# NHE-1 blockade reversed changes in calcium transient in myocardial slices from isoproterenol-induced hypertrophied rat left ventricle

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## ABSTRACT

We previously reported that left ventricular (LV) slices from isoproterenol (ISO)-induced hypertrophied rat hearts showed an increase of energy expenditure due to remodeling of $Ca^{2+}$ handling in excitation-contraction coupling, i.e., suppressed SERCA2a activity and enhanced $Na^{+}/Ca^{2+}$exchanger-1 (NCX-1) activity. $Na^{+}/H^{+}$ exchanger-1 (NHE-1) inhibitor (NHEI) has been demonstrated to exert beneficial effects in the development of cardiac remodeling. We hypothesized that a novel NHE-1 selective inhibitor, BIIB723 prevents remodeling of $Ca^{2+}$ handling in LV slices of ISO-induced hypertrophied rat hearts mediated by inhibiting NCX-1 activity. The significant shortening in duration of multi-cellular $Ca^{2+}$ transient in ISO group was normalized in ISO+BIIB723 group. The significant increase in amplitude of multi-cellular $Ca^{2+}$ waves (CaW) generated at high $[Ca^{2+}]_o$ of LV slices in ISO group was also normalized in ISO+BIIB723 group. However, the enhanced NCX-1 activity was not antagonized by BIIB723. We recently reported that ISO-induced down-regulation of a $Ca^{2+}$ handling protein, SERCA2a, was normalized by BIIB723. Therefore, it seems likely that BIIB723 normalized shortened multi-cellular $Ca^{2+}$ transient duration and increased CaW amplitude in LV slices mediated via normalization of SERCA2a activity. Furthermore, the results presented here suggest the multi-cellular $Ca^{2+}$ transient duration and CaW amplitude in LV slices might be better indices reflecting SERCA2a activity than SERCA2a protein expression level.



## Key Words

calcium transient; cardiac hypertrophy; excitation-contraction coupling; myocardial slice; $Na^{+}/H^{+}$ exchanger-1

**Introduction**

$Na^+/H^+$ exchanger (NHE) is an integral membrane glycoprotein, plays a key role in maintaining intracellular pH and $Na^+$ concentration and cellular volume. Recently, the link between cardiac NHE-1 activity and myocardial hypertrophy has been clearly established in elevated sympathetic nerve activity models. Long-term isoproterenol (ISO)-induced cardiac hypertrophy was prevented and NHE-1 protein expression was normalized by the inhibition of NHE-1 in rats [1]. NHE-1 also contributes to ISO-induced abnormal $Ca^{2+}$ handling associated with cardiac hypertrophy. Inhibition of NHE-1 ameliorates cardiac $Ca^{2+}$ handling impairment by up-regulation of SERCA2a protein expression and prevents the development of cardiac dysfunction in ISO-infused rats [2]. Furthermore, the development of cardiac hypertrophy and fibrosis associated with increased NHE-1 protein expression in $ß_1$-adrenergic receptor transgenic mice was prevented by NHE-1 inhibition [3].

Myocardial oxygen consumption related with the total $Ca^{2+}$ handling in excitation-contraction (E-C) coupling was increased in the left ventricular (LV) myocardial slice of ISO-induced hypertrophied rat heart [4]. Molecular and cellular physiological studies demonstrated that this increase was caused by functional increase in $Na^+$-$Ca^{2+}$ exchange (NCX1) activity [4]. This functional increase seems to be induced by attenuation of the intrinsic inactivation mechanisms associated with functional depression of SERCA2 induced by lower protein expression of phospho-$Ser^{16}$ PLB and SERCA2. On the other hand, LV myocardial oxygen consumption related with the total $Ca^{2+}$ handling in E-C coupling was not increased in ISO-induced hypertrophied rat whole heart preparation perfused with blood at a lower rate, 240 bpm pacing [5]. Lowering the heart rate enabled ISO-induced hypertrophied rat hearts to exert normal myocardial oxygen consumption related with the total $Ca^{2+}$ handling in E-C coupling.

