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Original Article

Assessment of Testing Methods of the Drug-Induced Repolarization Delay and Arrhythmias in an iPS-Derived Cardiomyocyte Sheet: Multi-Site Validation Study

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Abstract

A prospective comparison study across 3 independent research laboratories of a pure I_{Kr} blocker E-4031 was conducted by using the same batch of the human iPS cell-derived cardiomyocytes in order to verify the utility and reliability of our original standard protocol. Field potential waveforms were recorded with multi-electrode array system to measure the inter-spike interval and field potential duration. The effects of E-4031 at concentrations of 1 to 100 nM were sequentially examined every 10 min. In each facility, E-4031 significantly prolonged the field potential duration corrected by Fridericia's formula and caused early afterdepolarizations occasionally resulting in triggered activities, whereas it tended to decrease the rate of spontaneous contraction. These results were qualitatively and quantitatively consistent with previous non-clinical in vitro and in vivo studies as well as clinical reports. There were inter-facility differences in some absolute values of the results, which were not observed when the values were normalized as percentage change. Information described in this paper may serve as a guide when predicting the drug-induced repolarization delay and arrhythmias with this new technology of stem cells.

Keyword: E-4031, iPS cell-derived cardiomyocytes, Multi-site validation, Field potential, TdP

Introduction

The drug-induced proarrhythmia has been a major safety concern about the development of new drugs, leading to issue of ICH E14 and S7B guidelines in May 2005 (1,2). The guidelines have effectively reduced risks of new compound causing torsades de pointes, whereas non-clinical and clinical studies in the current approach remain still imperfect because they identify many drugs as being "positive" despite a lack of demonstrable proarrhythmic risk (3-6). In a recent workshop held in July 2013 by US Food and Drug Administration (FDA), the Cardiac Safety Research Consortium and the non-profit Health and Environmental Sciences Institute (HESI), a new paradigm was proposed and discussed, focusing on a comprehensive assessment of multi ion channel effects to determine actual proarrhythmic risk of drugs (7). This new approach will include a stem-cell technology that has the potential to improve the currently used assessment of cardiotoxicity; however, more work is required prior to the use of stem cell-derived cardiomyocyte models to accurately predict proarrhythmias in human (7).

There have been a large number of various studies with stem cell-derived cardiomyocytes examining electrophysiological effects of drugs (8-13). In an effort to further improve upon the assay system, this report describes a more simple and reliable protocol of an induced pluripotent stem (iPS)-cell derived, cardiomyocyte-sheet model. The extensive preliminary studies have confirmed that the protocol proposed in this paper could be optimal for assessing E-4031-induced repolarization delay and arrhythmias, and would qualitatively and quantitatively reflects its electropharmacological profile in human. This is a critically new finding and a significant improvement over the previous in vitro I_{Kr} assay systems including the hERG potassium channels and the papillary muscle of guinea pigs. In this study, in order to start verifying the reproducibility of our protocol, a prospective comparison study of E-4031 was conducted across 3 independent research laboratories with the same batch of the iPS cell-derived cardiomyocytes.

Methods

Cell culture and plating

Each facility (E, N, T) obtained the same batch (#1089404) of the cryopreserved human iPS cell-derived cardiomyocytes (iCells; Cellular Dynamics International (CDI), Madison, WI, USA). The cells were thawed in specially prepared medium (Plating Media; CDI), which were plated onto 0.1% gelatin-coated, 6-well tissue-culture plates (Becton Dickinson, Franklin Lakes, NJ, USA) at a density of 1.3-2.6 x 10^6 (E: 1.3 x 10^6, N: 2.0 x 10^6, T: 2.4-2.6 x 10^6) of cells per well. Two days after plating, Plating Media was replaced to specially prepared culture medium (Maintenance Media; CDI). Then, the culture medium was changed with fresh one every 2 days. The cells were cultured for 3.7±1.4 days (2-7 days) after thawing at 37°C with 5% CO_2 prior to re-plating.

