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EXPERIMENTAL STUDY

Chloroquine and cinchonine affect rat vascular smooth muscle tonus through calcium channels – *in silico* and *in vitro* approaches

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ABSTRACT
BACKGROUND: In the present study, two structurally similar alkaloids from trees of *Cinchona* genus, chloroquine and cinchonine, were examined for their vasorelaxant effects in a model of phenylephrine-induced smooth muscle contractions.
METHODS: Potential mechanisms of action associated with endothelial vasorelaxant compounds, voltage-gated Ca^{2+} channels (LTCCs), and inositol triphosphate receptors were examined in isolated rat aortic rings. Also, an *in silico* approach was used to predict the activity of the two test compounds.
RESULTS: Experimental results revealed that both chloroquine and cinchonine significantly decrease phenylephrine-induced smooth muscle contractions, although to a different extent. Evaluated mechanisms of action indicate that endothelium is not involved in the vasorelaxant action of the two tested alkaloids. On the other hand, voltage-gated Ca^{2+} channels were found to be the dominant way of action associated with the vasorelaxant action of chloroquine and cinchonine. Finally, IP3R is found to have only a small impact on the observed activity of the tested compounds.
CONCLUSION: Molecular docking studies predicted that chloroquine possesses a significant activity toward a suitable model of LTCCs, while cinchonine does not. The results of the present study point to the fact that great caution should be paid while administering chloroquine to vulnerable patients, especially those with cardiovascular disorders *(Tab. 3, Fig. 3, Ref. 28).* Text in PDF www.elis.sk
KEY WORDS: chloroquine, cinchonine, vascular smooth muscle, voltage-gated calcium channels.

Introduction

Chloroquine (CQ) (Fig. 1), which is routinely used as antimalarial, anti-amebiasis, cytostatic, and immunomodulatory drugs, as well as a possible anti-viral drug (towards coronavirus disease 2019 (COVID-19)) (1–3). This drug can have both proarrhythmic and antiarrhythmic activities via inhibition of voltage-gated Na, Ca, and K channels and channels expressed in SA node cells (1). By impeding normal autophagy flux, CQ could exacerbate ischemia or post-infarct remodeling (1). Other authors have conflicting results documenting that CQ inhibits aortic smooth muscle contraction through modulation of contractile systems and their regulatory proteins, independently from endothelium and voltage-gated channels (2). Chloroquine application has serious side effects such as gastrointestinal disturbances, itching, headache, visual disturbances, and cardiotoxicity (2). The mortality rate of malaria has increased recently due to widespread resistance of *Plasmodium* to main antimalarial drugs, including CQ, and invasive bacterial co-infections (4).

Cinchonine (Cin) (Fig. 1) is an alkaloid with antimalarial activity isolated from the bark of trees belonging to the *Cinchona* and *Ramijia* genera of the Rubiaceae family . The other three alkaloids found in cinchona bark extract are quinine, quinidine, and cinchonidine, and it is believed that the synergistic action of several alkaloids is the main reason for the extract's activity (5). Thus, such a combination of agents could be a promising solution for treating quinine-resistant *Plasmodium falciparum.* Cinchonine (Cin) is used to treat gastrointestinal parasite infections (schistosomiasis, amebiasis, dysentery, etc.) because of its lower toxicity and higher antiparasitic activity compared to quinine. It was documented that Cin decreases TLR2- and TLR4-mediated inflammatory responses in adipose tissue of animals with early symptoms of metabolic

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syndrome and could be used as an immunomodulatory agent. Another activity of Cin is based on its potential antiaggregatory effect on human platelets through the inhibition of Ca^{2+} influx and protein kinase (5). Both CQ and Cin can cause cinchonism, a syndrome that includes CNS and gastrointestinal disturbances which can vary from mild to severe symptoms (5).

