

Coronaviral Ion Channels as Target for Chinese Herbal Medicine

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ABSTRACT: A variety of viruses encode for proteins that can form ion channels in the membrane of infected cells. For example, the protein coded by the open-reading-frame 3a of SARS coronavirus (SARS-CoV) has been demonstrated to form a cation-selective channel that may become expressed in the infected cell, and its activation is then involved in virus release. Chinese herbal drugs that inhibit the ion channel formed by the 3a protein can be expected to inhibit virus release, and therefore they are a source for the development of novel therapeutic agents. Various drugs found in Chinese herbs are well known as anticancer agents and also have antiviral potency. In one study we tested some of them with respect to their potency to block the 3a channel. Application of the anthraquinone emodin was used as adjunct therapy in treatment of SARS, and we have demonstrated that it can inhibit the 3a ion channel as well as virus release with a $K_{1/2}$ value of approximately 20 μ M. Also the flavonols kaempferol and kaempferol glycosides may be potent inhibitors of the 3a channels. On the other hand, the flavonol quercetin seems not to be effective. In addition, the flavanone naringenin and the isoflavone genistein were ineffective in inhibiting 3a-mediated currents. Antiviral activity of the artemisinin derivative artesunate is well documented, but we did not detect any inhibition of 3a-mediated currents. We suggest that viral ion channels, in general, may be good targets for the development of antiviral agents, and that, in particular, emodin and kaempferol glycosides are good candidates for 3a channel proteins in coronaviruses.

KEY WORDS: Coronavirus, virus release, ion channel, anthraquinone, flavonol, flavanone, isoflavone, artesunate.

ABBREVIATIONS

BHV: Bovine herpes virus; **CMV:** cytomegalovirus; **CoV:** coronavirus; **EV71:** enterovirus 71; **HCV:** Hepatitis C virus; **HCMV:** human cytomegalovirus; **HSV:** Herpes simplex virus; **JEV:** Japanese encephalitis; **SARS:** severe acute respiratory syndrome

I. INTRODUCTION

For treatment of viral infections, drugs are developed that interfere with the viral life cycle (Figure 1). The virus attaches to the host cell, followed by incorporation of the virus, the

uncoating of the viral genome, transcription and translation, and assembly of new viral particles; finally the viruses are released from the host cell.¹ Inhibition of any of these steps could represent a potential target for antiviral drugs. Phytochemicals have gained enormous

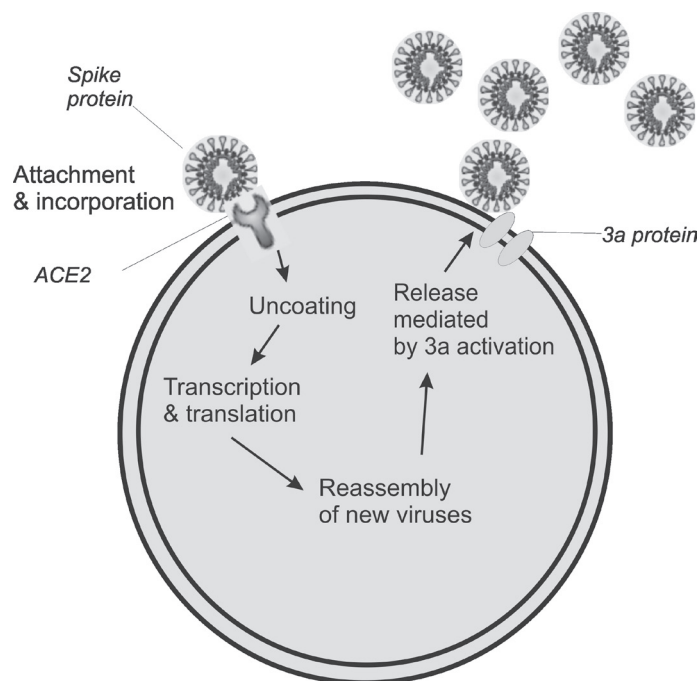


FIGURE 1. Viral life cycle of SARS CoV. Binding of viral spike protein with angiotensine-converting-enzyme-2 receptor of the host cell is followed by virus uptake, uncoating, and transcription and translation of the viral genome. After assembly of new viruses, 3a channel activity stimulates exocytotic release of the viruses from the host cell.

interest with respect to antiviral activities.² Among others, various flavonoids, anthraquinones, and artemisines were considered as potent candidates (Table 1).

