# Rhynchophylline from *Uncaria rhynchophylla* Functionally Turns Delayed Rectifiers into A-Type $K^+$ Channels

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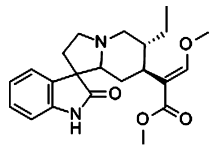
Rhynchophylline (**1**), a neuroprotective agent isolated from the traditional Chinese medicinal herb *Uncaria rhynchophylla*, was shown to affect voltage-gated $K^+$ (Kv) channel slow inactivation in mouse neuroblastoma N2A cells. Extracellular **1** (30 $\mu$M) accelerated the slow decay of Kv currents and shifted the steady-state inactivation curve to the left. Intracellular dialysis of **1** did not accelerate the slow current decay, suggesting that this compound acts extracellularly. In addition, the percent blockage of Kv currents by this substance was independent of the degree of depolarization and the intracellular $K^+$ concentration. Therefore, **1** did not appear to directly block the outer channel pore, with the results obtained suggesting that it drastically accelerated Kv channel slow inactivation. Interestingly, **1** also shifted the activation curve to the left. This alkaloid also strongly accelerated slow inactivation and caused a left shift of the activation curve of Kv1.2 channels heterologously expressed in HEK293 cells. Thus, this compound functionally turned delayed rectifiers into A-type $K^+$ channels.

The hook part of *Uncaria rhynchophylla* Miq. (Rubiaceae) has been used as a traditional Chinese medicine for cardiovascular and neurological diseases.[1,2] An active ingredient of *U. rhynchophylla* is rhynchophylline (**1**).[1,2] This alkaloid possesses antihypertensive and hypotensive properties,[3] which are likely to be attributed to the inhibitory effects of **1** on $Ca^{2+}$ entry in vascular smooth muscle.[4] Rhynchophylline exhibits neuroprotective functions and, for instance, attenuates ischemia-induced neuronal damage in the hippocampus.[5] Therefore, **1** may be of clinical potential in the therapeutic treatment of hypertension and stroke. Compound **1** also protects against glutamate-induced cytotoxicity in cultured rat cerebellar granule cells.[6] Furthermore, this alkaloid may improve memory impairment in mice.[7] The mechanisms for such neuroprotection are still unclear. Since modulation of ion channels has been implicated in neuroprotection,[8,9] it was considered worth examining whether or not **1** affects neuronal ion channels.



**1**

In neurons, voltage-gated $K^+$ (Kv) channels are important in repolarization by allowing $K^+$ efflux, thereby tuning neuronal excitability.[10] Kv channel blockers therefore alter neuronal firing. It has also been known that in neuronal apoptosis, surfacing of excess Kv channels causes cell death by allowing massive $K^+$ efflux.[11] Kv channel blockers therefore may rescue neurons from apoptosis.[8] It is possible that some of the effects of **1** on the nervous system may be via modulation of neuronal Kv channels. There is no previous report on the effects of **1** on neuronal Kv channels.

The Kv channel α-subunit (conducting pore) comprises four polypeptides clustering around a central pore; each polypeptide subunit possesses six transmembrane helices (S1−S6).[12] S4 is the voltage sensor, while between S5 and S6 is the P-loop; the P-loops from the four polypeptide subunits form the $K^+$ selectivity filter.[13,14] When Kv channels open in response to depolarization, the S6 helices, being part of the activation gate, swing open, allowing intracellular $K^+$ to gain access to the internal vestibule of the channel.[13,14] During depolarization, while the activation gate remains open, Kv channels immediately begin to inactivate. Kv channels manifest fast and/or slow inactivation. For the fast type (also termed N-type), the cytoplasmic N-terminus of certain Kv channels (Kv1.4, Kv3.1, and Kv4.2) forms a "ball-and-chain", plugging the opened Kv channel at the internal vestibule.[15] A slow type, also termed C-type inactivation, which may often span seconds, can occur in almost all Kv channels (with or without N-type inactivation).[16,17] The molecular mechanism of such slow inactivation is not completely understood, but could involve destabilization of the outer channel pore surrounding the selectivity filter[10] (also see review by Kurata and Fedida[18]).