There are various ways through which blood vessel tone is regulated. They involve α1 adrenergic receptors (α1AR), i.e., G protein-coupled receptors that activate inositol-1,4,5-trisphosphate (IP3), and rho-kinase (6). Another mechanism of tonus regulation is vasodilation through nitric oxide (NO), which is produced by one of three isoforms of nitric oxide synthase (neuronal, inducible, and endothelial isoforms) (7). Blood vessel tone is also regulated through voltage and ligand-gated Ca channels, which allow the entrance of Ca^{2+} into cytosol. When the cytosol Ca^{2+} concentration is increased, either through the influx of Ca^{2+} ions from the exterior or via intracellular release of Ca^{2+} from the sarcoplasmic reticulum storage, vasoconstriction occurs. In the vasculature, the voltage-gated calcium channel, referred to as L-type Ca^{2+} channels (LTCC), is one of the few crucial receptors that control the vascular tone by maintaining the membrane potential of the vascular smooth muscle cells (8). There are traditional antihypertensive drugs that act through one or several mentioned mechanisms. One of them is nifedipine, a calcium channel blocker, and the other include beta-blockers, diuretics, angiotensin-converting enzyme inhibitors, and angiotensin II receptor antagonists (9). On the other hand, some drugs provoke hypotension, as their side effect. It is known that hypotension was a common and prominent clinical feature of malaria caused by *P. falciparum* and *P. vivax*. However, it was concluded that it is not a direct repercussion of the effect of either the parasite or antimalarial drug on blood vessels (10).

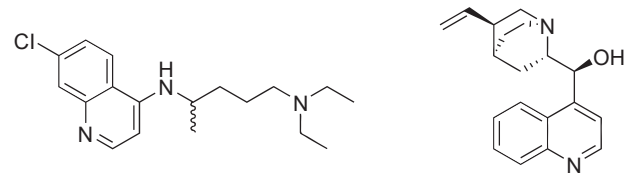
With previously mentioned facts in mind, this study aims to evaluate the impact of two antimalarial drugs on the vasculature tone of the isolated rat aorta and to study the involvement of Ca^{2+} channels in their activity. This was done by estimating the binding potentials of Cin and CQ on LTCC in an *in silico* docking experiment and was further corroborated in an *in vitro* experimental setting using isolated rat aortic rings. Also, the roles of LTCC, nitric oxide, and IP3 in the activity of Cin and CQ were estimated using isolated rat aortic rings and a specific antagonist.

Materials and methods

The experiments were approved by the local Ethics Committee and by the Ethics Committee of the Republic of Serbia (323-09-06862/2015-05/2). All experimental procedures with the animals were conducted in compliance with the declaration of Helsinki and European Community guidelines for the ethical handling of laboratory animals (EU Directive of 2010; 2010/63/EU).

Molecular docking

Molecular docking was performed using GOLD (2020.3 CSD release, Cambridge Crystallographic Data Center, Cambridge, UK) (23, 24). Default parameters were chosen for population size (100), selection pressure (1.1), number of operations (10.000),



chloroquine cinchonine

Fig. 1. Chemical structure of chloroquine and cinchonine.

number of islands (1), and niche size (2); operator weights for 'migrate' (0), 'mutate' (100), and 'crossover' (100) functions were applied. Default genetic algorithm settings were used for all calculations, and the ChemPLP scoring function was utilized. Structures of ligands at physiological pH were acquired from the ZINC database (25) as MOL2 files, while structures of the receptors were obtained as PDB files from the Protein Data Bank [PDB]. All docking experiments were validated by re-docking the co-crystallized ligands.

Drugs and chemicals

All chemicals used in this study were of analytical grade and were purchased from either Sigma Aldrich (USA) or Carl Roth (Germany). Both Cin and CQ (≥98.0% purity) were obtained from Sigma Aldrich, and a stock solution of 25 mg/ml was freshly prepared before the experiment.