Various viruses encode for proteins that form ion-selective channels in the infected cell.^{3–5} They all have important roles in the life cycle of the virus.⁶ In several cases it has been demonstrated that the viral channels become incorporated into the membrane of the host cell, and that activation of these channels seems to be essential for the virus release.^{7–9} Hence, inhibition of ion channel activation counteracts virus release; this may allow the infected body to build up or strengthen its own immune system. The viral ion channel is, therefore, a potential candidate for developing new antiviral drugs.

During first appearance of severe acute respiratory syndrome (SARS), approximately 50% of the patients in mainland China were treated successfully with Chinese herbal medicine in addition to Western medicine.^{10,11} By screening a large number of Chinese herbs, Ho et al.¹² identified the anthraquinone emodin of *Polygonaceae* to interrupt the viral life cycle (Figure 1) by blocking the interaction of the SARS coronavirus (CoV) spike (S) protein with the angiotensine-converting enzyme 2 (ACE2) of the host cell^{13,14} and thus reducing infectivity.

In this review we focus, as an example, on the SARS CoV 3a protein. The open reading frame (ORF) 3a of the CoV encodes for an ion-permeable channel, and its activity in the infected cell may influence virus release.⁹

TABLE 1. Drugs Tested with Respect to Their Effects on 3a-mediated Currents. In addition to the effects on 3a-mediated current are listed references on various antiviral as well as anticancer drugs.

Drug	3a Inhibition	Ref	Antiviral	Ref	Anticancer ref
Emodin	50% at 20 μ M	15, This work	CoV-SARS HSV JEV & EV71	12, 15 29, 30	31
Kaempferol	20% at 20 μ M	This work	HCMV	32	33,34
Kaempferol Glyco- sides	>50% at 20 μ M	This work	HCMV	32	34,35
Quercitin	Ineffective	This work	Influenza HSV-1	36,37	38,39
Naringenin	Ineffective	This work	Dengue virus HCV	40,41	42–45
Genistein	Ineffective	This work	BHV-1 Arenavirus Hemorrhagic fever Pirital virus	46, 47	48, ;49
Artesunate	Ineffective	This work	HCMV, CMV	26	43, 50–52

Because emodin was suggested to have antiviral activity,¹² we previously focused on this anthraquinone.¹⁵ Anthraquinones as well as various flavonoids and artemisines are well known to act also as anticancer drugs,^{16,17} but they have also been discussed as antiviral drugs.² Table 1 summarizes the drugs described in this review with respect to their effects on 3a-mediated currents, and documents by references various known antiviral effects and that all these drugs are well-known for their anticancer effects.

II. METHODS

To investigate functional characteristics of 3a protein, we used the *Xenopus* oocyte for heterologous expression and applied voltage-clamp techniques.^{9,15} For expression of 3a protein, oocytes were injected with 20 ng cRNA (at 1 ng/1 nl) 2 to 3 days before the experiments; uninjected oocytes served as controls. Results from water-injected oocytes did not show any significant difference from the uninjected cells.

Because the 3a-protein ion channel is highly permeable for K⁺, the external medium always was composed of 100 mM KCl; 1 mM MgCl was added as divalent cation salt, and the solution was buffered to pH 7.4 by 5 mM Hepes. In two-electrode voltage-clamp experiments, steady-state current-voltage dependencies were determined using TurboTec 03 voltage-clamp system and CellWorks software (NPI electronic, Germany). In patch-clamp experiments single-channel events were recorded in the on-cell mode using EPC9 and Pulse software (HEKA, Germany).

For the investigation of virus release from infected cells *Rhabdomyosarcoma* cells (RD cells) or FRhK-4 cells were used.^{9,15} For testing the effects of emodin on virus release, cells were first infected, and thereafter, they were incubated in various concentrations of emodin.

All stock solutions of drugs were made up in dimethylsulfoxide (DMSO). Emodine, naringenin, genestein, and kaempferol were purchased from Sigma-Aldrich (St. Louis, MO,

USA), and probes of emodin, and artesunate were purchased from Zelang Medicine Technology Com (Nanjing, China). The probes of the kaempferol glycosides were kindly provided by Prof. X. Hao and Dr. Y. Wang (Kunming, China), who provided juglanin, kaempferol 3-O- α -L-arabinofuranoside and afzelin, kaempferol-3-O- α -L-rhamnoside, and by Prof. A.R. Bilia (Florence, Italy), who provided tiliroside, kaempferol 3-O-(6''-pCm)-glucoside.

