Activities for Optimizing CiPA Recommended Protocols in Patch-Clamp Assay

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INTRODUCTION

Revising the ICH regulatory guideline, S7B, for the Non-clinical Evaluation of the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation by Human Pharmaceuticals) has been suggested and Comprehensive *In Vitro* Proarrhythmia Assay (CiPA) activities are being implemented. We previously studied the applicability of the CiPA-recommended voltage protocols for hERG, Cav1.2, and late Nav1.5 current patch clamping which was announced last year (this research was presented at the 2019 Japanese Safety Pharmacology Society annual meeting). As a result, inter-facility differences were found for some compounds in the hERG assay on an automated patch-clamp system, QPatch. Such differences were also noted in enhancement ability of ATX-II for late Nav1.5 current. This time, we investigated the factors for the inter-facility differences, and studied more appropriate protocols. In addition, we present the results of the assays by the CiPA-recommended protocols for each ion channel. Manual patch clamp and QPatch system were selected as the platforms. For the voltage protocols, the CiPA-recommended ones were followed whereas the composition of intra- and extracellular buffers were modified. Preparation procedures and application methods of test solutions (such as application duration or number of replicates) for the QPatch assay were examined. Test compounds were selected from each of the low-, intermediate-, and high-risk categories of the "training drugs" in the CiPA in silico model. We present the comparison results of our data by the CiPA-recommended protocols and the data given by the CiPA. We also discuss buffer compositions, preparation procedures or application methods for test solutions to reduce the inter-facility differences. We would like to show and discuss the more appropriate protocols including the detailed experimental methods, which are not given in the CiPA-recommended protocols, and points to be modified.

MATERIALS AND METHODS

◆ DRUGS

TdP Risk	High	Intermediate	Low
CiPA Drug	Bepridil	Astemizole	Ranolazine
	Dofetilide	Chlorpromazine	Verapamil
		Cisapride	

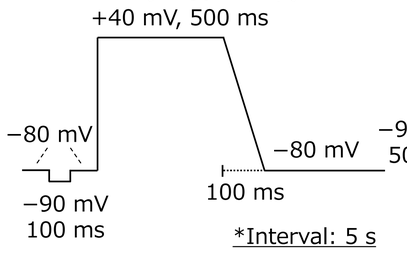
\+ Flecainide

◆ PLATFORMS

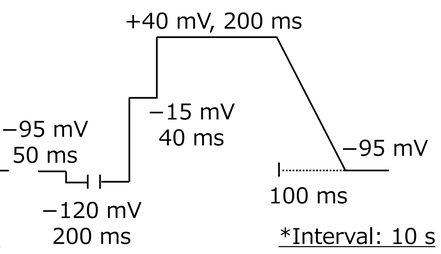
- Manual Patch Clamp System: Axopatch200B or EPC8 + pClamp
- Automated Patch Clamp System: QPatch and QPatch II

◆ VOLTAGE PROTOCOLS

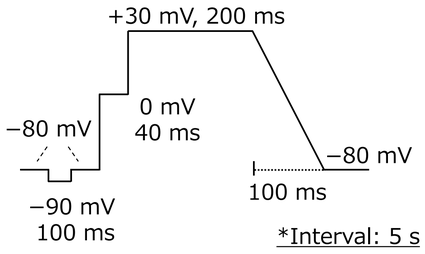
- hERG (IC_{50} Only)