Animals and housing

Four-month-old male and female Wistar rats, weighing 250–300 g, were selected for this study. They were fed with a standard rat chow diet and *ad libitum* water access, and kept under standard laboratory conditions, including room temperature of 20–22 °C and 12/12-hour light/dark cycle. The animals were obtained from the Vivarium of the Faculty of Medicine, University of Nis, Serbia. All experimental protocols were done in accordance with the Council of Europe Directive of 22nd September 2010 (Directive 2010/63/EU) and were pre-approved by the Animal Ethics Committee of the Faculty of Medicine, University of Nis.

Isolation of the thoracic aorta and mounting

After the sacrificed animal's thoracic cavity had been opened, thoracic organs were removed to expose the aorta. The entire aorta from the thoracic cavity was dissected and placed in a Petri dish containing Krebs solution ((mmol/L) NaCl 118.2, KCl 4.7, $CaCl_2$ x2H_2O 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 11.7, $NaHCO_3$ 25.0, and EDTA 0.026), where it was cleaned from the surrounding connective tissue and cut into smaller rings approximately 0.5–1 cm in length. Isolated segments of the thoracic aorta were mounted on a wire holder and placed in a tissue bath (10 mL) with Krebs solution maintained at 37 °C with 95% O_2 and 5% CO_2 (26).

Experimental procedure

After a 60-min equilibrium period, the aortic rings were contracted with phenylephrine (PE) (3 mmol/L) (Sigma–Aldrich)

(27), after which Cin and CQ were added in five increasing concentrations ranging from 10^{-7} to $5x10^{-3}$ mol/L for 5 min each. Tissue tension alterations were documented using a transducer (TSZ-04-E; Experimetria Ltd) and analyzed using the SPEl Advanced ISOSYS Data Acquisition System (Experimetria Ltd). The change in tension was expressed as a change in the percentage of the tension induced by PE.

Evaluation of the vasodilatory mechanism of action

Mechanism involving endothelium-derived vasodilator factors

In order to evaluate endothelium-derived vasodilator factors, i.e., nitric oxide (NO), the endothelium from the isolated rat thoracic aorta was removed mechanically by gently rubbing it off the isolated segment of a blood vessel with a stainless steel wire (14). The absence of endothelium was assessed by challenging the segment with acetylcholine (10^{-5} mol/L) after its pre-contraction with PE. The lack of relaxation was indicative of the absence of endothelium (14). Segments with the removed endothelium were treated in the same manner as those with intact endothelium, i.e., after PE steady contraction, Cin or CQ were added cumulatively starting from $5x10^{-5}$ to $5x10^{-3}$ M.

Further inhibition of NO production and its effect on vasorelaxation were assessed by inhibiting nitric oxide synthase (NOS) prior to PE application. This was achieved by pre-exposure of isolated aortic rings to L-NAME (30 μmol/L) 30 min prior to PE (15). After a steady contraction had been induced by PE, ascending concentrations of either Cin or CQ were added as described above. Tissue tension changes were monitored using the same equipment and the results are expressed as a change in the percentage of the tension induced by PE.

Mechanism involving voltage-gated cell membrane channels

To evaluate the involvement of cell membrane voltage-gated Ca^{2+} channels, rat thoracic aorta rings were exposed to a solution containing high K^+ (60 mmol/L) (28). After reaching a steady contraction of the isolated aortic ring, ascending concentrations of either Cin or CQ were added as described above. Tissue tension changes were monitored using the same equipment and the results are expressed as a change in the percentage of the tension induced by PE.

Tab. 1. GOLD docking scores.