III. CORONAVIRUS 3A PROTEIN FORMS AN ION CHANNEL IN THE MEMBRANE OF INFECTED CELLS

III.A. 3a Protein Expressed in *Xenopus* oocytes Induces a Ba^{2+} -Sensitive Current

Oocytes with expressed 3a protein exhibited pronounced currents⁹ that were not detected in uninjected control oocytes. These currents were inhibited by Ba^{2+} (Figure 2). At 10 mM Ba^{2+} , the current was completely inhibited; the $K_{1/2}$ value was approximately 2.7 mM.⁹ In the

presence of 10 mM Ba^{2+} the current was even smaller than in control oocytes, indicating that the control oocytes also exhibited some endogenous Ba^{2+} -sensitive current. Therefore, experiments on control oocytes and injected oocytes of the same batch were always performed in the absence and the presence of 10 mM Ba^{2+} , and the respective Ba^{2+} -sensitive currents were determined. For further analysis, the Ba^{2+} -sensitive endogenous current component was often subtracted from the total Ba^{2+} -sensitive current of injected cell of the same batch. This remaining current component was then considered as the current purely mediated by the 3a protein.

III.B. 3a-Protein-Induced Current Is Mediated by Ion Pores

The cell-attached patch-clamp configuration was applied to oocytes to search for 3a-protein-mediated single-channel events. Because the external bath solutions contained 100 mM K^+ , the membrane potential of the oocytes

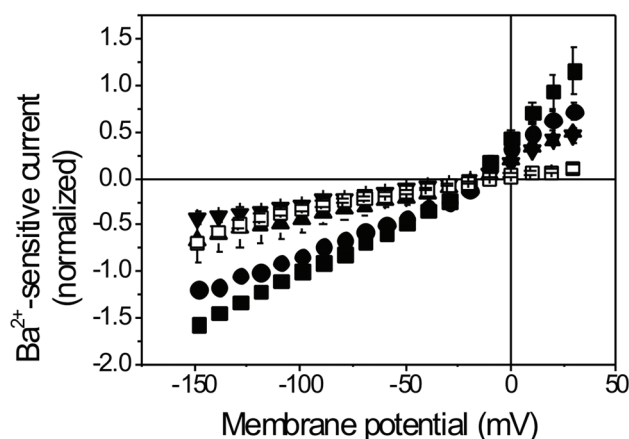


FIGURE 2. Voltage dependence of Ba^{2+} -sensitive current in uninjected control oocytes (open symbols) and in oocytes injected with 20 ng cRNA for 3a protein (filled symbols) in the absence (squares) and in the presence of 1 (circles), 5 (triangles up), and 10 (triangles down) mM BaCl_2 . External medium contained 100 mM KCl, 1 mM CaCl_2 and was buffered to pH=7.2 by 5 mM MOPS (Tris). Data points represent averages from 5 experiments \pm SEM. Based on data from Schwarz et al.¹⁵

was close to zero, and hence the potential across the membrane patch was close to the command potential. Figure 3a shows single-channel recordings from an oocyte that had been injected with cRNA for 3a protein. Non-injected control oocytes never exhibited this type of single-channel event. Histograms of the current amplitudes (Figure 3b) revealed single-channel currents of a few pAs. Figure 3c illustrates the current-voltage dependence with slope conductances of approximately 90 pS at the positive potentials and approximately 30 pS at the negative potentials.