However, whether changes in NCX1 activity and NHE-1 protein contribute to real $Ca^{2+}$ handling in E-C coupling, i.e., $Ca^{2+}$-transient of myocardial slices of ISO-induced hypertrophied rat heart LV, are not analyzed yet. In the present study, to clarify this, a new approach for

evaluating multi-cellular $Ca^{2+}$-transients and $Ca^{2+}$ waves (CaW) measured at multiple points of each LV myocardial slice is performed to compare between ISO-induced hypertrophied and normal rat hearts.

**Methods and Materials**

Experimental procedures followed the regulations of and were approved by the animal care and use committee of Nara Medical University.

***Animals and drug infusion***

Male Wistar rats weighing 250 to 370 g (8 to 10 weeks) were randomly divided into Vehicle (SA), NHEI, and ISO groups without or with pretreatment with NHEI. Delivery of drug was achieved by implanting an osmotic minipump (model 1003D, Alzet, Durect Corp, Cupertino, CA) subcutaneously in the neck under pentobarbital (50 mg/kg i.p.) anesthesia. Either ISO (2.4 $mg \cdot kg^{-1} \cdot day^{-1}$ for 7 days) or vehicle (0.1% ascorbic acid in saline 2.4 μl/day for 7 days) was infused subcutaneously [5, 6]. Rats were received an NHE-1 inhibitor, BIIB723 (Boehringer-Ingelheim pharmaceuticals, Inc, Ridgefield, CT, USA, 3.0 $mg \cdot kg^{-1} \cdot day^{-1}$) in drinking water 3 days before the start of ISO infusion [2].

***Animals and left ventricular myocardial slice preparation***

Male Wistar rats (n=98) were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and osmotic mini-pump was removed from the neck 7 days after the implantation. The heart and its slices were prepared as described before [7-10]. The whole heart was excised under perfusion with Tyrode solution oxygenated with 100% $O_2$ at 13°C for 5 min. The composition of Tyrode solution (in mM) was 136.0 NaCl, 5.4 KCl, 1.0 $MgCl_2$, 0.3 $NaH_2PO_4$, 1.8 $CaCl_2$, 10.0 glucose, and 5.0 HEPES, with pH adjusted to 7.4 with NaOH at 30°C. After the perfusion, both atria, the four valves including the connective tissue, the aorta and the pulmonary artery were removed from the heart. The heart was longitudinally cut into two or three pieces and each of the pieces was cut into 300 μm thick slices in parallel with the epicardium with a microslicer DTK-3000 (Dosaka EM, Kyoto, Japan). The slices with the thickness (300 μm) we chose seem to be

sufficiently oxygenated by diffusion, according to the published data and calculation [10]. This cut was chosen so that most fibers were parallel-sectioned to fiber orientation according to the known fiber architecture of the heart wall. We obtained 12-20 slices (average single-side surface area: approximately 30 $mm^2$) from each LV.

***$Ca^{2+}$ imaging***

The slices were stored in Tyrode solution oxygenated with 100% $O_2$ at 18℃ for 30 min. LV slices were incubated for 2-4 h at room temperature in Tyrode solution containing 10 μM fluo-3 acetoxymethyl ester (Dojindo, Kumamoto, Japan) and detergents (0.02% Pluronic F-127, Dojindo and 0.02% Cremophor EL, Sigma), after which changes in $[Ca^{2+}]_i$ were monitored using a digital imaging system (AQUACOSMOS, Hamamatsu Photonics, Shizuoka, Japan) mounted on an inverted microscope as previously reported [11]. Consequently, acute effects of isoproterenol were removed before starting experiments. The fluo-3-containing LV slices were illuminated at 488 nm, and the intensity of the fluorescent emission from the indicator at 515-565 nm was recorded. Digital $Ca^{2+}$ images (527 x 511 pixels) were normally collected at 8-ms intervals, and the intensity of the fluorescence at a given time ($F_t$) was usually normalized to the fluorescence intensity at the start ($F_0$), yielding relative values representative of the integrated $[Ca^{2+}]_i$ recorded at 2-5 points in a single slice during 25-30 stimuli. Stimulation consisted of 1-Hz (low frequency) rectangular pulses, 10 ms in duration and current of 1 mA (voltage: 1 V). Tyrode solutions were oxygenated with 100% $O_2$ and preheated to 40℃ in a water bath. Slices without any mechanical load were placed into the dish superfused with the prepared Tyrode solution and fixed with a pair of the wire stimulation electrodes. The dish was warmed with a Microwarm Plate DC-MP10DM (Kitazato, Tokyo, Japan) at 36℃.

