

## Full Paper

## Effects of pH on Nifekalant-Induced Electrophysiological Change Assessed in the Langendorff Heart Model of Guinea Pigs

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**Abstract.** Since information regarding the effects of pH on the extent of nifekalant-induced repolarization delay and torsades de pointes remains limited, we assessed it with a Langendorff heart model of guinea pigs. First, we investigated the effects of pH change from 7.4 to 6.4 on the bipolar electrogram simulating surface lead II ECG, monophasic action potential (MAP), effective refractory period (ERP), and terminal repolarization period (TRP) and found that acidic condition transiently enhanced the ventricular repolarization. Next, we investigated the effects of pH change from 6.4 to 7.4 in the presence of nifekalant (10  $\mu$ M) on the ECG, MAP, ERP, TRP, and short-term variability (STV) of MAP<sub>90</sub> and found that the normalization of pH prolonged the MAP<sub>90</sub> and ERP while the TRP remained unchanged, suggesting the increase in electrical vulnerability of the ventricle. Meanwhile, the STV of MAP<sub>90</sub> was the largest at pH 6.4 in the presence of nifekalant, indicating the increase in temporal dispersion of repolarization, which gradually decreased with the return of pH to 7.4. Thus, a recovery period from acidosis might be more dangerous than during the acidosis, because electrical vulnerability may significantly increase for this period while temporal dispersion of repolarization remained increased.

**Keywords:** nifekalant, acidosis, Langendorff, torsades de pointes, monophasic action potential

### Introduction

While nifekalant hydrochloride, a class III antiarrhythmic drug, has been clinically used to suppress life-threatening refractory fatal ventricular arrhythmias, the drug was reported to induce excessive QT-interval prolongation, resulting in the onset of torsades de pointes (TdP) ventricular tachyarrhythmia (1 – 6). These adverse events tended to occur during the recovery period from lethal conditions including severe systemic acidosis, acute renal failure, and/or electrolyte disturbance (3 – 6). Although nifekalant would terminate the ventricular arrhythmia, each organ remained exposed to the low pH blood until the deteriorated systemic circulation was completely recovered (3 – 7). It is well known that extracellular pH changes can affect the electric activity of cardiomyocytes not only by altering ion flux through pumps and exchangers in the cell membrane (8 – 9) but also by changing the extent of proton binding to various

ion channels (10). Moreover, it has been demonstrated by the patch clamp method in single cells that lowering extracellular pH can weaken the pharmacological effect of some  $I_{Kr}$  channel blockers, including dofetilide, azimilide, ibutilide, and quinidine (11 – 12).

Since the information was still lacking regarding the pH effects on the nifekalant-induced electrophysiological change and proarrhythmic risk, we assessed it with the Langendorff heart preparation which allows the control of extracellular conditions (13). We selected guinea-pig hearts for this study, since they have similar ionic channels to those in humans except for  $I_{to}$  (14). To precisely analyze the electrophysiological conditions of the ventricle, we measured the monophasic action potential (MAP) and bipolar electrogram simulating surface lead II electrocardiogram (ECG). Furthermore, by adopting an electrical pacing protocol, we measured the MAP duration and effective refractory period (ERP) at a heart rate of 200, 240, and 300 bpm. In this study, we initially analyzed the effects of pH on electrophysiological variables by changing the pH of perfusate from 7.4 to 6.4 and then to 7.4 again; next we examined the effects of pH on nifekalant-induced changes in each electro-

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physiological variable. To precisely estimate the pro-arrhythmic risk of nifekalant, we examined the temporal dispersion of ventricular repolarization with the beat-to-beat analysis method (15).

## Materials and Methods

All animal experiments in this study were approved by the Animal Research Committee for Animal Experimentation of Toho University (No. 12-51-205) and performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of Toho University and The Japanese Pharmacological Society.