Ligand	ChemPLP Score	
	CavAb (PDB: 5KMF)	CavAb (PDB: 5KMH)
(*S*)-chloroquine (*S*-QC)	73.8	55.4
(*R*)-chloroquine (*R*-QC)	72.7	55.1
Cinchonine (Cin)	55.3	49.2
(*R*)-bromoverapamil	–	66.0
(*S*)-verapamil	–	64.0
(*R*)-verapamil	–	58.8
(*R*)-nimodipine	68.1	–
(S)-nimodipine	64.9	–

Involvement of IP3 receptors

The involvement of IP3 receptors (IP3R) in the vasorelaxant actions of CQ and Cin were evaluated using a specific IP3R antagonist xestospongin C (21). Isolated arterial segments were incubated with xestospongin C (0.5 μM) during a 30-minute period, after which the aortic rings were challenged with PE. When the stable contraction was reached, aortic rings were treated, as described in the previous section, with ascending concentrations of either Cin or CQ, which showed a significant vasorelaxant potential. Tissue tension changes were monitored using the same equipment and the results are expressed as a change in the percentage of the tension induced by PE.

Statistical analysis

Data of results were expressed as mean values±standard deviation. Statistical significance was determined by ANOVA followed by Tukey's post hoc test for multiple comparisons using the statistical program GraphPad Prism 5.03 (GraphPad, San Diego, California, USA). Probability values (p) that were equal to or less than 0.05 were considered statistically significant.

Results

Molecular docking

It is important to note that CQ, verapamil, and nimodipine possess a chiral center each, and can therefore occur in enantiomeric forms. As the differences in pharmacological activities of individual enantiomers are not pronounced, all three drugs are administered as racemic mixtures; in our experiments, both enantiomers were considered. The results of the docking are summarized in Table 1. While, as expected, the best ligands for the structure of CavAb bound to (R)-bromoverapamil were (R)-bromoverapamil and both enantiomers of non-brominated verapamil, it should be noted that CQ enantiomers returned higher ChemPLP scores compared to nimodipine native ligand. Cin, on the other hand, scored the lowest for both examined conformations of CavAb (Tab. 1).

This mandated a closer look at the binding of CQ to the L-type calcium channel model. Figure 2 displays close contacts observed for (S)-chloroquine bound to CavAb, as well as those of (R)-nimodipine observed in the crystal structure. No hydrogen bonds are evident in either case, with (S)-chloroquine exhibiting π-stacking with Tyr1195, and an alkyl-π contact with Phe1141. (R)-nimodipine, on the other hand, can only act as a π-donor in a nonclassical hydrogen bond with a Tyr1195 phenol group.

Both CQ and Cin reduced PE-induced vascular smooth muscle contraction

The cumulative addition of CQ and Cin to PE-precontracted aorta smooth muscle cells leads to a concentration-dependent relaxation (Fig. 3). The relaxation observed in the case of CQ was more pronounced than in Cin-exposed tissue segments. Calculated IC_{50} for CQ was found to be 2.6×10^{-5}M (±0.5), while for the Cin IC_{50} was found to be 6.47×10^{-3}M (±0.3).