The electrical activity of cardiomyocytes was measured by using our original protocol. Briefly, the recording area of probes with 64 of recording electrodes (MED probe; MED-P515A, Alpha Med Scientific, Osaka, Japan) of MED64 System (Alpha Med Scientific) was coated with 2 μL of fibronectin (50 μg in 1 mL of distilled water), which was incubated at 37 °C for ≥1 h. The cells cultured in the 6-well tissue-culture plates were dispersed with 0.25% trypsin-EDTA or TrypLE Select, which were re-plated onto the MED probes at a density of 3 x 10^4 cells in a 2 μL of the culture medium. The cells were incubated at 37°C with 5% CO_2 for 2-18 h (E: 4-12 h, N: 2-18 h, T: 12-18 h) in moisture condition prior to filling each probe with 1 mL of the culture medium. The half volume or all of the culture medium of the probes was changed with the culture medium which had been warmed to 37°C every 2 days thereafter. The cells were cultured for 5.2±1.6 days (3 to 7 days) to obtain a sheet of cardiomyocytes with spontaneous and synchronous electrical automaticity.

Field potentials (FPs) assay

Maintenance Media was used as a culture medium throughout the experiment. Prior to the measurement of FPs, cardiomyocyte sheets were

equilibrated for ≥30 min in the CO_2 incubator in 1 or 2 mL of fresh culture media. After equilibration, the probes were kept at 36-37°C with thermo-control systems, and covered with a lid, through which aeration of 95% O_2/5% CO_2 gas was provided. FPs from spontaneously beating cardiomyocyte sheets were recorded and digitized at 20 kHz by using MED64 System. The stability and constancy of the waveforms, inter-spike interval and field potential duration (FPD) were confirmed for ≥20 min. FPD was defined as an interval from the initial sharp deflection to the peak of dome (8). Using the information obtained in this observation period, we selected 3-6 electrodes, which would be suitable for continuous monitoring of the FP configuration consisting of spike and dome. After recording the basal control state, the effects of 1, 3, 10, 30 and 100 nM of E-4031 were assessed by adding stock solution cumulatively to the culture medium to obtain target concentrations. The final concentration of DMSO was limited to be <0.6%, since DMSO in concentration of <0.6% has been reported hardly to affect any of the variables assessed in this study (8). At each concentration, the FP was recorded for ≥10 min and the last 30 beats were extracted as a dataset to analyze waveforms, inter-spike interval and FPDs according to the previous report (8). The datasets of concentrations were excluded from the statistical analysis, when early afterdepolarization and/or triggered activity were observed. Early afterdepolarization was defined as deflection occurring at the plateau of dome, and sharp deflection originating from early afterdepolarization was judged as a triggered activity. FPD was corrected with Fridericia's formula which was defined as the primary method of correction in this study (FPDcF = FPD/(inter-spike interval /1000)$^{1/3}$) (14). The values of inter-spike interval and FPDcF from the last 30 waves at each concentration were averaged.

Drugs and chemicals

E-4031 was obtained from WAKO (Osaka, Japan) or synthetized at Eisai Co., Ltd (Tsukuba, Japan). Gelatin was obtained from Sigma (St. Louis, MO, USA). Fibronectin was obtained from Becton Dickinson or Invitrogen (Carlsbad, CA, USA). Trypsin-EDTA and TrypLE Select were obtained from Invitrogen.

Data analysis and statistical assessment

In each experiment, one electrode that satisfied the following two conditions was chosen: 1) FP was recorded whole through the experiment, and 2) The amplitude of the dome was the largest. The data were expressed as mean±SE. The effects of the drug on inter-spike interval and FPDcF obtained in each facility were evaluated with paired t-test or one-way repeated-measures analysis of variance (ANOVA) followed by Contrasts for mean values comparison between the baseline value (0 nM) and others. Meanwhile, inter-facility variability was assessed with one-way factorial ANOVA followed by Fisher's test or unpaired t-test. A p value <0.05 was considered statistically significant.

Results

The effects of E-4031 in concentrations of 0, 1, 3, 10, 30 and 100 nM were examined in each facility, except that 1 nM was not performed in facility N. The number of preparations that can be used for the assessment of inter-spike interval, field-potential duration and categorical analysis decreased due to the onset of early afterdepolarization and/or triggered activity as the concentration of drug increased.

Inter-spike interval

The effects of the drug on the inter-spike interval (ms) are summarized in Fig. 1 (upper panel). The baseline values (0 nM) were 926±44 ms in facility E, 1,216±56 ms in facility N and 956±22 ms in facility T. Inter-facility difference was observed between N and E besides between N and T, which was not detected between E and T. No significant change from the respective baseline values was detected at 1, 3 or 10 nM in E and T, and at 3 nM in N. Inter-facility difference was observed at 3 nM between N and E besides between N and T, which was not detected at any concentration between E and T. Meanwhile, the effects of the drug on the inter-spike interval (%) are summarized in Fig. 1 (lower panel). The significant increase was observed at 3 nM in N, which was not detected at 1, 3 or 10 nM in E or T, although the similar trend was observed. Inter-facility difference was not detected at any concentration.