### *Preparation of Langendorff heart model of guinea pigs and perfusion solution*

Male guinea pigs ( $n = 14$ ) weighing 450 – 550 g were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and heparinized (100 U, i.p.) to protect the heart against microthrombi. The chest was opened at the sternum and the heart was quickly removed. The heart was cannulated through the aorta and perfused with Krebs-Henseleit solution maintained at  $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$  and aerated with a mixture of  $\text{O}_2$  (95%) and  $\text{CO}_2$  (5%) at a pressure of approximately 60 mmHg and a flow rate of 20 to 30 mL/min by using a Langendorff system (Physio-Tech, Tokyo). The Krebs-Henseleit solution contained 118 mM NaCl, 4.7 mM KCl, 11.0 mM glucose, 25 mM  $\text{NaHCO}_3$ , 1.2 mM  $\text{MgSO}_4$ , 2.5 mM  $\text{CaCl}_2$ , and 1.2 mM  $\text{KH}_2\text{PO}_4$ .

A bipolar transcardiac electrogram simulating surface lead II ECG was recorded with two electrodes; one attached onto the apex and the other connected to the base of the heart via a metal cannula. MAP was recorded with a contact electrode placed onto the epicardial surface of the left ventricle. The duration of MAP at a 90% repolarization level ( $\text{MAP}_{90}$ ) was measured. The heart was electrically paced at a cycle length of 300, 250, and 200 ms through a pair of bipolar electrodes contacted onto the right ventricle with rectangular pulses of 2-ms duration and approximately twice the threshold voltage to measure the  $\text{MAP}_{90}$  values; namely,  $\text{MAP}_{90(\text{CL}300)}$ ,  $\text{MAP}_{90(\text{CL}250)}$ , and  $\text{MAP}_{90(\text{CL}200)}$ , respectively. ERP of the right ventricle was assessed with programmed electrical stimulation. The pacing protocol consisted of 5 beats of basal stimuli in a cycle length of 300, 250, and 200 ms followed by an extrastimulus of various coupling intervals to obtain the ERP values; namely,  $\text{ERP}_{(\text{CL}300)}$ ,  $\text{ERP}_{(\text{CL}250)}$ , and  $\text{ERP}_{(\text{CL}200)}$ , respectively. The values of  $\text{MAP}_{90}$  minus ERP at the same pacing cycle length were calculated to estimate the extent of the terminal repolarization period (TRP), which is a reliable marker of electrical vulnerability of the ventricle (16). Starting in the late diastole, the coupling interval was shortened

in 5-ms decrements until refractoriness occurred. After the stabilization period, the position and contact pressure of all recording electrodes were not readjusted throughout the experimental period.

Beat-to-beat analysis of  $\text{MAP}_{90}$  was performed to estimate the extent of temporal dispersion of repolarization (15). Thirty-one consecutive beats of  $\text{MAP}_{90}$  under sinus rhythm were recorded for each treatment, and Poincaré plots with  $\text{MAP}_{90(n)}$  vs.  $\text{MAP}_{90(n+1)}$  were prepared. The mean orthogonal distance from the diagonal to the points of the Poincaré plot was determined as short-term variability ( $\text{STV} = \Sigma |\text{MAP}_{90(n+1)} - \text{MAP}_{90(n)}| / [30 \times \sqrt{2}]$ ). The mean distance to the mean of the parameter parallel to the diagonal of the Poincaré plot was determined as long-term variability ( $\text{LTV} = \Sigma |\text{MAP}_{90(n+1)} + \text{MAP}_{90(n)} - 2\text{MAP}_{90(\text{mean})}| / [30 \times \sqrt{2}]$ ). The coefficient of variation of  $\text{MAP}_{90}$  (CV) was calculated with the following equation: standard deviation / mean  $\times 100$  (%).

Four types of perfusion solution were prepared according to a previous report (17), namely, a normal pH solution (pH 7.4, normal Krebs-Henseleit solution), low pH solution (pH 6.4, modified Krebs-Henseleit solution with  $\text{NaHCO}_3$  content of 2.5 mM), normal pH solution with drug (10  $\mu\text{M}$  nifekalant hydrochloride dissolved in normal pH solution), and low pH solution with drug (10  $\mu\text{M}$  nifekalant hydrochloride dissolved in low pH solution).