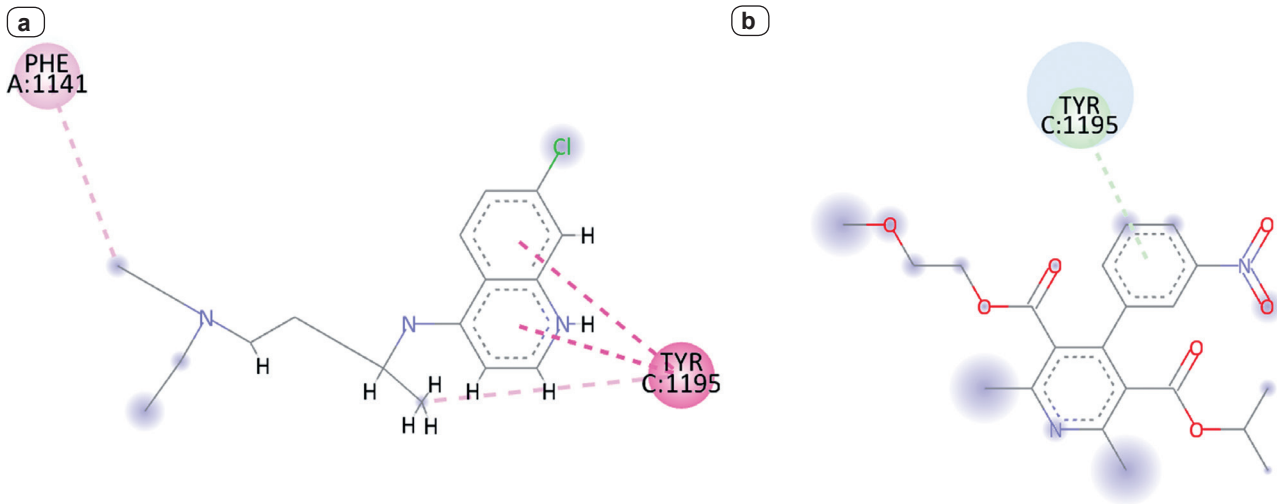


Fig. 2. Close contacts (<4 Å) of (S)-chloroquine docked into CavAb (a) and close contacts (<4 Å) of native (R)-nimodipine in CavAb (b).

Investigation of the underlying mechanism of action of CQ and Cin

For the evaluation of the mechanism of action, we opted for the concentrations that produced a notable statistically significant vasodilator effect on the intact blood vessels. In the first series of experiments, vasodilation actions of CQ and Cin were examined in denuded blood vessels pre-contracted with PE (Tabs 2 and 3). The activities of both CQ and Cin were unaffected by the removal of endothelium, and the percentage of relaxation was almost identical to that of the non-denuded blood vessels (Fig. 3). The same activity was observed for CQ and Cin when L-NAME was applied before the PE contraction (Tabs 2 and 3), where the vasorelaxant action of the test compounds was the same as without L-NAME (Fig. 3).

In the series of experiments evaluating the impact of LTCC on the cell membrane by the application of high K^+ solution, both CQ and Cin exhibited a statistically significant vasorelaxant activity (Tabs 2 and 3). The effect of CQ was found to be more pronounced than that of the Cin, as visible in the experiments with PE-induced contractions (Fig. 3). From the lowest applied concentration, CQ produced a significant vasorelaxant activity, however, its effects were less pronounced than in the case of the PE-induced contractions model (Tab. 2). On the other hand, Cin produced a significant vasodilatatory effect starting from 10^{-5} M, but likewise CQ, did not reach the effects observed in the case when contractions were evoked by PE (Fig. 3).

The activity of CQ was found to be statistically significantly altered in aortic segments exposed to xestospongin C (0.5 μM) and stimulated with PE (Tab. 2). Application of CQ in lower doses was only slightly decreased by the presence of IP3 blockage, while the higher doses were more affected (Tab. 2). The activity of Cin was not found to be affected by the presence of IP3R antagonist xestospongin C (0.5 μM) (Tab. 3) since the percentage of relaxation was almost identical to the observed activity of Cin without antagonist (Fig. 3).

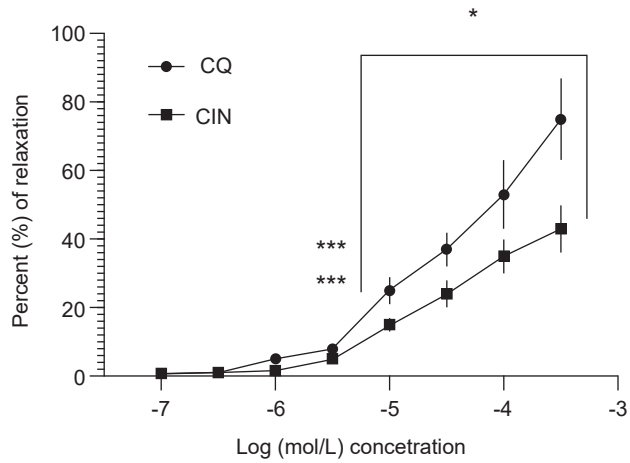


Fig. 3. Concentration-dependent relaxation of PE-precontracted aortic rings in the presence of CQ and Cin. Data are given as mean±SD (n=8) and mutually compared using ANOVA followed by Tukey's post hoc test. *** p < 0.05; *p < 0.001 vs control.