In the present study, we determined if **1** modulates Kv currents in mouse neuroblastoma N2A cells (which display delayed rectifier $K^+$ currents upon depolarization[19]) and Kv1.2 channels heterologously expressed in HEK293 cells. Kv1.2 channels are expressed in various neuronal tissues.[20,21] It was found that **1** acted extracellularly to drastically accelerate C-type inactivation of Kv channels. Furthermore, this alkaloid caused a left shift of the activation curve, thus lowering the activation threshold. Hence, **1** functionally converted delayed rectifiers into A-type $K^+$ channels, which are characterized by low activation thresholds and fast inactivation kinetics.[10] The mechanisms and pharmacological implications of these novel actions of **1** are discussed.

## Results and Discussion

**Rhynchophylline (1) Acted Extracellularly to Accelerate Current Decay.** Depolarization of N2A cells triggered an outward current, which decayed very slowly[19] (Figure 1A). Even when the cells were continuously pulsed with depolarizing voltages for more than 10 min, the current decay rate did not change significantly.[19] It should be noted that N2A cells did not display fast-inactivating A-type currents, as depolarizing N2A cells to +30 mV from a very low holding potential (−120 mV) did not trigger any fast-inactivating outward current (data not

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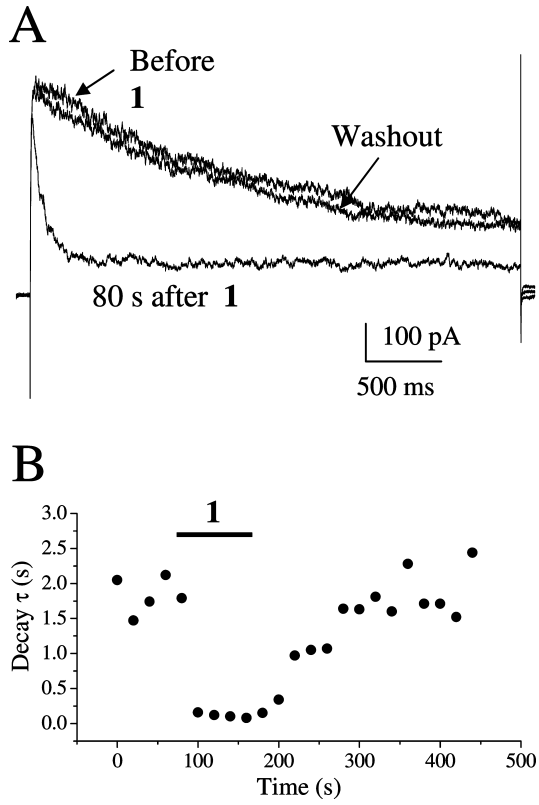


**Figure 1.** Rhynchophylline (**1**) accelerated slow inactivation of Kv currents in N2A cells. (A) Representative traces showing outward $K^+$ currents triggered by +30 mV before and after 30 $\mu$M **1** addition and after **1** washout. (B) Decay time constants as observed in (A) plotted against time. Similar results were obtained in three duplicate experiments.

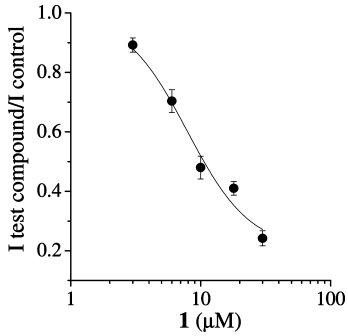


**Figure 2.** Inhibitory effects of **1** on Kv currents in N2A cells. With +30 mV stimulation, the steady-state Kv current (currents at the end of pulse) in the presence of **1** is normalized with the maximum steady-state current (in the absence of **1**) and then plotted against **1** concentration. The curve is fitted with the Hill equation. Results are means ± SEM from 3−11 cells.

shown). As shown in Figures 1A,B, extracellular application of 30 $\mu$M **1** accelerated the decay so that the decay time constant was drastically reduced (decay $\tau$ at equilibrium after test compound = 60.8 ± 9.9 ms, $n$ = 4; decay $\tau$ at time 0 = 1970 ± 101 ms, $n$ = 4; $p < 0.05$). The effect of **1** was fully reversible, as the current decay rate rapidly returned to pretest compound level upon washout (decay $\tau$ = 1905 ± 121 ms; peak current magnitude had 93.3 ± 5.5% recovery; $n$ = 4). Figure 2 shows the concentration-dependent effect of **1** on inhibiting the steady-state currents (currents at the end of pulse). An $IC_{50}$ of 7.9 $\mu$M was obtained and the Hill coefficient was 1.8, suggesting there might be more than one binding sites in the channels for alkaloid **1**.