### *Experiment 1: assessment of baseline electrophysiology under normal and acidic conditions*

After equilibration of  $> 30$  min of perfusion with the normal pH solution, the preparation was perfused with the low pH solution for 30 min and then re-perfused with the normal pH solution for 30 min. During each period of perfusion, ECGs under sinus rhythm and MAP signals at a cycle length of 300, 250, and 200 ms were recorded every 10 min. ERP of the right ventricle was assessed by programmed electrical stimulation. TRP was also calculated.

### *Experiment 2: assessment of electrophysiology under normal and acidic conditions after nifekalant exposure*

After equilibration of  $> 30$  min of perfusion with the normal pH solution, the preparation was perfused with the normal pH solution for 20 min as the basal control; then it was perfused with the low pH solution for 20 min, followed by the low pH solution with 10  $\mu\text{M}$  of nifekalant for 20 min; and it was finally perfused with the normal pH solution with 10  $\mu\text{M}$  of nifekalant for 20 min. At the end of each perfusion, ECG and MAP signals under sinus rhythm, and MAP signals and ERP at a basic cycle length of 300, 250, and 200 ms were recorded. TRP was also calculated.

### Drugs and chemicals

The following drugs were purchased: sodium pentobarbital (Kyoritsu Seiyaku Corporation, Tokyo) and sodium heparin (Ajinomoto Pharmaceuticals Co., Ltd. Tokyo). Nifekalant hydrochloride (MW = 441.91) was provided by Nacalai Tesque, Inc. (Kyoto). Nifekalant was dissolved in distilled water and small aliquots were added to each perfusion solution to obtain the desired final concentration. All other chemicals were commercial products of the available highest quality.

### Statistical analyses

Data are presented as the mean  $\pm$  S.E.M. The differences within a parameter were evaluated by one-way repeated measures analysis of variance (ANOVA). When a  $P$ -value was  $< 0.05$  by ANOVA, the protocol was judged as having affected the parameter. In this case, the statistical significance between the treatments was determined by contrast for mean values comparison, and  $P$ -value  $< 0.05$  was considered significant.

