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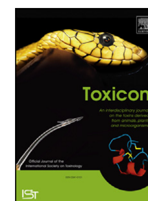

Short communication

Gambierol and n-alkanols inhibit *Shaker* Kv channel via distinct binding sites outside the K^+ pore

Evelyn Martínez-Morales [a,1], Ivan Kopljar [a,1,2], Jon D. Rainier [b], Jan Tytgat [c], Dirk J. Snyders [a], Alain J. Labro [a,*]

[a] Department of Biomedical Sciences, University of Antwerp, 2610 Antwerp, Belgium
[b] Department of Chemistry, University of Utah, 315 South 1400 East, Salt Lake City, UT 84112-0850, USA
[c] Toxicology & Pharmacology, Campus Gasthuisberg, Onderwijs en Navorsing 2, Herestraat 49, bus 922, KULeuven, B-3000, Belgium

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ABSTRACT

The marine polycyclic-ether toxin gambierol and 1-butanol (n-alkanol) inhibit *Shaker*-type K_v channels by interfering with the gating machinery. Competition experiments indicated that both compounds do not share an overlapping binding site but gambierol is able to affect 1-butanol affinity for *Shaker* through an allosteric effect. Furthermore, the *Shaker*-P475A mutant, which inverses 1-butanol effect, is inhibited by gambierol with nM affinity. Thus, gambierol and 1-butanol inhibit *Shaker*-type K_v channels via distinct parts of the gating machinery.

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1. K_v channels have drug/toxin binding sites outside the K^+ pore

Voltage-gated K^+ (K_v) channels are tetramers composed of α-subunits with six transmembrane segments S1-S6 (Long et al., 2005a). The S5-S6 segments assemble into the K^+ pore with a gate in the C-terminal region of S6 (Labro and Snyders, 2012). The S1-S4 segments form the voltage-sensing domains (VSDs) that move upon changes in the membrane potential (Long et al., 2005b; Bezanilla, 2000). An interaction between the S4-S5 linker and C-terminal region of S6 creates the electro-mechanical coupling that translates VSD movements into gate opening/closure (Blunck and Batulan, 2012). The ensemble of regions underlying voltage-dependent channel opening is termed the gating machinery.

Toxins and drugs can potentiate or inhibit K_v channels, which can have a therapeutic potential (Wulff et al., 2009). Gambierol is a polycyclic-ether toxin (MW = 757 g/mol) produced by the marine dinoflagellate *Gambierdiscus toxicus* and is related to ciguatoxins associated with ciguatera food poisoning (Lewis, 2006). Gambierol is a potent inhibitor of K_v1 and K_v3 channels (Cuypers et al., 2008; Kopljar et al., 2009), and has been shown to inhibit K^+ currents in native tissues (Ghiaroni et al., 2005; Schlumberger et al., 2010; Alonso et al., 2012; Perez et al., 2012; Cao et al., 2014). Gambierol most likely operates via a lipid accessible space located between the VSD and the lipid facing side of the pore forming S5 and S6 segments (Kopljar et al., 2009, 2016), a binding site that may correspond with that of the Psora-4 compound (Marzian et al., 2013). Similarly, n-alkanols such as 1-butanol (1-BuOH) act outside the K^+ pore affecting the electro-mechanical coupling (Barber et al., 2011; Bhattacharji et al., 2006; Zhang et al., 2013). Here we report that the *Shaker* channel, the prototypical K_v channel for exploring the gating mechanism, is sensitive to gambierol and show that gambierol and 1-BuOH target different parts of the gating machinery.

2. Gambierol and 1-BuOH do not compete as inhibitors of the *Shaker*-IR K_v channel

Gambierol-sensitive K_v1 and K_v3 channels contain an important

* Corresponding author. Laboratory for Molecular Biophysics, Physiology and Pharmacology, Department of Biomedical Sciences, University of Antwerp, Universiteitsplein 1, CDE T5.51 2610 Antwerp, Belgium.
E-mail address: alain.labro@uantwerpen.be (A.J. Labro).
[1] Both authors contributed equally.
[2] Current address: Discovery Sciences, Janssen Research and Development, 2340 Beerse, Belgium.