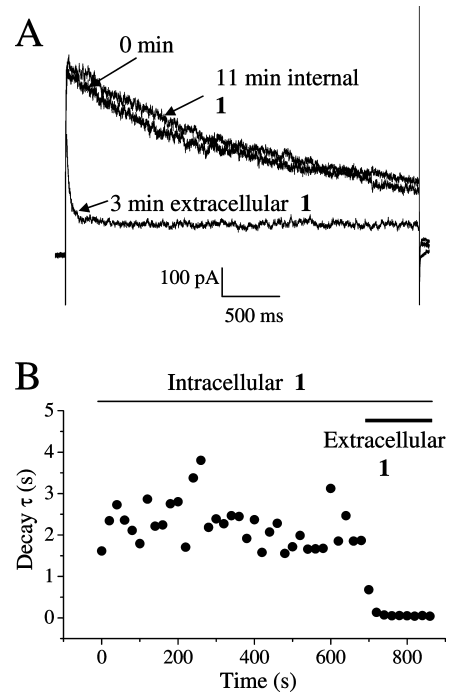


**Figure 3.** Intracellularly applied **1** did not affect slow inactivation of Kv currents in N2A cells. (A) Representative traces showing outward $K^+$ currents triggered by +30 mV just after whole-cell configuration was established, after 11 min of 30 $\mu$M **1** dialysis, and 3 min after bath application of 30 $\mu$M **1** to the same cell. (B) Decay time constants before and after **1** treatment as observed in (A) plotted against time. Similar results were obtained in another two duplicate experiments.

An 11 min intracellular dialysis of 30 $\mu$M **1** did not alter the decay rate (decay $\tau$ at time 0 and 11 min = 1567 ± 104 ms and 1607 ± 157 ms, respectively, $n$ = 3), while a subsequent bath application of 30 $\mu$M **1** caused a substantial acceleration of current decay (decay $\tau$ at equilibrium = 35 ± 9 ms, $n$ = 3) (Figures 3A, B). These data suggest that **1** acted extracellularly.

**Interaction of Rhynchophylline (1) with the Open State of the Kv Channel.** It was then determined if **1** interacts with the closed or open state of the Kv channel. An N2A cell was first depolarized with a +30 mV pulse to record the currents (Figure S1A, Supporting Information). The cell was subsequently exposed to 30 $\mu$M **1** for 4 min without being stimulated through depolarizing pulses. Thereafter, the cell was stimulated again with a +30 mV pulse, and the second current trace was recorded (Figure S1B). The peak current magnitude was not significantly changed (115 ± 13.1% of control; $p > 0.05$, $n$ = 4). Thereafter, repeated stimulation every 20 s resulted in a reduction of peak current magnitude at 6 min (Figure S1C, Supporting Information; 72.5 ± 9.1% of control; $p < 0.05$, $n$ = 4). Washout of **1** resulted in a 98 ± 14.1% recovery (Figure S1D). Since channel opening was required for inhibition of peak magnitude, the data suggested that **1** displays an open channel blockage of Kv channels.

**Rhynchophylline (1) is Not a Direct Channel Pore Blocker.** Whether **1** acts as a direct channel pore blocker was then examined. This alkaloid might reside at the outer channel pore and gain better access to a site at the outer vestibule (or selectivity filter) upon depolarization to account for the accelerated current decay. In other words, does **1** also have an affinity for a site at the pore of the opened channel? If the latter proposal is correct, the **1**−channel interaction is expected to correlate positively with channel openness and may be manifested as faster current decay with stronger depolarization. However, as shown in Figure 4A, decay time constants were voltage-independent in the absence or presence of 30 $\mu$M **1**. Further, as shown in Figure 4B, the percentage block of