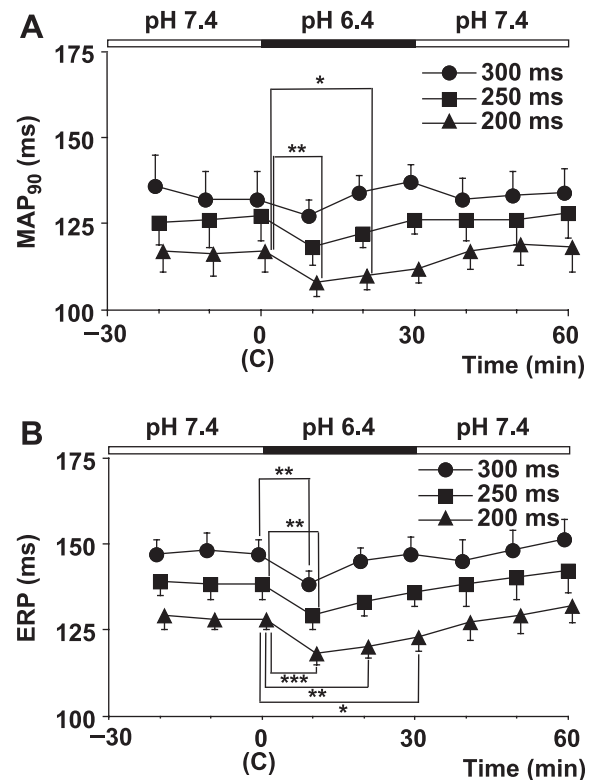
## Results

### Experiment 1: baseline electrophysiology under normal and acidic conditions

The time courses of changes in the MAP<sub>90</sub> and ERP are summarized in Fig. 1 ( $n = 6$ ) and typical waveforms of the ECG and MAP signals at each treatment are depicted in Fig. 2. Basal control values (C) of the MAP<sub>90(CL300)</sub>, MAP<sub>90(CL250)</sub>, and MAP<sub>90(CL200)</sub> were  $132 \pm 8$ ,  $127 \pm 7$  and  $117 \pm 6$  ms, whereas those of the ERP<sub>(CL300)</sub>, ERP<sub>(CL250)</sub>, and ERP<sub>(CL200)</sub> were  $147 \pm 4$ ,  $138 \pm 4$ , and  $128 \pm 3$  ms, respectively. The decrease in pH transiently shortened the MAP<sub>90(CL200)</sub>, and it also shortened the ERP at each basic pacing cycle length. These changes recovered to basal level within 30 min except for the ERP<sub>(CL200)</sub>, which remained shortened during the low pH perfusion as shown in Fig. 1B. These treatments did not affect TRP (data not shown). The morphology of T waves of the ECG under the sinus rhythm significantly changed after pH decreased as depicted in Fig. 2A, indicating a difficulty of precise measurement of QT interval. MAP amplitude remained decreased after lowering pH as depicted in Fig. 2, B and C. Arrhythmia was not induced by the programmed electrical stimulation during the assessment of ERP at any time point.

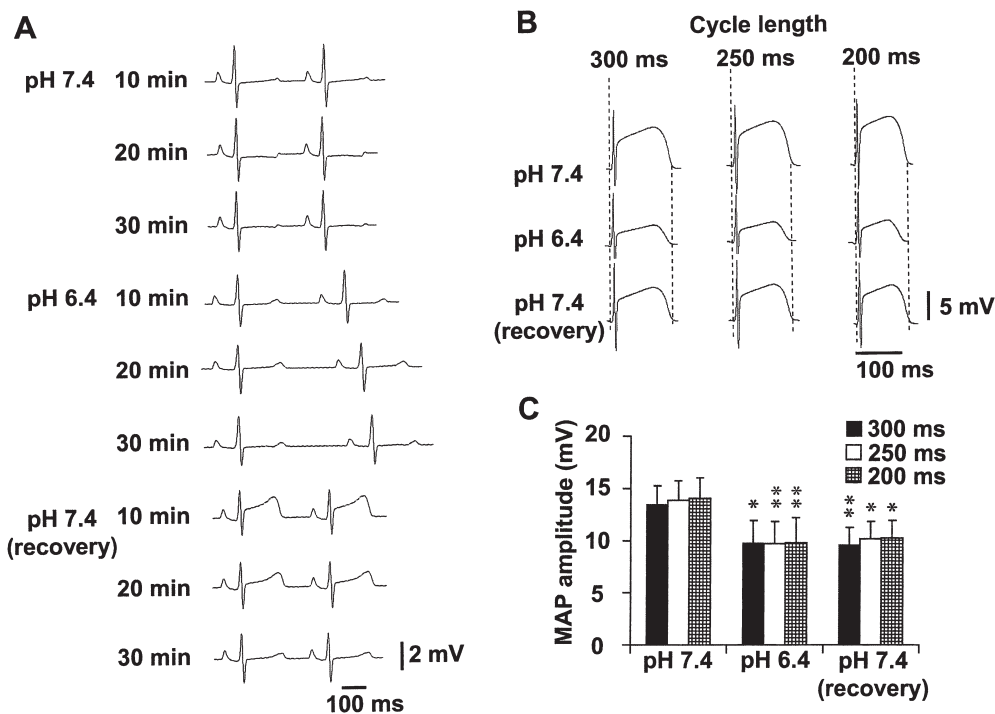
### Experiment 2: electrophysiology under normal and acidic conditions after nifekalant exposure

The time courses of changes in the MAP<sub>90</sub> and ERP are summarized in Fig. 3 ( $n = 8$ ), and those of typical waveforms of the ECG and MAP signals at pH 6.4 and 7.4 are depicted in Fig. 4. Basal control values (C)