III.C. 3a Protein Is Expressed at the Membrane of the Host Cell and Is Involved in Virus Release

Previously it was demonstrated that the 3a protein is expressed in the surface membrane of infected cells.⁹ The expression of the 3a protein in the infected cell is essential for virus release. Transfecting FRhK-4 cells with increasing amounts of siRNA targeting the 3a gene decreased the number of viral RNA copies in the incubation medium (Figure 4). The same dependency on siRNA amount was obtained for

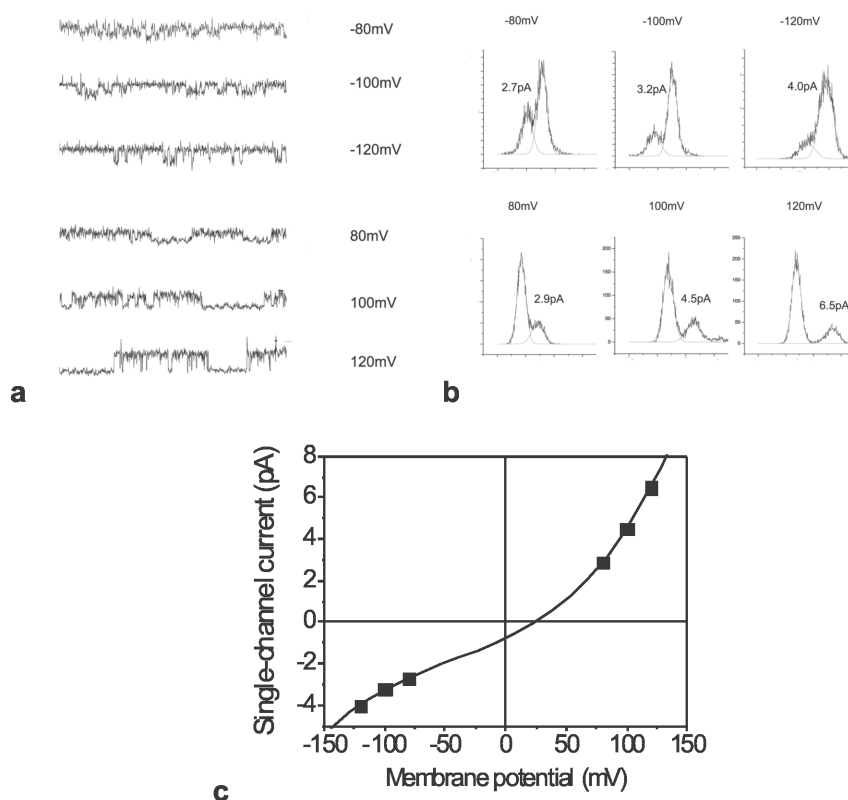


FIGURE 3. (a) Single-channel events at various command potentials (record length 500 ms) in an on-cell membrane patch of a cRNA-injected oocyte. At the negative potentials upward current deflections reflect channel openings, at the positive potentials downward deflections. (b): Histograms of open events at the different command potentials. (c): Current-voltage dependence based on the data shown on panel c.

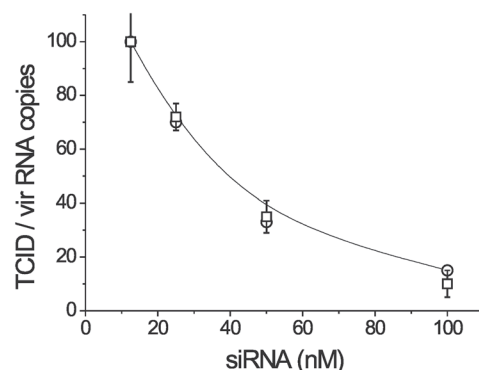


FIGURE 4. Decrease of tissue-culture infective dose (TCID₅₀, circles) and number of viral RNA copies (squares) in incubation medium of HCoV-OC43 infected cells. Data are based on results reported by Lu et al.⁹

the titre measured as tissue-culture infective dose by the method of Reed and Muench.¹⁸

The ORF 3a of SARS CoV is also called “new gene” localized between “spike and envelope gene” (SNE),¹⁹ and has also been identified in other coronaviruses.⁵ The amino acid sequence of the SNE 3a-like protein of HCoV-OC43 shows similarity of approximately 25% with that of the SARS CoV, except for the missing long cytoplasmic C terminus. The protein also forms an ion channel with the high permeability for K⁺.¹⁵ For our further investigation of 3a protein in the infected host cell, we could not use the SARS CoV because it required biosafety level 3. In our laboratory, with biosafety level 2, we instead used the coronavirus HCoV-OC43 for infecting RD cells. cRNA injection into *Xenopus* oocytes led to expression of the ion channels with essentially the same characteristics as the channels formed by the 3a protein of SARS CoV.¹⁵ In particular, the channel is also highly permeable for K⁺ ions and can completely be blocked by 10 mM Ba²⁺. Therefore, the electrophysiological data could be treated and analysed in the way described for the 3a channel of SARS CoV, and we suggest similar function and results.