***Drugs***

BIIB723, a gift from Boehringer-Ingelheim pharmaceuticals, Inc. (Ridgefield, CT, USA) was used as an NHE-1 inhibitor, cyclopiazonic acid (CPA) (Sigma; St. Louis, MO) was used as a SERCA2 inhibitor [8, 9], or SEA0400 (2-[4-[(2,5-difluorophenyl) methoxy] phenoxy]-5-

ethoxyaniline), a gift from Taisho Pharmaceutical Co. Ltd. (Saitama, Japan), was used as a bidirectional-mode NCX 1 inhibitor [12-14].

***Myocytes study***

Cell isolation. The rats (n=14) were deeply anesthetized by intraperitoneal injection of pentobarbital sodium (>0.1 mg/g body weight). LV myocytes were dissociated from SA (n=10 myocytes from 4 rats) and ISO group hearts without (n=18 myocyctes from 5 rats) or with pretreatment with NHEI (n=13 myocytes from 5 rats) as described in previous studies [15]. The dissociated myocytes were kept in a HEPES-buffered DMEM solution (ICN Biomedicals) and used for the patch clamp and fluorescence studies within 8 hrs. All procedures were approved by the Animal Research Committee of the Graduate School of Medicine, Kyoto University.

Solutions. The standard pipette solution contained (in mM) 30 CsOH, 40 aspartate, 20 TEACl, 40 EGTA, 5 MgATP, 33.8 $CaCl_2$, 1.19 $MgCl_2$, 10 HEPES, 50 NaOH (pH=7.2 with CsOH). Free $Ca^{2+}$ concentration was calculated to be 0.8 mM. Composition of bath solution for recording NCX current ($I_{NCX}$) was 145 NaCl, 2 $BaCl_2$, 3 $MgCl_2$, 5 HEPES, 0.2 EGTA (or 0 when adding 2 $CaCl_2$), 0.05 mM ouabain and 0.002 nicardipine (pH=7.4 with NaOH) according to a previous study [16].

Electrophysiology. The myocytes were voltage clamped using the whole cell method with an Axopatch 200B amplifier (Axon Instruments). Holding potential was -40 mV. Current-voltage (I-V) relationships were measured by applying ramp pulses and normalized by membrane capacitance. $I_{NCX}$ was induced by applying 2 mM $Ca^{2+}$ for 10 s at intervals of 30 s and determined as the difference current in the same manner as described in our previous studies [15, 16].

***Statistics***

All data were presented as mean ± SD. Differences between two mean values were evaluated by paired or non-paired Student's *t*-test. Multiple comparisons were performed by one-way ANOVA and Bonferroni's *t*-test or Dunnett's *t*-test, or two-way ANOVA. In all statistical tests,

P values less than 0.05 were considered statistically significant.

**Results and Discussion**

$Ca^{2+}$ images at I, II and III of averaged and normalized $Ca^{2+}$ transient were shown in Fig. 1A. $Ca^{2+}$ transient was recorded at 2-5 points in a single slice (Fig, 1B) during 25-30 stimuli. Averaging 25-30 $Ca^{2+}$ transient traces normalized to peak amplitude and $D_{20}$ (normalized $Ca^{2+}$ transient duration at 0.2 of intensity ratio) at one point was shown in Fig. 1C. From other 1-4 points, we obtained quite similar $Ca^{2+}$ transient and thus we averaged $Ca^{2+}$ transient recorded at all points in each single slice during 25-30 stimuli. Normalized averaged $Ca^{2+}$ transient trace, $D_{20}$ and diastolic calcium wave (CaW) under higher (10.8 mM) extracellular $Ca^{2+}$ concentrations ($[Ca^{2+}]_o$) was shown in Fig. 1D.