**Fig. 1.** Time courses of the effects of pH change on the electrophysiological variables. A) Monophasic action potential duration at a 90% repolarization level (MAP<sub>90</sub>). B) Effective refractory period (ERP). Extracellular pH was decreased from 7.4 to 6.4, and it then increased to 7.4. Data are presented as the mean  $\pm$  S.E.M. ( $n = 6$ ). \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ .

of the MAP<sub>90(CL300)</sub>, MAP<sub>90(CL250)</sub>, and MAP<sub>90(CL200)</sub> at pH 7.4 in the absence of nifekalant were  $131 \pm 7$ ,  $123 \pm 6$ , and  $113 \pm 5$  ms, whereas those of the ERP<sub>(CL300)</sub>, ERP<sub>(CL250)</sub>, and ERP<sub>(CL200)</sub> were  $144 \pm 5$ ,  $136 \pm 5$ , and  $126 \pm 5$  ms, respectively. The decrease in pH alone did not significantly affect the MAP<sub>90</sub> or ERP at 20 min, which was essentially consistent with the results of experiment 1 except for MAP<sub>90(CL200)</sub> and ERP<sub>(CL200)</sub>. The difference seems to be explained by the faster adaptation of cellular functions of the preparations in experiment 2 to acidic condition. The addition of nifekalant at pH 6.4 significantly prolonged both the MAP<sub>90</sub> and ERP at each pacing cycle length. The increase in pH from 6.4 to 7.4 in the presence of nifekalant further prolonged the MAP<sub>90</sub> and ERP at each pacing cycle length except for MAP<sub>90(CL300)</sub>, which tended to be prolonged but did not achieve statistical significance ( $P = 0.06$ ). The increments of MAP<sub>90</sub> at a pacing cycle length of 300, 250, and 200 ms were  $25 \pm 4$ ,  $25 \pm 4$ , and  $20 \pm 3$  ms, respectively, and there were no significant differences among them. The treatments did not affect TRP (data not shown). The T-wave morphology of ECG under the sinus



**Fig. 2.** Typical tracings of the ECG and MAP and the summary of MAP amplitude. Decrease in pH to 6.4 from 7.4 followed by an increase to 7.4 changed the morphology of the T wave during the sinus rhythm (A), whereas it reduced the amplitude of the MAP during the electrical pacing (B and C). \* $P < 0.05$  and \*\* $P < 0.01$ , compared with pH 7.4 in the absence of nifekalant. MAP: monophasic action potential.

rhythm significantly changed after pH increased in the presence of nifekalant as depicted in Fig. 4A, also indicating the difficulty of precise measurement of QT interval. MAP amplitude increased with elevating pH in the presence of nifekalant as depicted in Fig. 4B. Arrhythmia was not induced by the programmed electrical stimulation during the assessment of ERP at any time point.

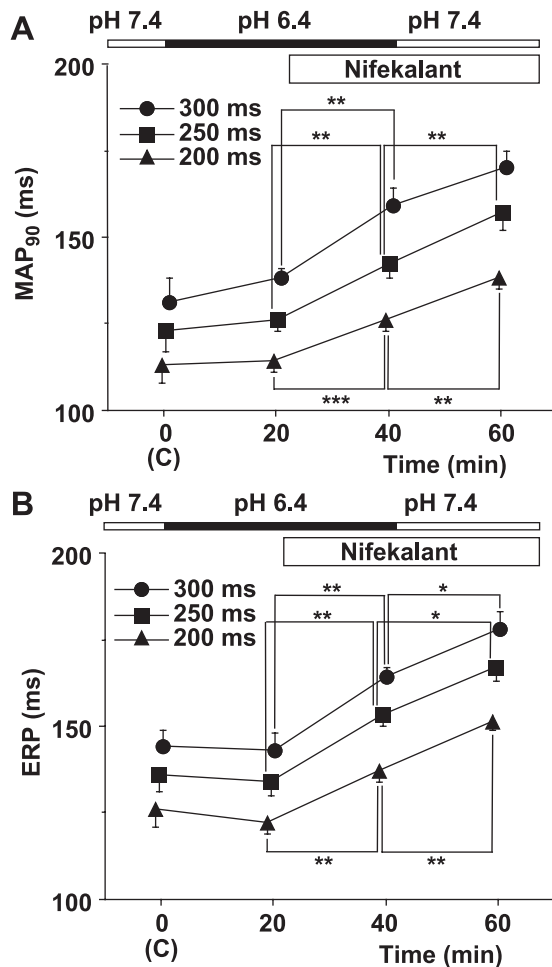
Typical tracings of Poincaré plots are depicted in Fig. 5A, and the time courses of the STV, LTV, and CV are summarized in Fig. 5B. While lowering pH from 7.4 to 6.4 in the absence of nifekalant did not significantly affect the STV, the administration of nifekalant in low pH solution significantly increased the STV. The normalization of pH in the presence of nifekalant gradually decreased the STV. Although LTV and CV tended to increase at pH 6.4 and/or pH 7.4 in the presence of nifekalant, these changes did not achieve statistical significance.