IV. INHIBITION OF THE VIRAL ION CHANNELS REDUCES VIRUS RELEASE

Though research on antiviral activity of phytochemical drugs has focused on the interference of virus entry to host cell and virus replication steps in the infected cell,² virus release and the involvement of viral ion channels has also recently attracted interest.^{5,6} Here we summarize some results of the effects of known antiviral phytochemicals on the ion-channel-forming 3a protein of coronavirus.

IV.A. Application of Emodin

The anthraquinone emodin (Figure 5) has been demonstrated to inhibit binding of SARS CoV S protein to angiotensin-converting enzyme 2 of the host cell with a K_{1/2} value of 200 μM though infectivity was inhibited by 80% at already 50 μM.¹² We found that, in addition, the 3a-mediated current was inhibited by emodin (Figure 5). The figure illustrates reversibility faster than the 1 minute needed for solution change. The inhibition of 3a-mediated current with a K_{1/2} value of only 20 μM (see Figure 9)¹⁵ might account for the discrepancy between inhibition of virus binding and infectivity by contributing to reduced infectivity with higher

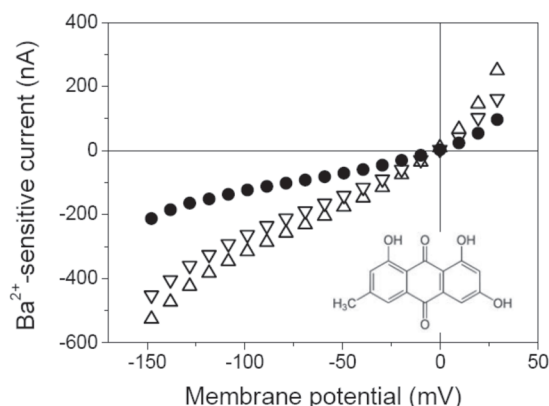


FIGURE 5. Voltage dependence of Ba^{2+} -sensitive current in a 3a-protein-expressing oocyte before (triangles up), during (filled circles), and after (triangles down) application of $50 \mu\text{M}$ emodin. The inset shows the structure of emodin.

efficacy than the S-protein–angiotensin-converting-enzyme-2 interaction.

With the same dependence on concentration of emodin, i.e., with $K_{1/2}$ value of only $20 \mu\text{M}$, release of human CoV-OC43 was inhibited.¹⁵ The current mediated by the 3a-like protein was also inhibited to 50% by $20 \mu\text{M}$ emodin.¹⁵ These findings support the view of antiviral activity of emodin against coronaviruses by blocking 3a ion channels, the activation of which is prerequisite for virus release from the infected host cell.

In addition to emodin, we tested several flavonoids that were reported to have strong antiviral activity² to determine whether they could block the 3a ion channel. These drugs included the flavonols kaempferol (Figure 6a) and quercetin (Figure 7a), the flavanon naringenin (Figure 7b) and the isoflavone genistein (Figure 7c).

IV.B. Application of Flavonols

Among the flavonols, kaempferol had high antiviral activity, and effects on the intracellular events (see Figure 1) were favored as an explanation.²⁰ We found that $20 \mu\text{M}$

kaempferol inhibited 3a-mediated current by approximately 20% (Figure 6a). Even stronger effects were detected with various kaempferol glycosides. Tiliroside (Figure 6b) produced a block of approximately 50% at $20 \mu\text{M}$ (Figure 6b); a similar degree of inhibition was obtained with only $10 \mu\text{M}$ of afzelin (Figure 6c). Furthermore, $10 \mu\text{M}$ of juglanin (Figure 6d) even gave nearly complete block of 3a-mediated current, and 25% inhibition was already detectable at $2.5 \mu\text{M}$ (Figure 6d).

We tested the effect of another flavonol, quercetin, which was reported to act also as an effective drug against virus infections including SARS CoV.²¹ We found that the 3a-mediated current was not significantly affected by $10 \mu\text{M}$ quercetin (Figure 7a).