Tab. 2. Percentage of relaxation exerted by chloroquine (CQ) associated with different mechanisms.

	Control	5 x 10^{-5}	10^{-5}	5 x 10^{-4}	10^{-4}	5 x 10^{-3}
Mechanism involving endothelium-derived vasodilator factors						
Denuded blood vessel	0±1.8	10±2**	29±4.2*	41±3.3*	59±6.2*	79±4.5*
L-NAME (30 μM)	0±2.3	9±1.5**	28±3.1*	40±5.2*	55±8.9*	83±2.5*
Mechanism involving voltage-gated cell membrane channels						
K^+-induced contraction	3±1.7	8±1.1**	26±3*	33±4.4*	42±8.5*	65±9.1*
IP3R involvement						
Xestospongin C (0.5 μM)	1±0.6	9±2.3**	23±3.5*	37±5.1*	49±6.2*	74±8.8*

**p < 0.05; * p < 0.001 vs control

Tab. 3. Percentage of relaxation exerted by cinchonine (Cin) associated with different mechanisms.

	Control	5 x 10^{-5}	10^{-5}	5 x 10^{-4}	10^{-4}	5 x 10^{-3}
Mechanism involving endothelium-derived vasodilator factors						
Denuded blood vessel	0±1.8	10±1.4**	16±2.2*	24±2.1*	38±4.6*	41±4.8*
L-NAME (30 μM)	0±2.3	7±3.2**	17±3.1*	25±3.5*	35±4.4*	40±5.2*
Mechanism involving voltage-gated cell membrane channels						
K^+-induced contraction	3±1.7	4±0.7	17±1.5*	21±4.2*	31±7.1*	35±5.4*
IP3R involvement						
Xestospongin C (0.5 μM)	1±0.6	5±1.1**	16±2.7*	22±1.5*	37±2.9*	42±4.8*

**p < 0.05; * p < 0.001 vs control

Discussion

Vascular smooth muscle tension is regulated through several mechanisms and α1AR is among the frequently targeted ones in the pursuit of discovering new vasoactive drugs (6). The mechanism underlining these receptors involves few pathways resulting in the production of several secondary messengers, all leading to smooth muscle contraction and tension increase. Among the secondary messengers responsible for smooth muscle contraction in the present study, the LTCC and IP3 were investigated as possible targets for the two antimalarial drugs. Vasoconstriction induced by PE application is mediated via the IP3 mechanism, thus the role of this signaling pathway was evaluated (11). Also, the role of NO as a vasodilatory molecule was studied in denuded blood vessels and NOS-inhibited segments.

Previous studies performed *in vitro* on isolated guinea pigs' and human blood vessels found out that CQ, as an agonist of taste-sensing type 2 receptors, induces strong endothelium-independent relaxation evoked by PE application (12). The effective concentrations of CQ were in the range from 10^{-6} to 10^{-3} M, which is similar to those applied in the current study and aligns with the findings of the effects of CQ on PE pre-contracted rat aortic rings (Fig. 2). Also, Cin has been previously confirmed to have vasodilatory properties, which is the rationale for the hair growth-promoting action of this compound (13). The claims related to the action of Cin were also confirmed in an i*n vitro* setting using a PE-induced contraction model (Fig. 2).The results also point to the fact that, to a lesser extent than CQ, Cin influences PE-induced contraction of the isolated non-denuded rat aortic segments.