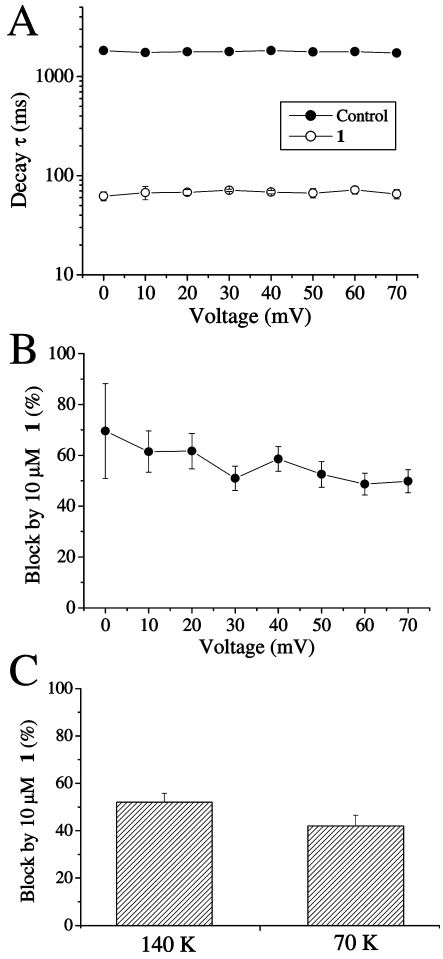


**Figure 4.** Block by **1** was voltage-independent and not affected by intracellular $K^+$ concentration in N2A cells. (A) Decay time constants in the presence or absence of 30 $\mu$M **1** are plotted against voltages. (B) Percentage block of steady-state Kv currents by 10 $\mu$M **1** plotted against the applied voltages. (C) Percentage block of steady-state Kv currents by 10 $\mu$M **1** in the presence of 70 or 140 mM intracellular (i.e., pipet) $K^+$. Results are means ± SEM from 5–6 cells.

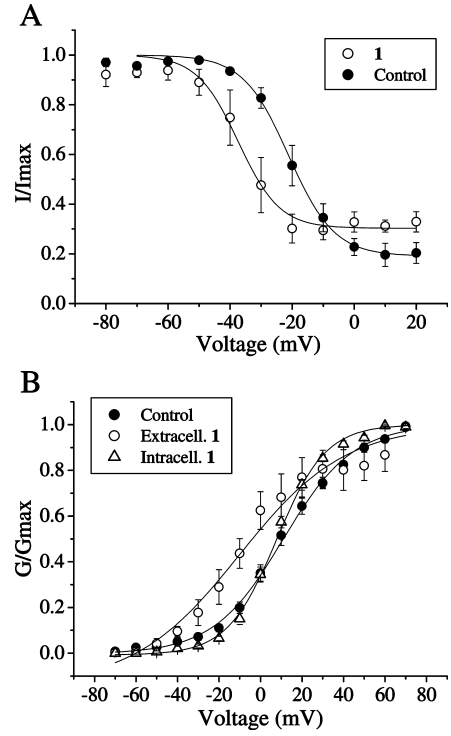


**Figure 5.** Rhynchophylline (**1**) caused left shifts in both steady-state inactivation curves and activation curves. (A) Steady-state inactivation experiments performed in cells treated with and without 30 $\mu$M **1**. In these experiments, a dual-pulse protocol was used in which a test pulse step of +70 mV was preceded by a long prepulse (10 s) of different potentials. The test pulse currents were normalized to the largest test pulse current and plotted against the prepulse voltages. Results are means ± SEM from 5 cells of each group. (B) Voltage-dependence of activation: Kv currents were stimulated with increasing depolarization (from a holding potential of −70 mV and then 10 mV increments), and conductance ($G$) is calculated as described in the Experimental Section. Conductances in the control group and test compound groups (bath and pipet applications) are normalized with their respective maximum conductance ($G_{max}$) and then plotted against the applied depolarization voltages. The curves are fitted with the Boltzmann equation. Results are means ± SEM from 4−10 cells.

steady-state Kv currents by 10 $\mu$M **1** (a concentration producing approximately a half-block, see Figure 2) was independent of the applied voltages, suggesting that **1**−channel affinity did not appear to be correlated with the degree of channel opening. To confirm that **1** did not block by directly occluding the channel pore, the effect of intracellular $K^+$ concentration on **1** action was examined. If **1** directly occludes the pore, then this alkaloid and intracellular $K^+$ would encounter each other in the pore itself. Thus, the lower the intracellular $K^+$ concentration, the greater the block. Reducing the intracellular $K^+$ concentration to 70 mM (with 140 mM sucrose added to keep the intracellular solution iso-osmotic) would be expected to enhance the effects of **1**. This did not significantly affect the % block by 10 $\mu$M **1** (Figure 4C), suggesting that **1** is unlikely to be a direct pore blocker.