IV.C. Application of Naringenin and Genistein

The flavanon naringenin and the isoflavon genistein are known for their antiviral potency.^{22–24} Neither naringenin nor genistein exhibited any significant modulation of 3a-mediated current (Figure 7b and 7c).

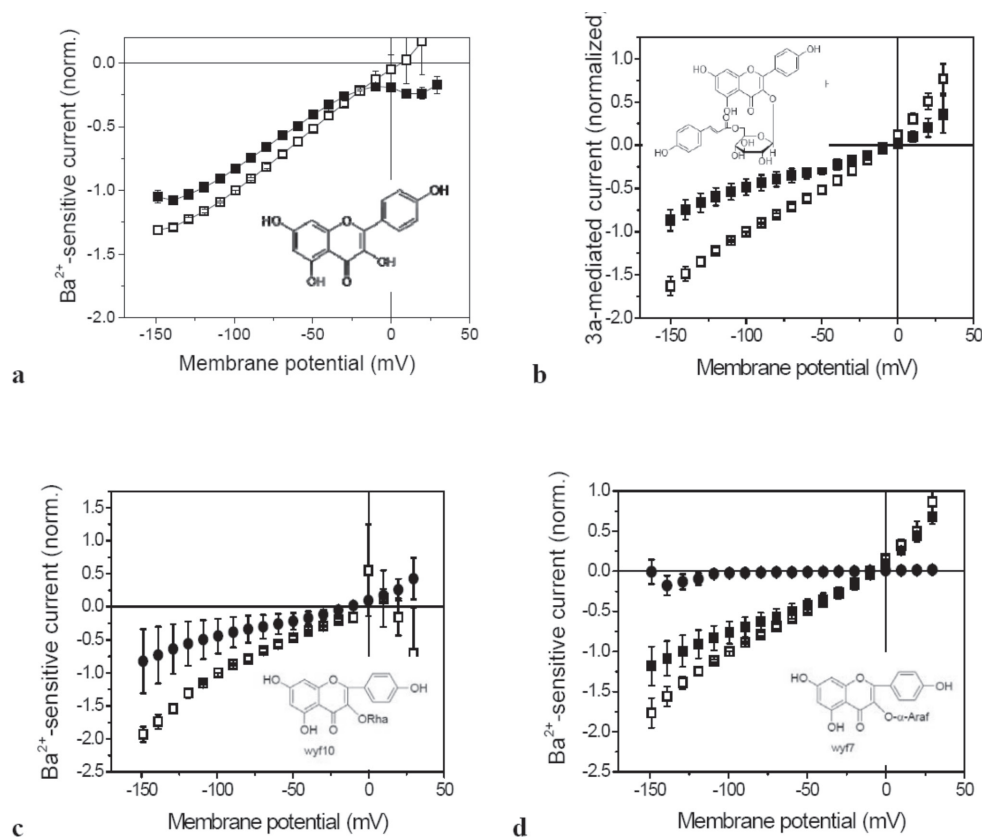


FIGURE 6. Effect of kaempferol and kaempferol glycosides on voltage dependence of Ba^{2+} -sensitive currents in oocytes expressing 3a protein. Open squares refer to data in the absence of drug, filled symbols in the presence of drug. The insets show the structures of the respective drug. (a) kaempferol (20 μM), (b) tiliroside (20 μM). (c) afzelin (10 μM), (d) juglanin (circles: 10 μM , squares: 2.5 μM). Data points represent averages from 4–7 experiments \pm SEM.

IV.D. Application of Artesimines

Artesimines are well known as components of moxa in Chinese medicine. In recent years it was demonstrated that artemisinin and artesunate, a semisynthetic derivative of artemisinin, has activity against various diseases, including malaria and cancer.²⁵ Interestingly, the bioactivity of these drugs is even broader and also includes inhibition of various viruses;²⁶ therefore, we were interested in determining whether artesunate can inhibit the 3a protein.

Artesunate at a concentration of 20 μM had no effect on Ba^{2+} -sensitive current. Figure

8 shows the total Ba^{2+} -sensitive current and illustrates that neither the endogenous current component nor the 3a-mediated current were affected. At the more negative potentials, the 3a-mediated current is the dominating Ba^{2+} -sensitive component; at less negative and positive potentials, it is the endogenous Ba^{2+} -sensitive current.