Prolongation of field-potential duration

The effects of the drug on the FPDcF (ms) are summarized in Fig. 2 (upper panel) and typical tracings of field potential before and after the drug treatment are depicted in Fig. 3. The baseline values (0 nM) were 430±12 ms in E, 443±5 ms in N and 320±15 ms in T. Inter-facility difference was detected between T and E besides between T and N, which was not detected between E and N. FPDcF was prolonged at 3 and 10 nM in E, and at 10 nM in T, which tended to be prolonged at 3 nM in N without statistical significance. Inter-facility difference was detected at 1, 3 and 10 nM between E and T, and at 3 nM between N and T.

Inter-facility difference was not detected at any concentration between E and N. Meanwhile, the effects of the drug on the FPDcF (%) are summarized in Fig. 2 (lower panel). FPDcF was prolonged at 3 and 10 nM in E, and at 10 nM in T, whereas it tended to be prolonged at 3 nM in N without statistical significance. Inter-facility difference was not detected at any concentration.

Incidence of early afterdepolarization or triggered activity

The incidence of early afterdepolarization or triggered activity is summarized in Table 1 and typical tracing of field potential with triggered activity is depicted in Fig. 3. Early afterdepolarization or triggered activity was induced at ≥10 nM in N, and at ≥30 nM in E and T.

Categorical analysis of FPDcF

The results of categorical analysis of absolute FPDcF (ms) are summarized in Table 2. At the baseline and 1 nM, all FPDcF in each facility were categorized in ≤480 ms, whereas FPDcF of ≥500 ms was observed at ≥3 nM in N and at ≥10 nM in E, which was not observed in T.

The results of categorical analysis of ΔFPDcF are summarized in Table 3. At 1 nM all ΔFPDcF in E and T were categorized in ≤60 ms. ΔFPDcF of >60 ms was observed at ≥3 nM in E and N, and at ≥10 nM in T.

Discussion

In this study, a prospective comparison study of E-4031 was conducted with the same batch of the human iPS cell-derived cardiomyocytes in order to start verifying the reproducibility of our original standard protocol across 3 independent research laboratories. We demonstrated that the protocol can be reliable in detecting the drug-induced repolarization delay and arrhythmias with high reproducibility.

E-4031 tended to show a negative chronotropic effect at concentrations of ≥3 nM; however, a significant change was detected only at 3 nM in facility N when assessed by percentage change (Fig. 1). The more potent negative chronotropic effect was observed by higher concentrations of E-4031 in each facility, although we did not perform the statistical analyses on inter-spike interval at concentrations of ≥10 nM in facility N and ≥30 nM in facilities E and T because of the limited number of experiment (n=0-3). These results are in good accordance with a previous observation in patients with supraventricular tachyarrhythmias (15), in which E-4031 at a plasma concentration of 4.85±1.35 ng/ml (11 nmol/L) modestly prolonged RR interval, but it did not achieve statistical significance. Meanwhile in the single sinoatrial nodal cells of rabbits, E-4031 at a concentration of 100 nM suppressed or blocked the spontaneous activity (16), and moreover in the Langendorff-perfused whole hearts of guinea pig, E-4031 at concentrations of 30-300 nM or 5 μM significantly reduced the heart rate (17, 18). Thus, our testing method using the human iPS cell-derived cardiomyocytes can be considered to be more sensitive than currently available in vitro non-clinical models in detecting the E-4031-induced negative chronotropic effect.

E-4031 caused early afterdepolarization and/or triggered activity in a concentration-related manner as shown in Fig. 3 and Table 1. In previous studies using the Langendorff-perfused rabbit heart, 0.5 μM of E-4031 induced early afterdepolarization and triggered activity (19,20). Also, in the human embryonic stem cell-derived cardiomyocyte clusters, 1 μM of E-4031 induced early afterdepolarization in half of the clusters (11). Meanwhile, in the human

engineered heart tissue sheet made of the human embryonic stem cells, 10 nM of E-4031 was reported to induce arrhythmias (12). Thus, our testing method as well as the previous human engineered heart tissue sheet is considered to have higher sensitivities than the human cardiomyocyte clusters or the Langendorff-perfused rabbit heart in detecting E-4031-induced early afterdepolarization and/or triggered activity.