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threonine residue in S6 (Kopljar et al., 2009), which is conserved in *Shaker* (T469). Therefore, we expected the *Shaker* channel to be sensitive. In this study we used the fast inactivation removed *Shaker*-IR channel, which was transiently expressed in HEK293 cells and whole-cell ionic currents were recorded with the patch-clamp technique (20 h after transfection). Patch-clamp setup and data acquisition/analysis were similar as described previously (Martinez-Morales et al., 2015). During recordings the cells were continuously superfused with a bath solution (in mM): NaCl 130, KCl 4, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 10, Glucose 10, adjusted to pH 7.35 with NaOH. The intracellular patch-pipette solution contained: KCl 110, K_4BAPTA 5, K_2ATP 5, $MgCl_2$ 1, HEPES 10, adjusted to pH 7.2 with KOH. Application of 300 nM gambierol to *Shaker*-IR resulted indeed in approximately 80% current inhibition (Fig. 1A). This observation differs from a previous study, which used *Xenopus* oocytes as expression system, reporting *Shaker* to be less sensitive (Cuypers et al., 2008). Since gambierol is highly lipophilic the use of HEK293 cells instead of *Xenopus* oocytes is a likely explanation for the different response. Similarly, K_V1.2's gambierol affinity depends on the expression system used (Konoki et al., 2015).

A valine substitution for T469 reduced, as expected, gambierol sensitivity (Fig. 1B–C). However, this *Shaker*-IR-T469V mutant was still inhibited by 1-BuOH suggesting that both compounds have different binding determinants. To investigate this further, we performed competition experiments and compared the experimental data with the predicted level of inhibition using an allotopic (non-competing) or syntopic (competing) binding model (Jarvis and Thompson, 2013). Experiments were done with concentrations near the IC_{50} values as in these conditions the largest difference between both models is expected; thus we used 50 mM 1-BuOH and 100 nM gambierol, respectively. Both compounds were applied to the cells using a pressurized perfusion system as described previously (Kopljar et al., 2009; Martinez-Morales et al., 2015). For each experiment (number of cells analyzed $n = 7$), we determined first the amount of current inhibition by 50 mM 1-BuOH (58.0 ± 2.6%) and 100 nM gambierol (58.5 ± 3.0%) alone.