V. CONCLUSION

Activity of the 3a protein results in ion-channel gating, allowing small cations to cross the membrane; the channel conductance

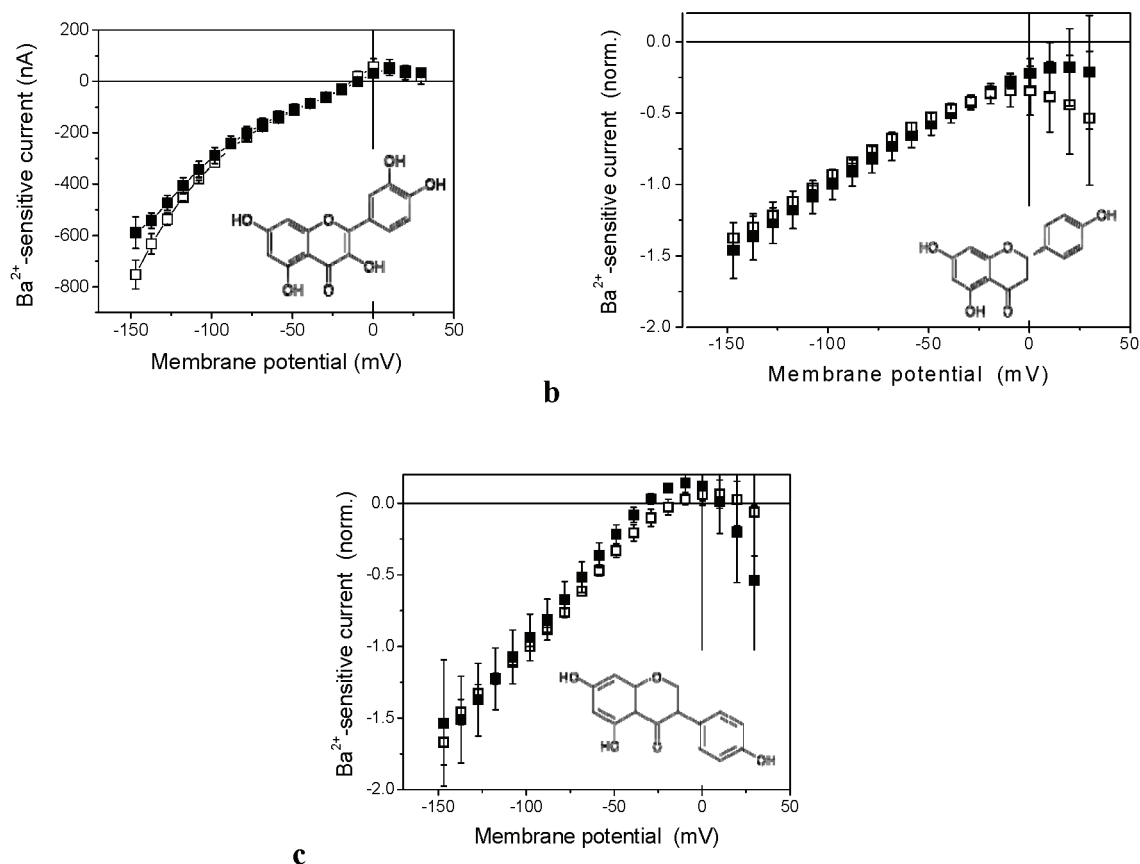


FIGURE 7. Effect of (a) quercetin, (b) naringenin and (c) genistein on voltage dependence of Ba^{2+} -sensitive currents in oocytes expressing 3a protein. Open squares refer to data in the absence of drug, filled symbols in the presence of drug. The insets show the structures of the respective drug which were applied at a concentration of 20 μM . Data points represent averages from 6–10 experiments \pm SEM.

exhibits slight outward rectification (Figure 3c). Though the channel shows selectivity for K^+ , Na^+ can also penetrate with slightly lower permeability.⁹ As a consequence, activity of channel openings leads to membrane depolarization, and activation of L-type Ca^{2+} channels;²⁷ an elevation of intracellular Ca^{2+} can result.²⁸ This could account for the 3a-protein-dependent release of CoV from infected cells via exocytosis. Therefore, inhibition of 3a channel activity can block virus release, offering the body the chance

to adjust its immune system to counteract the viral attack.