Each averaged $D_{20}$ of SA, NHEI, ISO and NHEI+ISO group under normal (1.8 mM) and higher (7.2 mM) $[Ca^{2+}]_o$ was summarized in Table 1. Mean $D_{20}$ in ISO (129.6 ± 14.6 msec) was significantly ($P<0.05$) shorter than that in SA (140.6 ± 17.9 msec). Mean $D_{20}$ in NHEI+ISO (135.9 ± 12.2 msec) was significantly ($P<0.05$) longer than that in ISO. At 7.2 mM $[Ca^{2+}]_o$, mean $D_{20}$ did not show significant differences among the four groups, but CaW was detected in a part of myocytes of each single slice. Mean maximal amplitude of CaW in ISO was significantly ($P<0.05$) larger than that in SA. Mean maximal amplitude of CaW in NHEI+ISO was significantly decreased compared to that in ISO. NHEI attenuated ISO-induced CaW exaggeration (Table 1), although NHEI did not attenuate ISO-induced CaW exaggeration at 10.8 mM $[Ca^{2+}]_o$ (data not shown).

It was confirmed that the higher expression of NHE protein in ISO group than that in SA group was suppressed by an NHEI, BIIB723 [2] as in long-term ISO-induced cardiac hypertrophy. NCX current was measured in SA, ISO and NHEI+ISO myocytes. Increases in NCX current in ISO was confirmed as shown previously [4]. However, NCX current in ISO+NHEI was not different from that in ISO (Fig. 2), suggesting that NHE does not contribute to changes in $Ca^{2+}$ transient mediated via suppression of NCX activity.

We have recently reported that ISO induced down-regulation of a $Ca^{2+}$ handling protein, SERCA2 and the down-regulation was normalized by BIIB723 [2]. Shorter mean $D_{20}$ in ISO than in SA seems due to enhanced SERCA2a activity in compensation for moderate down-regulation of SERCA2a protein, although the duration of $Ca^{2+}$ transients in long-lasting hypertrophy with chronic heart failure was longer than normal and another NHE inhibitor treatment normalized the duration of $Ca^{2+}$ transients [16]. Normalized D20 in NHEI+ISO seems likely to be related to normalized SERCA2a activity due to normalized SERCA2a protein level [2]. These results were obtained under normal $[Ca^{2+}]_o$. At 10.8 mM $[Ca^{2+}]_o$, a significantly longer mean D20 in ISO than at 1.8 mM $[Ca^{2+}]_o$ seems due to the failure of the above compensation for moderate down-regulation of SERCA2a protein (Table 2).

CaW corresponds to multi-cellular diastolic calcium oscillations induced by rapid pacing in the guinea pig whole heart preparations [17]. This calcium oscillation is caused by $Ca^{2+}$ release from the sarcoplasmic reticulum [17]. Effects of a selective NCX1 inhibitor, SEA0400 and a SERCA2 inhibitor, CPA on $D_{20}$ and CaW in SA and ISO groups at normal (1.8 mM) and higher (10.8 mM) $[Ca^{2+}]_o$ were summarized in Table 2.

Significantly ($P < 0.05$) shortened $D_{20}$ in ISO group was only slightly widened by SEA0400. At 10.8 mM $[Ca^{2+}]_o$, each stimulus elicited diastolic CaW. CaW in ISO group was only slightly increased by SEA0400. These results indicated a less contribution of activation of NCX1 to $D_{20}$ and CaW in ISO group.

CPA significantly ($P < 0.05$) widened $D_{20}$ in SA and ISO mediated via suppression of SERCA2a activity. The CaW in ISO and SA was abolished by CPA, indicating that $Ca^{2+}$ stored in the sarcoplasmic reticulum was depleted.