## Discussion

This study was designed to assess the effects of pH on the nifekalant-induced repolarization delay and on proarrhythmic risk. The concentration of nifekalant (10  $\mu$ M) used in this study was determined based on our preliminary experiments in order to better characterize

the pharmacological profile of nifekalant, although it is higher than the clinically effective plasma concentration [approximately 0.8  $\mu$ g/ml (1.8  $\mu$ M)] in patients with inducible sustained ventricular arrhythmias (18). As depicted in Figs. 2A and 4A, the precise analysis of the effects of each treatment on the repolarization process was difficult in the ECG recordings because of the ambiguity in T-wave terminals, which indirectly supports the utility of the currently used MAP recording/pacing technique. Also, the present results indicate that the CV and LTV may be less sensitive in estimating the temporal dispersion of repolarization than the STV as shown in Fig. 5B.

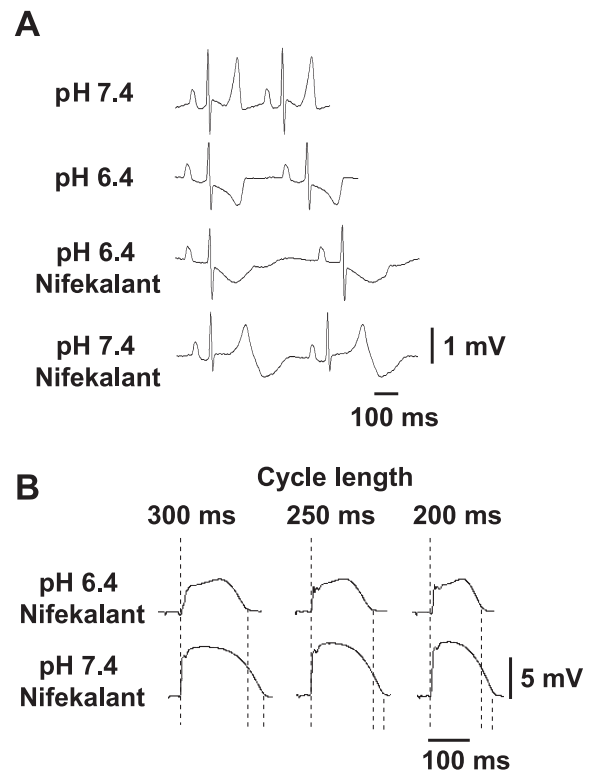
In experiment 1, we analyzed the effects of pH on the electrophysiological variables in the absence of nifekalant. When the preparation was perfused with pH 6.4 solution, the phase 2 amplitude of MAP decreased, and MAP duration was transiently shortened as depicted in Fig. 2B. This decrease in phase 2 amplitude of MAP is consistent with previous reports (9, 19, 20), which has been ascribed to a decrease in plateau-phase  $\text{Ca}^{2+}$  influx (19, 21). Meanwhile, with regard to the shortening of MAP duration, some studies reported that a decrease in extracellular pH shortens the action potential duration (22–25), but the others described opposite results (26, 27). It has been reported that under acidic condi-