E-4031 prolonged the FPDcF in a concentration-related manner as shown in Figs. 2 and 3. A wide variety of analyses have been performed to clarify the effects of E-4031 on repolarization process as summarized in Table 4 (12,15,17-26). The effects of E-4031 in these previous reports are directionally the same as the currently observed results, although their potency varied greatly. Thus, the present findings suggest that the sensitivity of current testing method to detect drug-induced repolarization delay can be considered to be comparable to hERG assay and human subjects, but it may be higher than in vivo and in vitro animal models.

Since 82% of the basal FPDcF were ≤450 ms which is upper limit of normal range of QTc in human subjects, we examined the repolarization delays with the categorical analysis described in ICH E14 guideline. FPDcF >500 ms and/or ΔFPDcF >60 ms were detected at concentrations of ≥3 nM in some preparations (Tables 2 and 3), indicating that 3 nM of E-4031 will be a critical concentration for inducing the excessive QT-interval prolongation by this testing method.

The major purpose of this study was to clarify the extent of the inter-facility difference in sensitivity and reliability of this new testing method. The concentrations of E-4031 that caused early afterdepolarization and/or triggered activity were close to each other among the 3 facilities; however, there were some variations in the basal absolute values of inter-spike interval and FPDcF. Since we used the same batch of the cardiomyocytes, these differences might be induced by small inter-facility variability in the net culture period, the cell density on recording electrodes and/or experimental temperature. It should be noted that there was no difference in inter-spike interval or FPDcF among the 3 facilities when compared using percentage change.

In conclusion, we demonstrated that the use of the standardized protocol

for the iPS cell-derived cardiomyocyte sheets can minimize inter-facility difference in detecting the drug-induced repolarization delay and arrhythmia. While further studies are needed to establish the currently proposed protocol including the assessment of variability across batches, more reference compounds and clinical predictability, information described in this paper may help predict the potential of the drug-induced repolarization delay and arrhythmias with this new technology. Also, methodological work for high-throughput evaluation is now ongoing.

Conflict of interest statement: The authors declare no conflicts of interest.

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Figure legend

Fig. 1

Summary of the results showing the actual measurement values (upper) and their percentage changes (lower) in inter-spike interval (ISI) of E-4031 in human iPS cell-derived cardiomyocytes in each facility. Each value represents the mean±S.E. of 6 preparations for facility E and facility N and 5 preparations for facility T. Values in parentheses represent the number of the preparations. Asterisk indicates significant difference between the facilities, whereas closed symbol represents significant change from respective baseline value.

Fig. 2

Summary of the results showing the actual measurement values (upper) and their percentage changes (lower) in FPDcF of E-4031 in human iPS cell-derived cardiomyocytes in each facility. Each value represents the mean±S.E. of 6 preparations for facility E and facility N and 5 preparations for facility T. Values in parentheses represent the number of the preparations. Asterisk indicates significant difference between facilities, whereas closed symbol represents significant change from respective baseline value.

Fig. 3

Typical tracings of the field potential from each facility. Upper traces show the field potential at control (Control); middle traces indicate the prolongation of field potential duration (FPD) with 3-30 nM of E-4031; and lower traces represent the onset of early afterdepolarization or triggered activity with 10-100 nM of E-4031 (arrow). ISI: inter-spike interval.

Table 1. Incidence of E-4031-induced early afterdepolarization or triggered activity.

Concentration (nM)	Facility						All	
	E		N		T			
0	0%	(0/6)	0%	(0/6)	0%	(0/5)	0%	(0/17)
1	0%	(0/6)	NT		0%	(0/5)	0%	(0/11)
3	0%	(0/6)	0%	(0/6)	0%	(0/5)	0%	(0/17)
10	0%	(0/6)	83%	(5/6)	0%	(0/5)	29%	(5/17)
30	100%	(6/6)	100%	(6/6)	40%	(2/5)	82%	(14/17)
100	100%	(6/6)	100%	(6/6)	100%	(5/5)	100%	(17/17)

NT: Not tested

Note: Numerator in parentheses shows the number of preparations exerting early afterdepolarization or triggered activity, whereas denominator indicates the total number of preparations assessed.

Table 2. Summary of categorical analysis of absolute FPDcF values.

