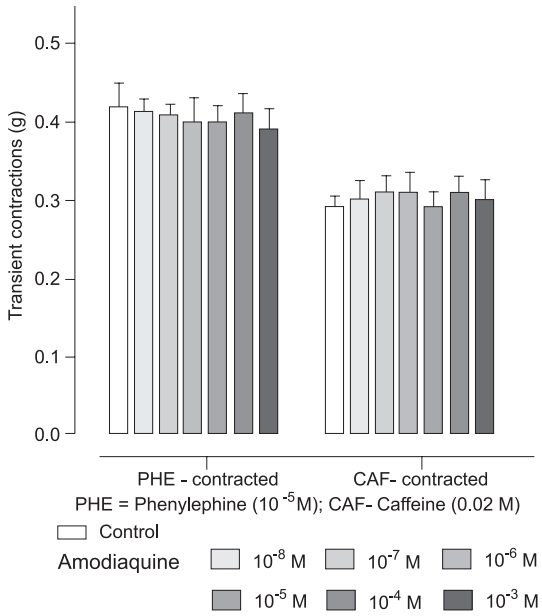

Figure 6. Effect of amodiaquine (10^{-8} – 10^{-3} M) on phenylephrine (10^{-5} M) and caffeine (0.02 M) induced transient contractions in endothelium-denuded mesenteric rings in Ca^{2+}-free medium containing EGTA

of muscarinic receptors located on the epithelium of blood vessels (29). Furthermore, there may be need to perform experiments to confirm the involvement of muscarinic receptors in the bradycardic effect shown by AMQ. In agreement with this study, Ngouesse et al. (12) observed bradycardia in 16 of 20 AMQ-treated patients on day 2 of administration that corresponds to the time when maximal cumulative plasma concentration of AMQ was reached.

In the present study, AMQ concentration-dependently reduced the contractions induced by phenylephrine or KCl in endothelium-intact and -denuded arterial rings with statistically similar E_{max} and pD_2 values, indicating that endothelium is not required for the relaxation induced by this antimalarial. Furthermore, AMQ was found to be effective in relaxing the endothelium-denuded rings preconstricted with both phenylephrine and KCl. Precisely, the IC_{50} of AMQ for the inhibition on phenylephrine- and KCl-induced contractions were $1.34 \pm 0.04 \times 10^{-5}$M and $5.88 \pm 0.05 \times 10^{-5}$ M, respectively. It is well known that KCl induces smooth muscle contraction through the activation of voltage-dependent calcium channels and subsequent release of calcium from sarcoplasmic reticulum (30, 31) whereas phenylephrine-induced vasoconstriction is mediated by the stimulation of G-proteins coupled to α-adrenoceptors (32). Thus, both contractile agents produce a significant increase in intracellular calcium concentration through calcium

entry. Therefore, it can be suggested that the vasorelaxant effects observed for AMQ may be due to an endothelium-independent mechanism that could be linked to the blockade of calcium influx into the vascular smooth muscle cells (VSMCs).

Another aspect investigated in this study was whether AMQ-induced vasorelaxation could be related to inhibition of Ca^{2+} influx from extracellular medium. It was observed that AMQ at 10^{-4} M and 10^{-3} M decreased $CaCl_2$-induced contraction in Ca^{2+}-free medium containing KCl. Taken together, these results support the notion that AMQ can block Ca^{2+} influx through Ca^{2+} channels on VSMCs. This observation does not rule out the possibility that AMQ reduces the sensitivity of the contractile filaments to Ca^{2+}. This led to the investigation on whether AMQ could exert its vasorelaxant effects by interfering with the release of intracellular calcium, $[Ca^{2+}]_i$ *via* the phosphoinositide-dependent or -independent pathway following receptors activation. In Ca^{2+}-free media containing EGTA, AMQ did not interfere with the transient contractions induced by either phenylephrine or caffeine. It seems likely that the vascular effect of AMQ does not involve the reduction in Ca^{2+} release from intracellular stores, there may be need to confirm this observation using inhibitors of intracellular Ca^{2+}-release.

The opening of K^+ channels in the cell membrane of smooth muscle cells in arteries increases K^+ efflux causing membrane potential hyperpolarization, which leads to vasodilation (23). Tetraethylammonium (TEA) (10^{-3} M), a non-selective blocker of large and intermediate conductance Ca^{2+}-activated K^+ channels (BK_{Ca}), produced a displacement of the concentration-response curve for AMQ to the right, probably indicating the participation of K^+ channels in the vasorelaxation action induced by this antimalarial. Vascular smooth muscle cells are known to express different types of K^+ channels (33). Agents that block these channels are useful tools for exploring the role of a particular K^+ channel. In the present study, both glibenclamide (10^{-5} M) and 4-aminopyridine (10^{-3} M) produced rightward displacement of the concentration-response curves for AMQ, suggesting that K^+ channels that are sensitive to glibenclamide and 4-aminopyridine may be involved in the vasorelaxation induced by AMQ. In contrast, ouabain (10^{-4} M), an inhibitor of Na^+-K^+ ATPase (19, 34) did not affect the concentration-response curves for AMQ. Therefore, Na^+-K^+ ATPase may not be involved in the vasorelaxant effect elicited by AMQ.

In conclusion, the vasorelaxation-induced by AMQ occurred through an endothelium-independent mechanism that may involve the blockade of extracellular Ca^{2+} influx by interfering with voltage-operated channels. In addition, the relaxant effect of AMQ may be linked to K^+ channels that are sensitive to 4-aminopyridine, tetraethylammonium and glibenclamide. However, further studies are required on the cardiac evaluation of different types of antimalarials in order to ensure their safety.

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