**Rhynchophylline (1) Acted on the Inactivation Gate.** The results above are incompatible with the proposal that **1** directly blocks at the outer pore mouth. This compound may indeed accelerate the current decay by accelerating the closing of the inactivation gate. To examine how **1** may affect Kv channel inactivation, we investigated if it could affect the steady-state inactivation of the Kv currents (Figure 5A). In the presence of **1**, the steady-state inactivation curve was shifted to the left by 14 mV ($V_{1/2}$ = −20.9 ± 2.1 mV and −34.6 ± 2.6 mV in the absence and presence of **1**, respectively; $p < 0.05$). However, **1** did not alter the slope factor (6.4 ± 1.1 and 3.7 ± 2.4 in the absence and presence of **1**, respectively; $p > 0.05$).

Rhynchophylline (**1**) accelerated the closing of the inactivation gate, but whether it affected the recovery of the inactivation gate was unknown. A dual-pulse protocol was employed to examine the recovery of the Kv currents (Figure S2, Supporting Information). The recovery was best fitted to a double-exponential function. The Kv currents in the absence and presence of **1** recovered at similar rates, suggesting that this alkaloid does not retard the recovery of the inactivation gate.

**Rhynchophylline (1) Caused a Left Shift of the Activation Curve without Affecting Activation Kinetics.** It was next investigated if **1** affected the activation gating of the Kv currents. This substance caused a left shift in the activation curve (Figure 5B) ($V_{1/2}$ = 10.8 ± 2.4 mV and −6.5 ± 7.4 mV in the absence and presence of **1**, respectively; $p < 0.05$). Rhynchophylline also increased the slope factor (16.1 ± 1.1 and 23.2 ± 4.5 in the absence and presence of **1**, respectively; $p < 0.05$). It is unknown if **1** acts intracellularly or not at the activation gate to alter these gating behaviors. Intracellularly applied **1** (using a pipet), however, did not significantly affect the activation curve ($V_{1/2}$ = 7.7 ± 1.6 mV; slope factor = 11.8 ± 0.4; $p > 0.05$ compared with control). These results suggest that **1** acted extracellularly to affect activation gating.

The effects of **1** on activation kinetics were also studied and analyzed (Figure S3, Supporting Information). Representative traces are shown, illustrating the early kinetics of Kv currents before and after addition of 30 $\mu$M **1**. It was noted that the currents reached their peaks sooner in the presence of this alkaloid. However, it was evident that **1** did not affect the rate of activation. Apparently, C-type inactivation, being accelerated by **1**, prevented the currents from reaching their peaks.

**Rhynchophylline (1) Accelerated Inactivation Gate Closing and Caused a Left Shift of the Activation Curve of Kv1.2 Channels Heterologously Expressed in HEK293 Cells.** It was then determined whether or not **1** can exert similar effects on Kv1.2 gating as in the delayed rectifiers in N2A cells. As shown in Figure S4A (Supporting Information), extracellular application of **1** accelerated current decay drastically (decay $\tau$ at equilibrium after test compound = 30 ± 4.5 ms, $n = 4$; decay $\tau$ at time 0 = 2703 ± 633 ms, $n = 4$; $p < 0.05$). Alkaloid **1** caused a left shift in the activation curve (Figure S4B) ($V_{1/2} = -0.1 \pm 2.2$ mV and $-16 \pm 6.3$ mV in the absence and presence of **1**, respectively, $n = 5$; $p < 0.05$). However, **1** did not affect the slope factor (16.3 ± 1.8 and 19.4 ± 2.1 in the absence and presence of **1**, respectively; $n = 5$; $p > 0.05$).

While there have been reports describing the beneficial effects of **1** on the central nervous system, the mechanisms are not fully understood.[5−7] As Kv channels are critically implicated in neuronal apoptosis,[8,11] we investigated if **1** affects Kv channels. Here it is reported for the first time that **1** may modulate the gating behaviors of Kv1.2 channels and delayed rectifier $K^+$ channels in N2A cells.