Concentration of E-4031 (nM)	FPDcF (ms)	Facility						All	
		E		N		T			
0	≤450	67%	(4/6)	83%	(5/6)	100%	(5/5)	82%	(14/17)
	>450	33%	(2/6)	17%	(1/6)	0%	(0/5)	18%	(3/17)
	>480	0%	(0/6)	0%	(0/6)	0%	(0/5)	0%	(0/17)
	>500	0%	(0/6)	0%	(0/6)	0%	(0/5)	0%	(0/17)
1	≤450	50%	(3/6)	NT		100%	(5/5)	73%	(8/11)
	>450	50%	(3/6)			0%	(0/5)	27%	(3/11)
	>480	0%	(0/6)			0%	(0/5)	0%	(0/11)
	>500	0%	(0/6)			0%	(0/5)	0%	(0/11)
3	≤450	17%	(1/6)	17%	(1/6)	100%	(5/5)	41%	(7/17)
	>450	50%	(3/6)	50%	(3/6)	0%	(0/5)	35%	(6/17)
	>480	33%	(2/6)	17%	(1/6)	0%	(0/5)	18%	(3/17)
	>500	0%	(0/6)	17%	(1/6)	0%	(0/5)	6%	(1/17)
10	≤450	17%	(1/6)	0%	(0/1)	80%	(4/5)	42%	(5/12)
	>450	0%	(0/6)	0%	(0/1)	20%	(1/5)	8%	(1/12)
	>480	17%	(1/6)	0%	(0/1)	0%	(0/5)	8%	(1/12)
	>500	67%	(4/6)	100%	(1/1)	0%	(0/5)	42%	(5/12)
30	≤450	-	(0/0)	-	(0/0)	100%	(3/3)	100%	(3/3)
	>450	-	(0/0)	-	(0/0)	0%	(0/3)	0%	(0/3)
	>480	-	(0/0)	-	(0/0)	0%	(0/3)	0%	(0/3)
	>500	-	(0/0)	-	(0/0)	0%	(0/3)	0%	(0/3)

FPDcF = FPD/(inter-spike interval /1000)$^{1/3}$ NT: Not tested

Note: Numerator in parentheses shows the number of preparations exerting respective FPDc values in each category, whereas denominator indicates the total number of preparations assessed.

Table 3. Summary of categorical analysis of ΔFPDcF values.

Concentration of E-4031 (nM)	ΔFPDcF (ms)	Facility						All	
		E		N		T			
1	≤30	83%	(5/6)	NT		100%	(5/5)	91%	(10/11)
	>30	17%	(1/6)			0%	(0/5)	9%	(1/11)
	>60	0%	(0/6)			0%	(0/5)	0%	(0/11)
3	≤30	67%	(4/6)	67%	(4/6)	100%	(5/5)	76%	(13/17)
	>30	0%	(0/6)	17%	(1/6)	0%	(0/5)	6%	(1/17)
	>60	33%	(2/6)	17%	(1/6)	0%	(0/5)	18%	(3/17)
10	≤30	0%	(0/6)	0%	(0/1)	60%	(3/5)	25%	(3/12)
	>30	17%	(1/6)	0%	(0/1)	20%	(1/5)	17%	(2/12)
	>60	83%	(5/6)	100%	(1/1)	20%	(1/5)	58%	(7/12)
30	≤30	-	(0/0)	-	(0/0)	33%	(1/3)	33%	(1/3)
	>30	-	(0/0)	-	(0/0)	33%	(1/3)	33%	(1/3)
	>60	-	(0/0)	-	(0/0)	33%	(1/3)	33%	(1/3)

ΔFPDcF: Increase from baseline in FPDcF NT: Not tested

Note: Numerator in parentheses shows the number of preparations exerting respective ΔFPDc values in each category, whereas denominator indicates the total number of preparations assessed.

Table 4. Summary of nonclinical and clinical reports regarding the effects of E-4031 on the repolarization markers.