All of the phytochemical drugs described in this work are well known for their anticancer activity, but various antiviral effects have also been reported (Table 1). Here we have summarized results demonstrating that some of them may at least contribute to the antiviral effect against CoV by blocking 3a-mediated current and virus release.

Though Figure 9 summarizes that the flavonol quercetin, flavanon naringenin, the

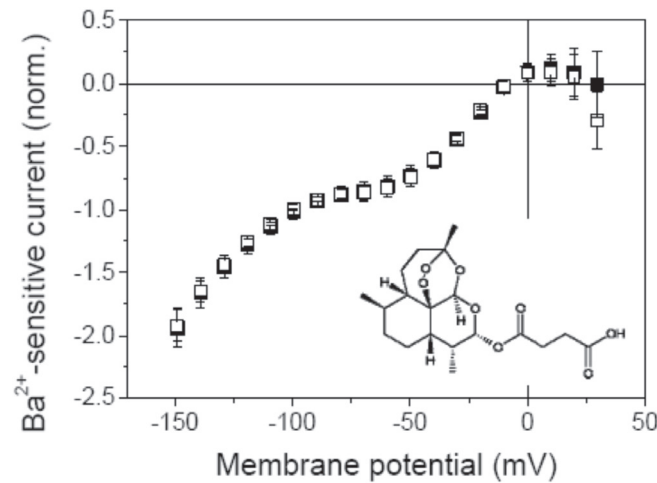


FIGURE 8. Effect of artesunate on voltage dependence of Ba²⁺-sensitive currents in oocytes expressing 3a protein. Open squares refer to data in the absence of drug, filled in the presence of drug. The insets show the structures of the drug which was applied at a concentration of 20 μ M. Data points represent averages from 11 experiments \pm SEM.

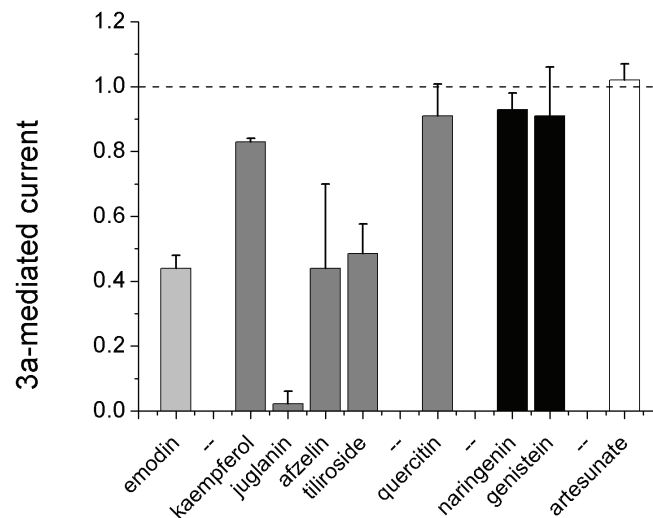


FIGURE 9. Effect of different herbal drugs in the bath medium on 3a-mediated current at -100 mV. Data are normalized to the 3a-mediated current in the absence of drug, and represent averages of 5–10 measurements \pm SEM. For juglanin, afzelin and quercetin the concentrations were 10 μ M, for all the other drugs 20 μ M.

isoflavon genistein as well as artesunate do not affect the activity of the 3a protein, the anthraquinone emodin and the flavonol kaempferol exhibit clear inhibition of the 3a-mediated current; even more potent inhibitors are the kaempferol glycosides,

suggesting the importance of the sugar residues.

Because 3a channel activity is prerequisite for virus release,⁹ inhibition of the 3a-channels results in inhibition of coronavirus release. We were able to demonstrate for emodin¹⁵

the correlation of 3a-channel inhibition and inhibition of virus release, and hence, the other drugs affective in 3a-channel inhibition can also be expected to exhibit antiviral activity via inhibition of virus release from the infected host cells.

In conclusion, we suggest that emodin and kaempferol are promising bases for the development of new antiviral drugs. In particular, the glycosides of kaempferol seem to be highly potent. The fact that these drugs not only block the 3a channel, thus inhibiting virus release, but that they interfere also with other steps of the viral life cycle is in accord with the current research emphasis on the importance of multitarget drugs.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical assistance from Heike Biehl, Heike Fotis and Huiming Du during the various steps of this project. We also thank Prof. Quanbao Gu for helpful discussions.

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