Rhynchophylline (**1**) has been shown before to inhibit cardiac $K^+$ channels: mild blockade of delayed rectifiers and very weak block of HERG channels ($IC_{50}$ of about 773 $\mu$M).[23,24] No data were provided as to whether **1** modulates the gating of these channels. In this report, two effects of **1** on the Kv currents of N2A cells were described: a left shift of the activation curve (hence a lowering of activation threshold) and a drastic acceleration of Kv current decay. These dual effects are likely to be mediated by the binding of **1** to extracellular site(s) of the Kv channels, since intracellular dialysis of this compound had no effects at all (Figures 3 and 5B). Therefore, extracellular binding of **1**, intriguingly, modulated the intracellular activation gate. Such modulation only affected the activation threshold, but not the activation kinetics (Figure S3).

Since **1** did not act intracellularly, a direct block at the internal vestibule is unlikely. There are two possibilities to account for the accelerated current decay: (1) a direct, yet rate-limiting, block at the outer pore mouth and (2) enhancement of the C-type inactivation gate. We consider the first possibility to be less likely. Alkaloid **1** acted as an open channel blocker (Figure S1). The proposal of a direct and open channel block may envisage a drug−channel affinity site available upon depolarization (or channel opening). Thus, **1**−channel interaction is expected to correlate positively with the degree of depolarization. However, **1**-induced current decay rate and the percent blockage by **1** were voltage-independent (Figure 4A,B). Further, it was reasoned that if **1** directly occludes the pore, then **1** and $K^+$ would encounter each other inside the pore itself. Thus, it is expected that the lower the intracellular $K^+$ concentration, the greater the block of $K^+$ efflux. However, the percent blockage by 10 $\mu$M **1** was not significantly affected by drastically reducing the intracellular $K^+$ concentration (Figure 4C).

Taken together, the data obtained are therefore incompatible with **1** as a direct channel pore occluder. Since **1** caused a 14 mV left shift in the steady-state inactivation curve (Figure 5A), it is more likely that this alkaloid enhanced the C-type inactivation gate. The molecular mechanisms for C-type inactivation of Kv channels are not fully understood, but it is believed that they involve destabilization of the selectivity filter (or domains around it), hence restricting $K^+$ fluxes.[10,18] Recent evidence supports a mechanistic model in which the interaction strength (via hydrogen bonds) between residues in the selectivity filter and the adjacent pore helix is crucial for C-type inactivation.[25] Thus, in the Kv1.2 channel, mutating Val370 into Glu370 renders a strong interaction with Asp379 possible; such interaction acts as a molecular spring to distort the selectivity filter.[25] Whether such interaction (or similar interactions) is augmented by **1** warrants future investigation. Intriguingly, this compound did not slow the channel recovery rate (Figure S2, Supporting Information), indicating that there might be a very fast drug unbinding following repolarization.

A number of synthetic gating modifiers have been reported to affect the inactivation gate of Kv channels. KN-93, acting in an extracellular manner, has been known to enhance slow inactivation of a number of Kv channels.[26,27] In contrast to **1**, KN-93 did not cause a significant left-shift in the activation curve (although causing a significant decrease in its Boltzmann slope factor).[27] We recently also reported that HMJ-53A, a novel compound, substantially enhances the closing of the inactivation gate, without affecting the activation gate of Kv channels in N2A cells.[19] Interestingly, there are several lipid molecules that could affect both activation and inactivation gates. Linoleic acid, a dietary fatty acid, accelerates slow inactivation of Kv1.5 and Kv2.1 by acting extracellularly but not intracellularly.[28] In a similar manner to **1**, linoleic acid also affects activation gating by causing a left shift in the activation curve.[28] Also of physiological importance is arachidonic acid, which reportedly induces fast inactivation of otherwise noninactivating Kv channels (Kv1.1 and 3.1), thus functionally converting delayed rectifiers into A-type $K^+$ channels.[29] Furthermore, arachidonic acid also accelerates activation of Kv1.1.[30]

Besides the above-mentioned naturally occurring lipids, rhynchophylline (**1**) could be employed as a novel pharmacological intervention agent that modifies activation and inactivation gatings in such a manner as to functionally turn delayed rectifier $K^+$ channels into A-type $K^+$ channels (low activation thresholds and fast inactivation). Such modulation may profoundly affect cell excitability. In addition, the drastic acceleration of current inactivation would limit the amount of $K^+$ efflux; such a property of **1** may be advantageous with regard to treatment of neuronal apoptosis, in which excessive loss of cytosolic $K^+$ via Kv channels becomes lethal.[11]

