LB51

Automated electrophysiology combined with photoaffinity labeling indicates distinct blocking and modulator binding sites on voltage gated sodium channels

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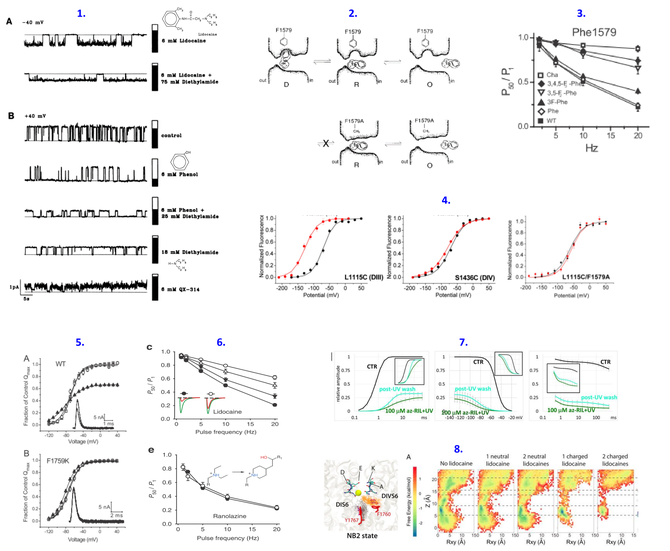
Background:

Hints of two kinds of inactivated state inhibition in the literature:

1. Zamponi & French	*Biophys J*	*1993*	**"fast"** *vs.* **"slow"** block	**Aromatic ring** (phenol) produces slow block **Charged N⁺** (diethylamine) produces fast block
2. Kimbrough & Gingrich	*J Physiol*	*2000*	**"rapid"** *vs.* **"discrete"** block	**F1579** is needed for **discrete** block
3. Ahern, Eastwood, Dougherty & Horn	*Circ Res*	*2008*		**F1579** needed for $V_{1/2}$ **shift** & **use-dependent inhibition** unmodulated block without F1579
4. Muroi & Chanda	*JGP*	*2009*		**F1579** needed for **DIII S4** $V_{1/2}$ **shift** (modulation)
5. Hanck, Fozzard, Lipkind & Sheets	*Circ Res*	*2009*	**"lipophilic"** vs. **"voltage-sensor inhibition"**	**F1579** needed for voltage-sensor inhibition (modulation)
6. Pless, Galpin, Frankel & Ahern	*Nature Comm*	*2011*	**"cation-pi"** vs. **"non-cation-pi"**	**F1579** needed for $V_{1/2}$ **shift** & **use-dependent inhibition** unmodulated block without F1579
7. Lukacs, Földi, ... & Mike	*Sci Rep*	*2018*	**"block"** *vs.* **"non-blocking modulation"**	Conduction is possible with fully occupied binding sites. Part of the binding sites must be "**non-blocking modulatory**".
8. Nguyen, DeMarco, ... & Yarov-Yarovoy	*PNAS*	*2019*	**F1760** – binding site **Y1767** – binding site	**2** distinct pore lumen **binding sites** for lidocaine Neutral lidocaines may **allow conduction**

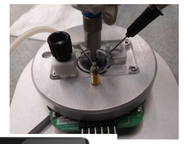


All these data are consistent with the hypothesis that two distinct binding sites exist for small sodium channel inhibitors:

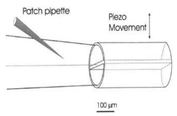
- **F1579 BS** – **All sodium channel inhibitors cause modulation**
  – **Large or charged ones also cause channel block**
  – **Small neutral ones only cause non-blocking modulation**
- **non-F1579 BS** – **All sodium channel inhibitors cause channel block**

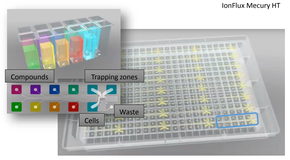
Methods:

- **WT** and **F1579A** mutant **rNav1.4** expressing CHO cells
- Nanion's **Port-a-Patch** + **310 nm UV LED** controlled by the voltage-clamp amplifier
- Different timing of UV pulses
- Photoactivatable analog of riluzole
- **IonFlux Mercury** – Special protocol for maximal information content
- Manual patch clamp + **Ultrafast solution exchange**