**Fig. 3.** Time courses of the effects of pH change on the electrophysiological variables after nifekalant exposure. A) Monophasic action potential duration at a 90% repolarization level (MAP<sub>90</sub>). B) Effective refractory period (ERP). Extracellular pH was decreased from 7.4 to 6.4, then 10  $\mu$ M of nifekalant was added, and finally the pH was increased to 7.4 in the presence of nifekalant. Data are presented as the mean  $\pm$  S.E.M. ( $n = 8$ ). \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ .

tions, H<sup>+</sup> directly binds to  $I_{Kr}$  and  $I_{Ca}$  channels, resulting in their current inhibitions (11, 19, 20, 28). Inhibition of  $I_{Kr}$  channels causes the prolongation of MAP duration, whereas inhibition of  $I_{Ca}$  channels leads to its shortening. Thus, MAP duration can change in both directions, which depends on the balance in the extent of inhibitions between  $I_{Kr}$  and  $I_{Ca}$  channels. In addition, acidosis may decrease the ATP production, which opens ATP-dependent K<sup>+</sup> channels, leading to the shortening of MAP duration (23, 29). Therefore, that variability in the response of MAP duration to acidosis among the studies may depend on the differences in methodologies and experimental conditions and/or species differences (29).

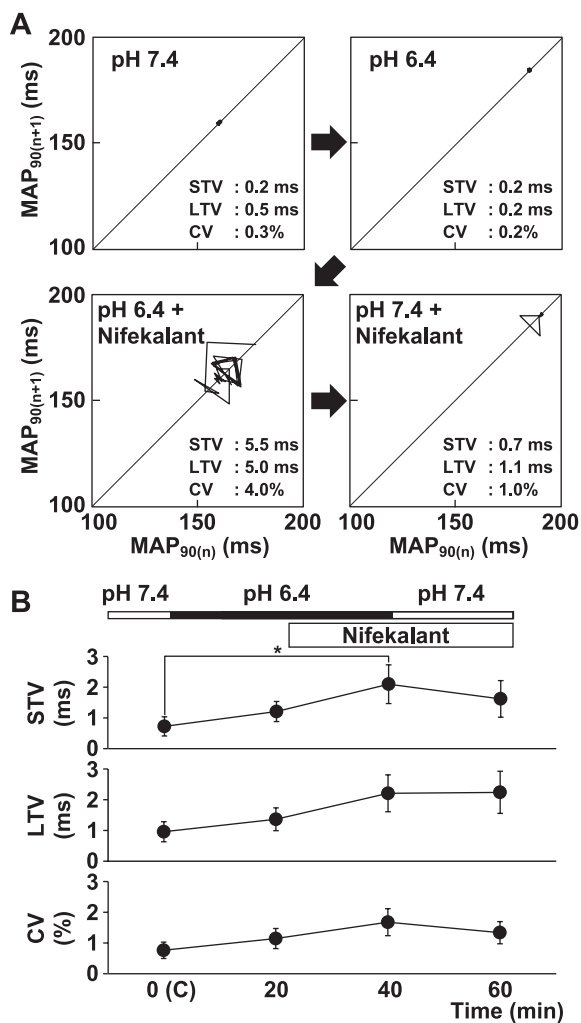
In experiment 2, the increase in pH from 6.4 to 7.4 significantly prolonged the MAP<sub>90</sub> and ERP in the



**Fig. 4.** Typical tracings of the ECG (A) and monophasic action potential (B) at pH 6.4 and 7.4. Increase in pH from 6.4 to 7.4 dramatically changed the morphology of the T wave during the sinus rhythm (A), whereas it increased the amplitude of the monophasic action potential (B).