- Late Nav1.5



- Cav1.2

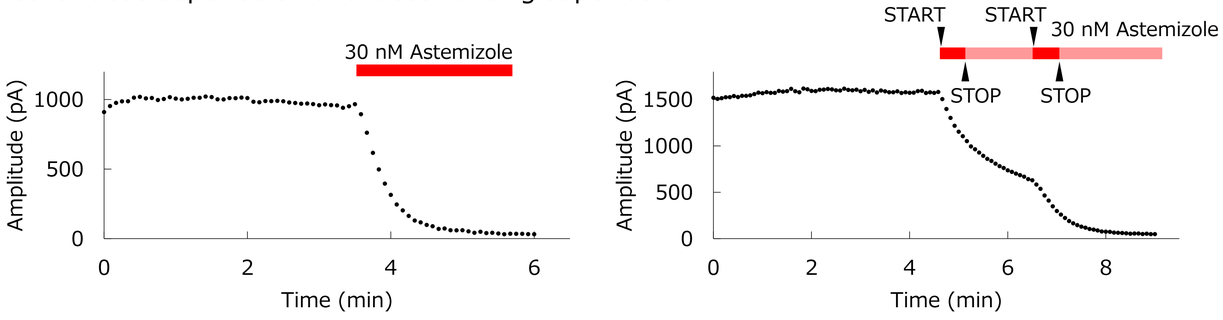


RESULTS

◆ hERG CURRENT

- Optimizing Application Methods

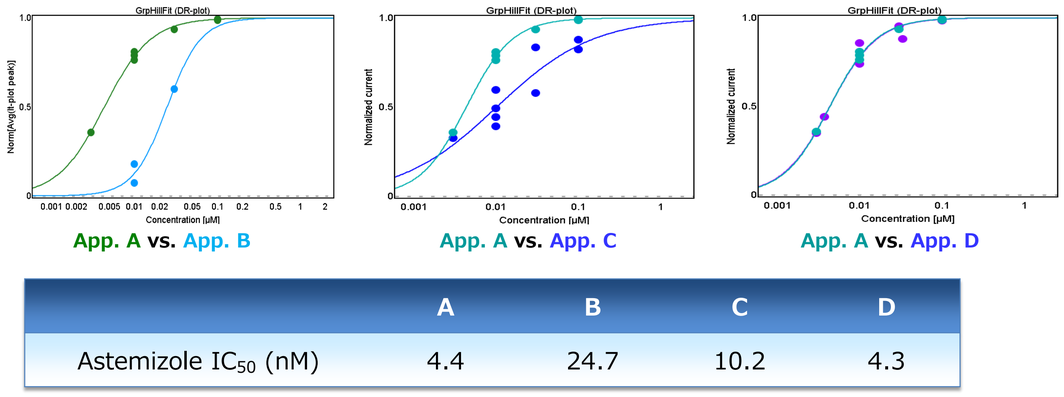
➢ In manual patch clamping, the reactivity of sticky compounds could be variable between continuous superfusion and discontinuing superfusion.



➢ To optimize the application method in QPatch, we confirmed the difference in effects of astemizole in the following patterns.

App. A: 8 time additions (2 repetition × 5 μL)
App. B: 1 time addition (2 repetition × 5 μL)
App. C: 1 time addition in large volume (4 repetition × 5 μL)
App. D: 1 time addition in large volume (4 repetition × 5 μL) + 7 time additions (2 repetition × 5 μL)

➢ Results



App. A vs. App. B　　App. A vs. App. C　　App. A vs. App. D

	A	B	C	D
Astemizole IC_{50} (nM)	4.4	24.7	10.2	4.3

➢ To mimic the continuous superfusion, we selected a method of adding 8 times (1 concentration) or 4 times (2 concentrations) at every 20 pulses.

- Inter-facility Comparison

Drug	Site A			Site B			Site C		
	IC_{50} (μM)	Hill	*n*	IC_{50} (μM)	Hill	*n*	IC_{50} (μM)	Hill	*n*
Bepridil	0.26	1.9	6-7	0.28	1.3	6	0.031	1.3	6
Dofetilide	0.015	1.5	6-8	0.011	1.8	6	0.0095	1.2	6
Astemizole	0.026	1.4	6-9	0.026	1.3	6	0.0042	1.2	6
Chlorpromazine	1.7	2.0	6-7	0.85	1.6	6	0.17	1.2	6
Cisapride	0.078	1.6	6-10	0.061	1.3	6	0.0080	0.93	6
Ranolazine	9.0	0.92	6	9.0	0.91	6	3.5	0.89	6
Verapamil	0.90	2.1	7-10	0.63	1.2	6	0.13	1.1	6
Flecainide	1.8	1.0	7-8	1.2	0.98	6	0.44	0.82	6

Drug	Site D			Site E			Site F		
	IC_{50} (μM)	Hill	*n*	IC_{50} (μM)	Hill	*n*	IC_{50} (μM)	Hill	*n*
Bepridil	0.10	1.6	7-8	0.11	1.4	4-12	0.046	0.92	5
Dofetilide	0.014	1.8	6-8	0.0094	1.0	3-7	0.0098	1.1	5
Astemizole	0.0066	1.0	6-9	0.0075	1.2	11-16	0.00085	0.90	5
Chlorpromazine	0.50	1.3	6-8	0.46	1.4	5-9	0.47	0.97	5
Cisapride	0.019	0.93	6-11	0.016	1.3	8-12	0.030	1.0	5
Ranolazine	5.2	0.89	7-9	8.4	0.89	2-3	6.0	0.99	5
Verapamil	0.35	0.90	6-8	0.36	1.2	2-3	0.66	1.1	5
Flecainide	0.59	0.90	6-8	–	–	–	0.74	1.0	5

*The platform of site F is manual patch clamp

➢ The results on the QPatch were almost similar in all the facilities, showing comparable results to manual patch-clamp results. Exceptionally, results of some compounds varied.