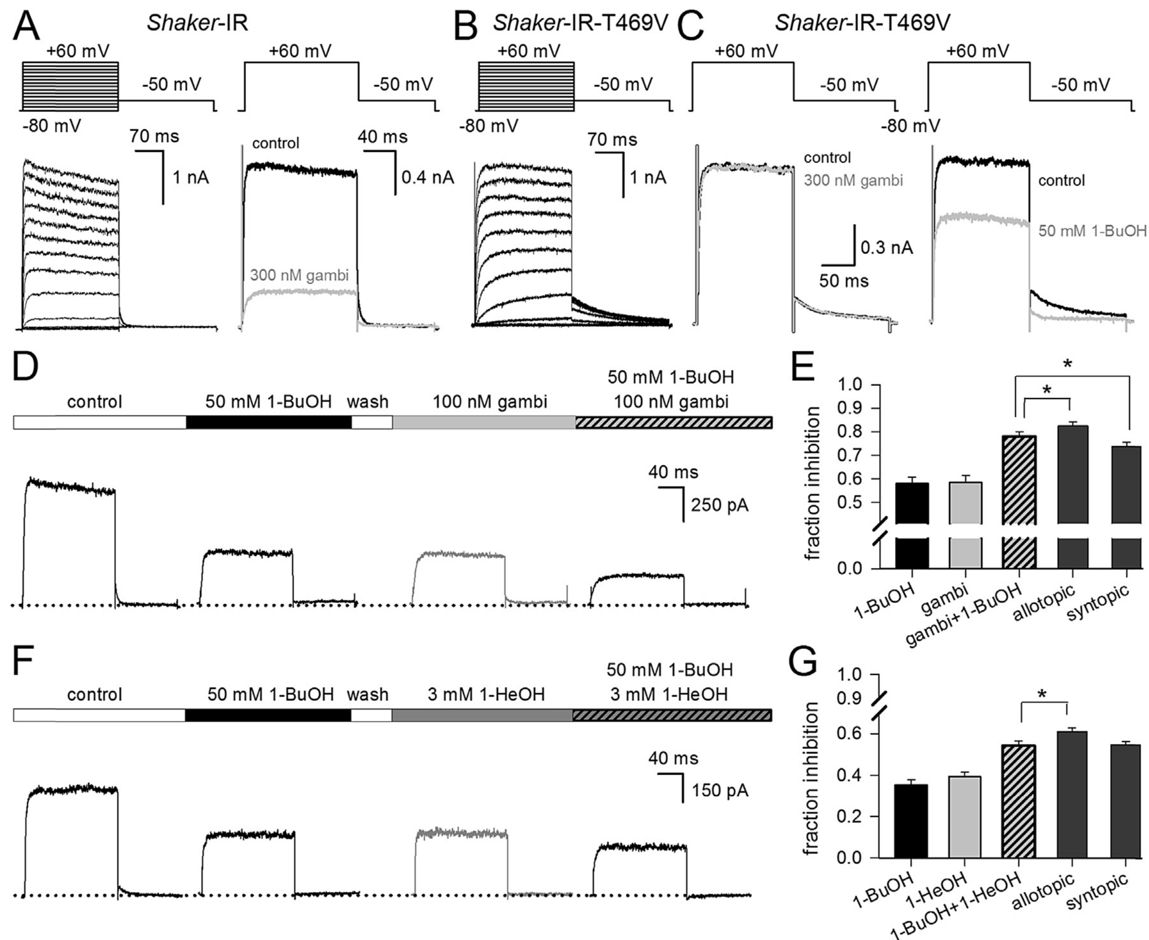

Fig. 1. Gambierol and 1-BuOH do not compete for inhibiting *Shaker*-IR. **A**, Left, ionic currents of *Shaker*-IR channels recorded at 22 °C and elicited with the pulse protocol shown on top. Right, currents of *Shaker*-IR in control conditions (black trace) and upon steady-state inhibition by 300 nM gambierol (gambi, gray trace). **B**, Representative currents of the *Shaker*-IR-T469V mutant elicited with the pulse protocol shown on top. **C**, Steady-state currents of *Shaker*-IR-T469V in control conditions (black trace) and upon application of either 300 nM gambierol (left recordings, gray trace) or 50 mM 1-BuOH (right recordings, gray trace). **D**, Sequentially recorded steady-state currents of *Shaker*-IR elicited by applying a 150 ms long +40 mV depolarization from a holding potential of −80 mV. After the depolarizing step the membrane potential was briefly repolarized to −45 mV to elicit a deactivating tail current. To reach steady-state conditions, depolarizations were repetitively applied with an inter-pulse interval of 10 s. The bar on top illustrates the sequential addition of 1-BuOH and/or gambierol. Below, representative currents recorded from left to right: in control conditions, upon steady-state inhibition by 50 mM 1-BuOH, steady-state inhibition by 100 nM gambierol after washout of 1-BuOH, and finally the current inhibition by the mixture (100 nM gambierol + 50 mM 1-BuOH). **E**, Bar chart shows the average reduction in current amplitude at +40 mV ± S.E.M. (obtained from recordings as shown in D, $n = 7$) after applying 50 mM 1-BuOH, 100 nM gambierol and the mixture gambi+1-BuOH. Fraction inhibition was calculated by normalizing the steady-state current in presence of drug/toxin to the current amplitude in control conditions. The expected inhibition according to an allotopic or syntopic model was calculated as described in the text. Note, the experimentally obtained inhibition with the mixture differed statistically (using paired t-tests) from the predicted value of either model (*, $p < 0.05$). **F**, Steady-state currents of *Shaker*-IR recorded upon sequential addition of 50 mM 1-BuOH, 3 mM 1-HeOH after washout of 1-BuOH, and the mixture 50 mM 1-BuOH + 3 mM 1-HeOH. **G**, Bar chart shows the fractional reduction in current amplitude at +40 mV ± S.E.M. ($n = 5$) after applying 50 mM 1-BuOH, 3 mM 1-HeOH and the mixture. The inhibition obtained with the mixture differed only statistically from the predicted value of an allotopic model (*, $p < 0.05$).

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