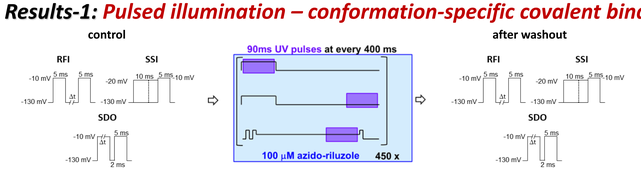


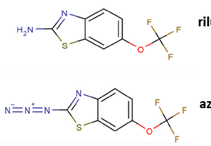
IonFlux Mecury HT



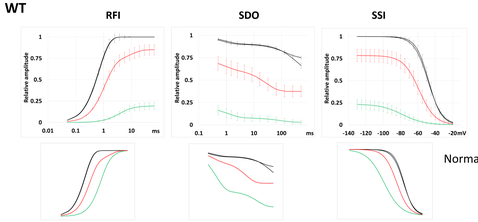


Results-1: Pulsed illumination – conformation-specific covalent binding:





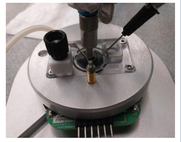
WT



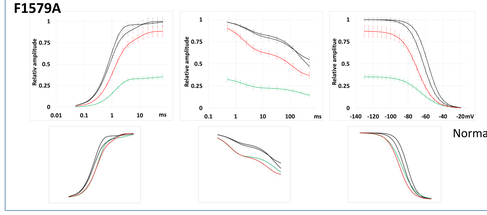
After washout all ligands still present are **covalently bound**.
the shift in **SDO**, **RFI** and **SSI** curves is **not** due to binding/unbinding dynamics but to genuine modulation of gating

Covalent binding to **resting** and **inactivated** conformation of **WT** channels.
***Resting* – weak block + weak modulation**
***Inactivated* – strong block + strong modulation**

Normalized curves show the extent of modulation



F1579A



Covalent binding to **resting** and **inactivated** conformation of **F1579A** channels.
***Resting* – weak block + weak modulation**
***Inactivated* – strong block + *weak* modulation**

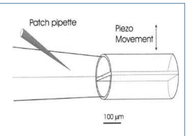
Normalized curves

Summary of azido-riluzole data

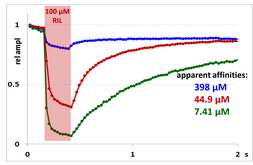
	WT resting	WT inactivated	F1579A resting	F1579A inactivated
low affinity **block**	+		+	
moderate affinity **block**		+		+
high affinity **modulation**		+		

Results-2: Ultrafast solution exchange:

The 3-Pulse Train PROTOCOL was repeated every 26 ms.
There was **10 ms at -120 mV** between two trains



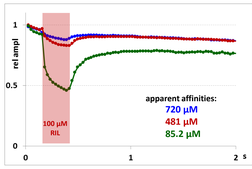
WT



Binding does not equal channel block.

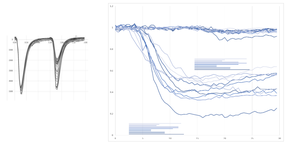
- Dissociation of riluzole is relatively slow, as reflected by the **third pulse**
- At the **first pulse** minimal block was seen, even when riluzole was present
- **2 ms** was clearly not enough for dissociation (see **second pulse**)
- **10 ms** was enough for almost complete recovery
- Could riluzole "completely dissociate **between 2 and 10 ms** in each cycle?