Model	Method	Marker	Change (%)	Concentration (nM)	Reference
hERG		IC_{50}		7.7	(21)
Guinea pig	Ventricular myocytes	APD_{90}	26	5,000	(22)
		APD_{90}	71	100	(23)
	Papillary muscles	APD_{70}	9 - 68	30 - 300	(23)
		APD_{90}	10	20	(24)
		APD_{30-90}	10	7	(24)
	Langendorff heart	QTc	5 - 27	3 - 300	(18)
		MAP_{90}	3 - 18	3 - 300	(18)
		QTc	26	5,000	(17)
Rabbit	Langendorff heart	QT	51	500	(19)
		MAP_{90}	50	500	(19)
Dog	In vivo (Anesthetized)	QT	57	6.2	(25)
		QTc	10	5.1	(24)
	In vivo (Conscious)	QTc	10	19.2	(24)
Monkey	In vivo (Conscious)	QTc	10	3.1	(24)
Human		QTc	14	12.1	(15)
		QTc	4.7 - 15	5.1 - 27	(26)
iPS cell	Single cell	APD_{90}	40 - 70	30 - 100	(20)
		APD_{90}	5 - 11	10 - 1,000	(12)
		APD_{30-90}	15 - 29	10 - 1,000	(12)

Fig.1

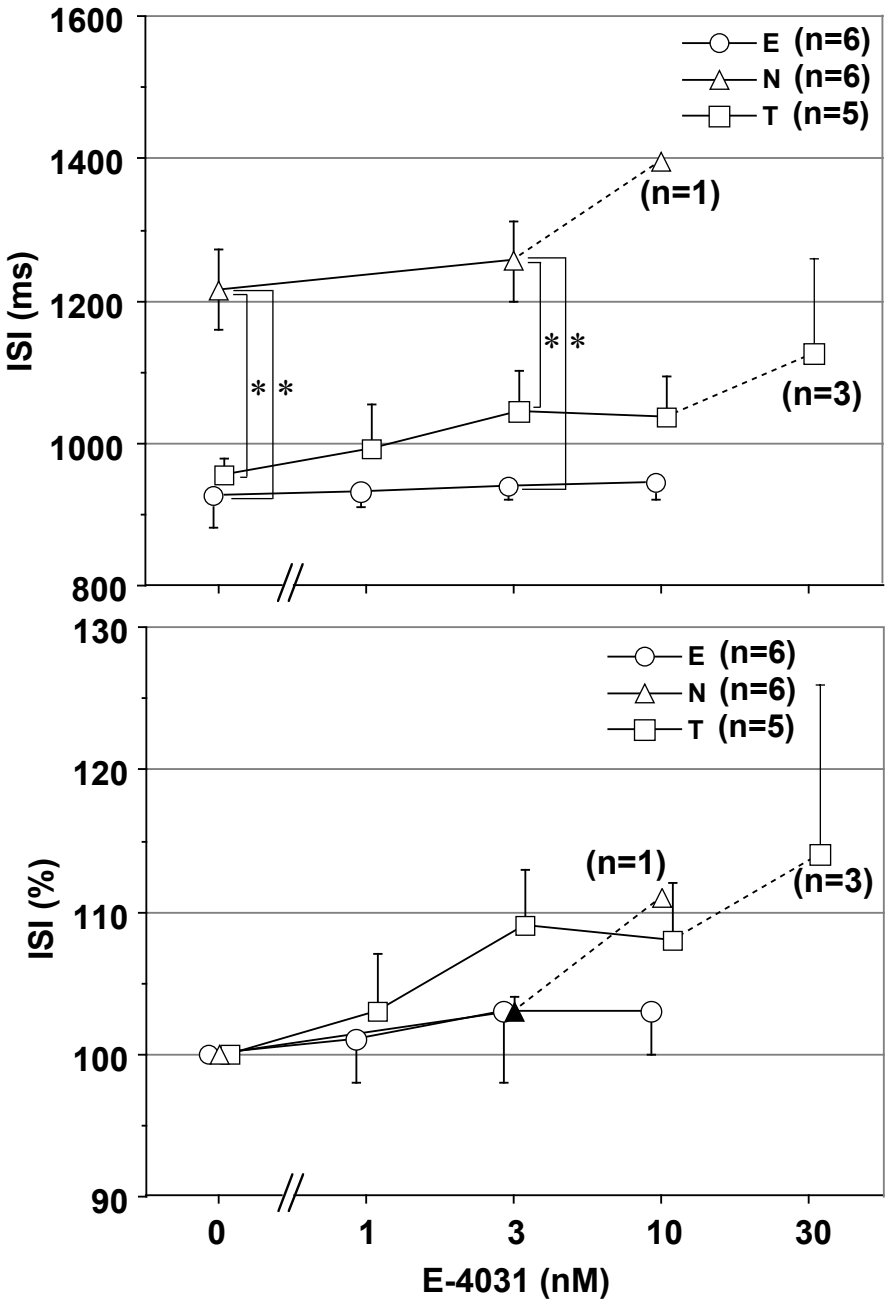

1600
1400
1200
1000
800
ISI (ms)
E (n=6)
N (n=6)
T (n=5)
(n=1)
(n=3)
* *
* *
130
120
110
100
90
ISI (%)
E (n=6)
N (n=6)
T (n=5)
(n=1)
(n=3)
0
1
3
10
30
E-4031 (nM)