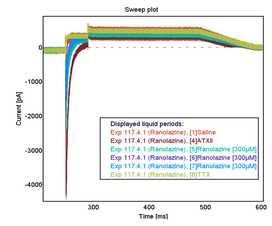
- Investigation of Preparation Method

➢ We investigated dilution methods of the DMSO solution of compounds. However no influence of the difference in the dilution procedure was noted (data not shown).

◆ LATE Nav1.5 CURRENT

- Comparison of Intracellular Solutions for Late-current Enhancing Ability

➢ With the CiPA-recommended intracellular solution, the late current enhanced by ATX-II was often insufficient. Since the membrane resistance showed lower values than usual (200-400 MΩ) in such cases, high membrane resistance seems to be required for enhancement of the late current. Thus we used F^--containing intracellular solution in the investigation.

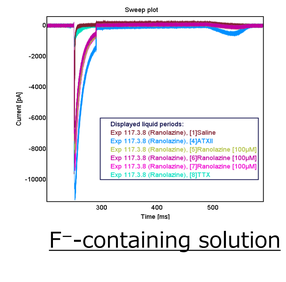


CiPA solution

➢ With the F^--containing intracellular solution, sufficient late current could be observed in the depolarizing-pulse and ramp-down phases at a high rate.

➢ F^--containing intracellular solution (in mM): 10 NaCl, 135 CsF, 5 EGTA, 10 HEPES, pH7.3

➢ We roughly examined the effect of fluoride on the gating property of Nav1.5 channel, and no notable effect was found (data not shown).



F^--containing solution

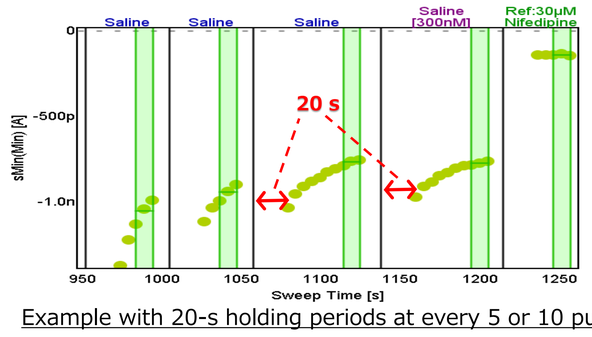
- Effects of Difference in Intracellular Solutions

Drug	CiPA Solution (Site A)					F^--containing solution (Site C)				
	−15 mV Step		Ramp down		*n*	−15 mV Step		Ramp down		*n*
	IC_{50} (μM)	Hill	IC_{50} (μM)	Hill		IC_{50} (μM)	Hill	IC_{50} (μM)	Hill	
Bepridil	1.9	1.5	1.2	0.92	3-5	1.6	2.5	0.71	1.6	6
Astemizole	1.7	1.8	1.1	1.3	3-6	0.52	1.8	0.31	1.9	6
Chlorpromazine	1.9	2.6	1.3	1.9	3-4	1.8	2.3	0.69	2.1	6
Ranolazine	52	1.7	29	1.7	7-8	40	1.2	17	1.2	6
Verapamil	14	1.4	6.3	1.3	5	14	0.87	3.7	0.87	6
Flecainide	1.5	1.4	1.3	1.0	3	3.9	1.5	2.2	1.2	6

➢ The IC_{50} was similar with the F^--containing intracellular solution and the CiPA-recommended one; however, the value for astemizole differed by approximately 3 times.

◆ Cav1.2 CURRENT

➢ In Cav1.2 current measurement on the QPatch, notable rundown was seen. This seemed to have been caused by the short pulse intervals (5 s). Setting stimulation-holding periods could control rundown.



Example with 20-s holding periods at every 5 or 10 pulses

CONCLUSIONS

✓ In assessment of hERG current, it became clear that the difference in application methods of the test solution leads to the difference in the current-suppression effect. For the QPatch, the recommend to apply the test solution of 1 concentration multiple times to mimic the continuous superfusion.

✓ In assessment of the late Nav1.5 current, necessity of high seal resistance was suggested for enhancement by ATX-II. The F^--containing intracellular solution, with which seal resistance was higher than the CiPA-recommended one, enables assessment of the late current at good success rate.

✓ The reason for those inconsistent results for the hERG and late Nav1.5 currents will be studied in the future.

✓ In Cav1.2 current, notable rundown was seen; this seemed to have been caused by short (5 sec) interval pulses set by the CiPA recommended protocol. Such rundown could be controlled by setting longer holding period at a certain number of pulses.

✓ We will continue this research activity to optimize protocols for assessment of hERG, late Nav1.5, and Cav1.2 currents, and present the achievement.