presence of nifekalant. There have been several clinical case reports describing that nifekalant-induced excessive QT-interval prolongation and TdP during the recovery period from lethal conditions (2–7). In the present experiment with the Langendorff heart preparation, we used pH changes to mimic such pathophysiology in the heart. As summarized in Fig. 3, MAP duration was prolonged less greatly by nifekalant under acidic conditions, which is in accordance with a previous study of guinea-pig ventricular myocytes with the patch clamp method (30), describing that extracellular acidification (pH 6.4) strongly attenuated the effect of nifekalant on the open probability of  $I_{K1}$  channel activity when compared with its effect at pH 7.4. As the pK<sub>a</sub> of nifekalant is 7.9, in that paper (30) they speculated that the proportion of ionized to un-ionized form would be greater at pH 6.4 than that at pH 7.4, resulting in a decrease in accessibility to  $I_{K1}$  channels via the lipid membrane. Another possible explanation is that an increase in protons may interrupt the binding of nifekalant to  $I_{Kr}$  channels (10). Indeed, it is known that there are multiple proton-binding sites in the extracellular domain of the hERG channel, and extracellular protons rapidly and reversely bind to





**Fig. 5.** Beat-to-beat analysis of repolarization. A) Typical tracings of Poincaré plots. B) Time courses of the STV, LTV, and CV. STV: short-term variability, LTV: long-term variability, and CV: coefficient of variation. Data are presented as the mean  $\pm$  S.E.M. ( $n = 8$ ). \* $P < 0.05$ .

these sites, affecting both hERG activation and deactivation (10). It is possible that the binding of extracellular protons might have attenuated the effects of nifekalant by modulating its binding property. This explanation is indirectly supported by a report that acidic conditions do not affect hERG  $IC_{50}$  of amiodarone, since amiodarone is known to bind to a different site of hERG channels compared with other  $I_{Kr}$  blockers whose  $IC_{50}$  was increased under acidic conditions (11). Thus, the attenuation of pharmacological effects of nifekalant under acidic conditions as observed in this study might be due to the decrease in accessibility to  $I_{K1}$  channels by increased ionization of nifekalant and/or the interruption of the binding of nifekalant to  $I_{Kr}$  channels by proton binding.

Finally, we investigated the electrical instability of

repolarization with the beat-to-beat analysis method, since the Langendorff heart preparation model of guinea pig has been known to be less sensitive in directly detecting the drug-induced TdP (31). The STV of  $MAP_{90}$  was the longest at pH 6.4 in the presence of nifekalant. The increase in the STV indicates enhanced “temporal” dispersion and is reported to be closely associated with the onset of early afterdepolarizations and R-on-T-type premature ventricular contractions resulting in the onset of TdP (15). Also, in the present study the  $MAP_{90}$  was prolonged, while the TRP remained unchanged by pH normalization at each basic cycle length of 300, 250, and 200 ms in the presence of nifekalant. This observation reflects an increase in electrical vulnerability, in which some cardiomyocytes may have more chance to easily respond to the irregular stimulation from adjacent cells (16). Thus, the most dangerous period for nifekalant-induced TdP would be the recovery period from acidosis because electrical vulnerability may significantly increase during this time while temporal dispersion of repolarization remained increased.

There are some limitations in this study. In clinical cases with systemic acidosis, multiple pathological factors, including hypoxia, reperfusion injury, superoxide radicals, and immune-inflammatory responses, may play important roles in the development of nifekalant-induced QT prolongation and TdP. In order to begin to explore such complex cellular and molecular mechanisms, we assessed the effects of “pH change” on the electrophysiological variables in the hearts in the absence or presence of nifekalant. Based on the current findings, the experiments are now on-going to clarify the effects of the other factors on the nifekalant-induced electrophysiological responses.

In conclusion, the present study with the Langendorff heart model of guinea pigs indicates that the MAP recording/pacing technique may be an effective way to better analyze the nifekalant-induced electrophysiological responses in the heart, that pharmacological effects of nifekalant can be attenuated under acidic conditions, and that nifekalant may induce TdP during the recovery period from systemic acidosis through the increased electrical vulnerability of ventricle and the remaining temporal dispersion of repolarization.

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## Conflicts of Interest

The authors declare no conflicts of interest.

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