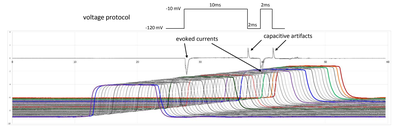
F1579A



- **Modulation** of recovery **is absent** in the F1579A mutant, as seen in the response to the **second pulse**
- Delayed recovery of the **third pulse** is also lost.
- **F1579** is responsible for **high affinity binding** and **modulation**
- However, at inactivated conformation, the drug can still bind with moderate affinity

Measuring the onset rate of inhibition by riluzole

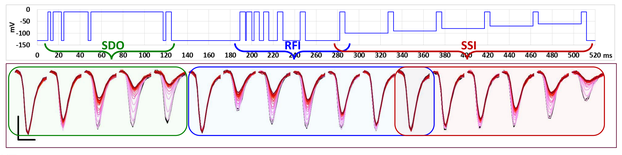




Onset is fast (τ = 4.87 ms), but delayed

Drug association is not manifested in channel block, only in modulation

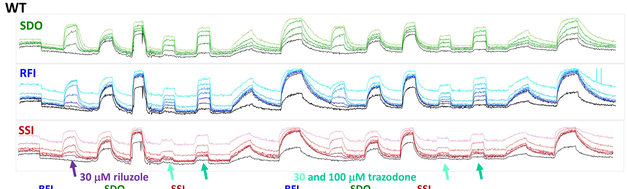
Results-3: Monitoring changes in gating kinetics:



The PROTOCOL consists of 17 depolarizations
It is designed to collect **SDO**, **RFI** and **SSI** data with **1 s resolution**.

EVOKED CURRENTS at each depolarization.
10 consecutive control currents are shown in **black**
20 consecutive currents after the start of **30 µM riluzole** perfusion are shown in **light to dark red**

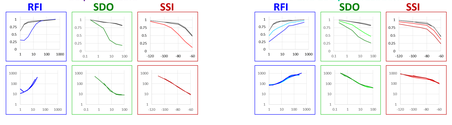
WT



PLOT OF PEAK AMPLITUDES during an **1684 s** experiment.
(1648 x 17 points)

Drug applications:
30 µM riluzole,
10 and **30 µM imipramine,**
30 and **100 µM trazodone,**
10 and **30 µM chlorpromazine.**

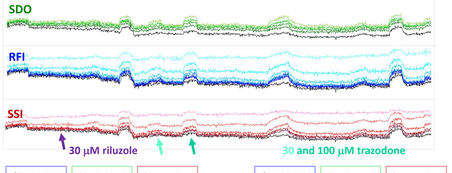
Each drug application is repeated twice.



SDO, RFI and SSI plots for control and drug,
shown on the example of riluzole and trazadone

APPARENT AFFINITY values calculated for each of the 17 evoked currents.
It shows the dynamics of drug binding/unbinding,
as well as modulation of gating kinetics.

F1579A mutant



SDO, RFI and SSI plots for control and drug,
shown on the example of riluzole and trazadone

APPARENT AFFINITY values:
F1579A mutation completely abolished the effect of **30 µM riluzole**
The effect of **trazodone**, on the other hand was not much affected.

Conclusions:

Results-1 section:

- We provided experimental evidence for two distinct binding sites at sodium channels
- Using covalently bound azido-riluzole we could exclude the effect of state-dependent dynamic association/dissociation
- The **F1579 BS** has high affinity, and is responsible for most of the modulation
- For certain drugs (especially small neutral ones) binding causes **non-blocking modulation**
- For large or charged molecules binding causes block and modulation
- An alternative **moderate affinity** binding site exists
- It is only accessible at inactivated conformation
- It causes minimal modulation in the case of azido-riluzole

Results-2 section:

- The most plausible mechanism for the action of riluzole is **non-blocking modulation**
- The ability of riluzole to cause **modulation is abolished in the F1579A mutant**
- Riluzole is able to reach its binding site **with a time constant of ~5 ms**, but its effect is only manifested in modulation, not block
- Like azido-riluzole, riluzole is also able to bind to a **moderate affinity non-F1579 BS**

Results-3 section:

- We are able to assess major properties of gating at **1 s resolution**
- Drug onset & offset kinetics on the time scale of **seconds**, as well as drug effects on gating kinetics on the time scale of **milliseconds** can be followed
- Different mechanisms of inhibition can be identified
- Interaction with F1579 can be tested
- The example of trazodone: **Modulation** is possible **at a non-F1579 BS** as well

Funding: Hungarian Brain Research Program (KTIA-NAP-13-2-2014-002) GINOP-2.3.2-15-2016-00051