## Experimental Section

**Chemicals, Cell Culture, and Transfection.** Rhynchophylline (**1**) (>99.0% purity by HPLC) was purchased from Matsuura Pharmaceuticals (Nagoya, Japan). N2A and HEK293 cells were grown at 37 °C in 5% $CO_2$ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and penicillin-streptomycin (100 units/mL, 100 $\mu$g/mL) (Invitrogen).[19] For heterologous expression of Kv1.2 channels, pcDNA3.1-Kv1.2 (from Prof. H. Gaisano, University of Toronto) and pEGFP (as marker, from Clontech, Palo Alto, CA) were transiently transfected into HEK293 cells using TurboFect (Invitrogen) according to the manufacturer's instructions.

**Electrophysiology.** Electrophysiological experiments were performed as previously reported.[22] N2A and HEK293 cells were voltage-clamped in the whole-cell configuration. Thin-walled borosilicate glass tubes (o.d. 1.5 mm, i.d. 1.10 mm, Sutter Instrument, Novato, CA) were pulled with a micropipet puller (P-87, Sutter Instrument) and then heat polished by a microforge (Narishige Instruments, Inc., Sarasota, FL). The typical pipet resistance filled with intracellular solution, containing (mM) 140 KCl, 1 $MgCl_2$, 1 EGTA, 10 HEPES, and 5 MgATP (pH 7.25 adjusted with KOH), was 4−7 MΩ. The bath solution contained (mM) 140 NaCl, 4 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES (pH 7.4 adjusted with NaOH). The currents were recorded using an EPC-10 amplifier with Pulse 8.60 acquisition software and analyzed by Pulsefit 8.60 software (HEKA Electronik, Lambrecht, Germany). Data were filtered at 2 kHz and sampled at 10 kHz. After a whole-cell configuration was established, the cells were held at −70 mV and subject to various protocols as detailed in the text and the figure legends. All experiments were performed at room temperature (~22 °C).

Concentration−response curves for **1** inhibition of steady-state Kv currents were fitted by the Hill equation: $I_{\text{test compound}}/I_{\text{control}} = 1/\{1 +$

$([\mathbf{1}]/K_d)^n\}$ where $I_{\text{test compound}}$ is the steady-state current (currents at the end of pulse) in the presence of **1**, $I_{\text{control}}$ is the steady-state current in the absence of **1**, [**1**] is the concentration of **1** in the bath, $K_d$ is the apparent dissociation constant, and $n$ is the Hill coefficient.

Curves showing voltage dependence of activation in Kv channels are usually generated using tail current analysis. However, as the Kv currents in the presence of **1** inactivated very quickly, tail current analysis was not considered accurate enough. Therefore, Kv currents were stimulated with increasing depolarization, and conductance ($G$) was calculated as $G = I/V - V_r$, where $V_r = (RT/zF)\ln(K_o/K_i)$.

$V$ is the applied voltage, $V_r$ is the reversal potential of $K^+$, $I$ is the current, $R$ is the universal gas constant, $T$ is the temperature, $z$ is the ion valency (+1 in this case), and $F$ is the Faraday constant. $K_o$ and $K_i$ represent bath and pipet $K^+$ concentrations, respectively.

Data for voltage dependence of activation and steady-state inactivation were fitted by the Boltzmann equation: $G/G_{max} = 1/\{1 + \exp[(V_{1/2} - V)/k]\}$ (for fitting voltage dependence of activation), or $I/I_{max} = 1/\{1 + \exp[(V - V_{1/2})/k]\}$ (for fitting steady-state inactivation), where $V_{1/2}$ is the half-maximal activation potential (for voltage dependence of activation) or the half-maximal inactivation potential (for steady-state inactivation), and $k$ the slope factor.

**Statistical Analysis.** Data are presented as means ± SEM. The unpaired or paired Student $t$ test was used where appropriate to compare two groups, and a value of $p < 0.05$ was considered to represent a significant difference.

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**Supporting Information Available:** Figures showing additional electrophysiological experiments conducted on **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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