Fig.2

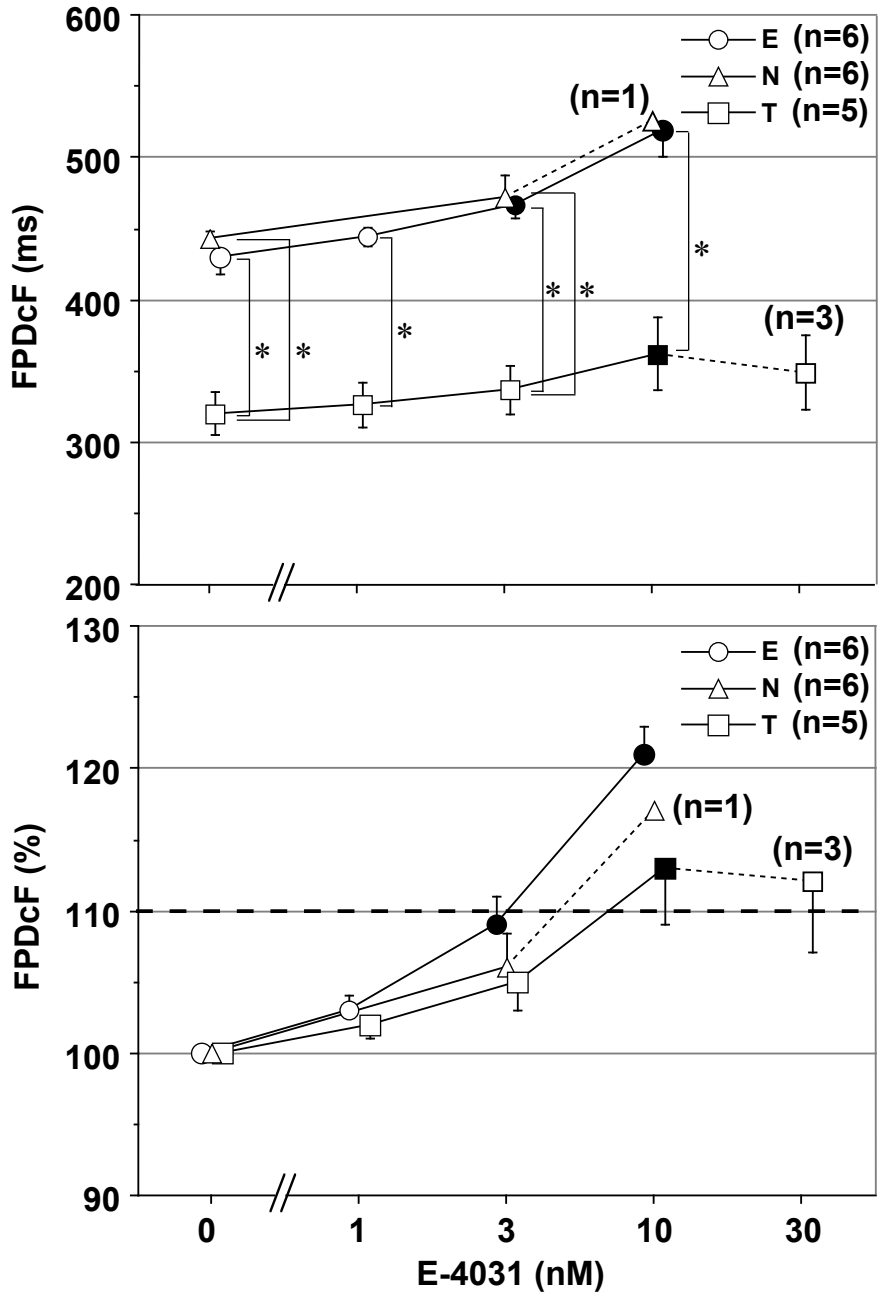
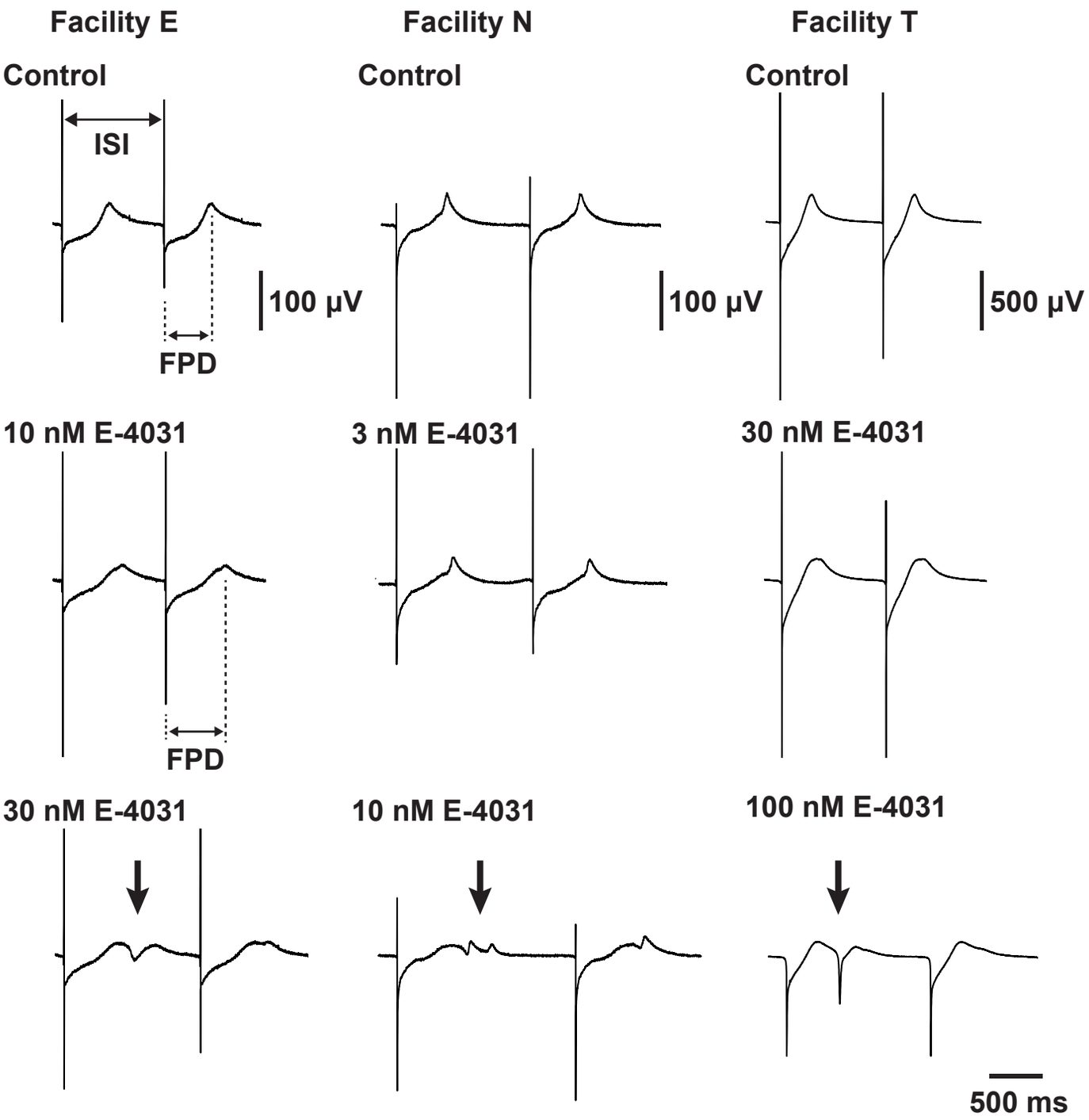

600
500
400
300
200
FPDcF (ms)
E (n=6)
N (n=6)
T (n=5)
(n=1)
(n=3)
*
130
120
110
100
90
FPDcF (%)
0
1
3
10
30
E-4031 (nM)

Fig. 3


Facility E
Facility N
Facility T
Control
Control
Control
ISI
FPD
100 µV
100 µV
500 µV
10 nM E-4031
3 nM E-4031
30 nM E-4031
FPD
30 nM E-4031
10 nM E-4031
100 nM E-4031
500 ms