Elimination of the NO source was achieved in the set of experiments where the blood vessels were denuded (endothelium removed by steel wire) (14) or pretreated with an NOS inhibitor, L-NAME (15). The activities of CQ and Cin were not affected by these two procedures, thus the role of NO in the vasorelaxant activity of the two tested compounds could be ruled out. Vasorelaxant effects of CQ in the PE-precontracted blood vessel segments were not endothelium-dependent nor were they associated with NO production/liberation (Tab. 2), as shown also by the results of the previous study (12). Also, the activity of Cin was affected neither by the removal of endothelium nor by blockage of NOS (Tab. 3).

A very useful tool for studying how L-type calcium channels interact with drugs was provided by Catterall and co-workers in the form of the crystal structure of CavAb, a calcium-permeable version of a bacterial sodium channel obtained by mutation (16). This macromolecule acts in many ways like a voltage-gated calcium channel, as it is inhibited by classical LTCC blockers (17). CavAb was crystallized along with its inhibitors, most notably (R)-bromoverapamil (Br-verapamil) and nimodipine. The reported crystal structures were useful for assessing any affinity CQ and Cin might have for LTCC. Molecular docking studies predicted that CQ possessed a significant activity toward CavAb, a suitable model of LTCC, and might indeed act as an LTCC blocker. Conversely, Cin is not predicted to have a significantly high binding activity toward LTCC (Tab. 1).

To a certain extent, the findings of molecular docking studies correlate with the findings involving K^+-induced vasoconstriction which is known to be mediated via voltage-gated Ca^{2+} channels (18). Namely, high K^+-induced tension was decreased significantly by adding CQ and to a lesser extent by Cin. These data indicate that the activity of CQ is significantly associated with LTCC but do not exclude the possibility of the involvement of some other Ca^{2+} channels. Interestingly, CQ in a concentration of 100 μM combined with LTCC blocker nifedipine produced an even greater vasorelaxation effect compared to nifedipine in a concentration of 10 μM alone.[12] These results suggest that CQ might possess some other mechanism of action that induces relaxation or affects other Ca^{2+} channels that could be associated with several locations within the smooth muscle cell. Although Cin was found to modulate ileum tissue contraction through the Ca^{2+}-related mechanism (6), the same effect was not detected in the vascular smooth muscle. The differences in the activity might be related to different Ca^{2+} channels expressed in different smooth muscle cells, e.g., gastrointestinal as opposed to vascular (19, 20).

In the subsequent set of experiments, we used xestospongin C as an IP3R inhibitor which prevents the contraction mechanism mediated through these receptors and is in tight relation with the release of Ca^{2+} from intracellular deposits, e.g., from the sarcoplasmic reticulum (21). The results of the present study indicate that, in higher doses, CQ potentially impacts IP3R, thus preventing the efflux of Ca^{2+} from the sarcoplasmic reticulum to the cytoplasm, while the same effect was not observed for Cin (Tabs 2 and 3). The activity of IP3 is tightly regulated by intracellular Ca^{2+} concentrations. Thus, if a concentration is too low (less than 0.01 μM), the function of the receptors is decreased[22]. By decreasing the influx of Ca^{2+} ions through LTCC, CQ could decrease intracytoplasmic concentrations of Ca^{2+} causing the inhibition of IP3R. This is another mechanism proposed in the pursuit of explaining the effects of CQ on the developed tension in rat aortic smooth muscle cells induced by PE application.

Conclusions

The results of the docking experiments suggest that some of the observed activity of CQ could be attributed to its binding to

the L-type calcium channel or similar targets, while the same is less possible for Cin. The *in vitro* experimental setting revealed that the activity of CQ, and to a lower extent also the action of Cin, are mediated through voltage-gated calcium channels. The involvement of IP3 receptors in the development of vascular tension in the presence of test compounds was found to be less important than that of membrane calcium channels. Also, in association with the observed activities of both CQ and Cin, our results do not exclude other possible mechanisms of action not investigated herein.

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