In normal rat hearts, 80-87% $Ca^{2+}$ recirculates intracellulary [18, 19], i.e., the SERCA2a has a main role in $Ca^{2+}$ handling, although the NCX1 will become more important after inhibition of the SERCA2a [20, 21]. In ISO groups, SERCA2a activity is enhanced in compensation for moderate down-regulation of SERCA2a protein under normal $[Ca^{2+}]_o$. In NHEI+ISO group,

SERCA2a down-regulation is normalized associated with unchanged NCX1 activity. Therefore, it seems likely that SERCA2a activity returns to the normal heart level (see Table 1)[2].

An NHEI, BIIB723 normalized shortened $Ca^{2+}$ transient duration at intensity ratio of 0.2 ($D_{20}$) in myocardial slices from isoproterenol-induced cardiac hypertrophy mediated via normalization of SERCA2a activity. CaW in ISO under high $[Ca^{2+}]_o$ was exaggerated by down-regulation of SERCA2a protein and was suppressed by NHEI simultaneous treatment mediated via normalization of SERCA2a protein expression. The results presented here suggest multi-cellular $Ca^{2+}$ transient duration and CaW generated at high $[Ca^{2+}]_o$ in LV slices might be better indices reflecting SERCA2a activity than SERCA2a protein expression level.

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**Figures**

Fig. 1. $Ca^{2+}$ transients of left ventricular slices were recorded at 2-5 points in a single slice (B). A: Real $Ca^{2+}$ imaging at I, II and III phase in averaged $Ca^{2+}$ transient trace at 2-5 points during 25-30 stimuli under normal $Ca^{2+}$ concentration (C) and 10.8 mM $Ca^{2+}$ concentration (D). D20: normalized $Ca^{2+}$ transient duration at 0.2 of intensity ratio; CaW: diastolic calcium wave maximum amplitude. Upper sequential $Ca^{2+}$ imaging from 1-5 in D at 100 msec interval showed CaW propagation. Arrowheads indicate high $Ca^{2+}$ fronts.

Fig. 2. I-V relationship of NCX current (INCX) measured in myocytes. The current density normalized to the membrane capacitance was significantly larger in Isoproterenol-induced hypertrophy (ISO)(open circles: n=18) than in saline infused control (SA)(open circles: n=10). However, INCX in ISO + a $Na^+/H^+$ exchanger-1 (NHE-1) inhibitor (NHEI), BIIB723 (open squares: n=13) was not different from that in ISO. *: $P < 0.01$, ISO vs. SA myocytes. #: $P < 0.05$, ISO+NHEI vs. SA myocytes.

Table 1. Effects of NHEI on D20 and CaW in SA and ISO goups under 1.8 mM and 7.2 mM $Ca^{2+}$ conditions.

| | Group | D20 | CaW |
|---|---|---|---|
| 1.8 mM $Ca^{2+}$ | SA (n=11) | 140.6 ± 17.9 | 0 |
| | NHEI (n=6) | 134.2 ± 12.6 | 0 |
| | ISO (n=11) | 129.6 ± 14.6* | 0 |
| | NHEI+ISO (n=9) | 135.9 ± 12.2# | 0 |
| 7.2 mM $Ca^{2+}$ | SA (n=6) | 153.7 ± 19.0 | 0.020 ± 0.027 |
| | NHEI (n=6) | 147.2 ± 13.7 | 0.016 ± 0.025 |
| | ISO (n=7) | 136.9 ± 15.2 | 0.044 ± 0.034* |
| | NHEI+ISO (n=6) | 150.4 ± 20.3 | 0.017 ± 0.027# |

*, $P < 0.05$ vs. SA; #, $P < 0.05$ vs. ISO. n: number of hearts. Under 1.8 mM $Ca^{2+}$, data were averaged from 33-62 slices and under 7.2 mM $Ca^{2+}$, data were averaged form 16-18 slices in each group.

Table 2. Effects of SEA0400 and CPA on D20 and CaW in SA and ISO groups under 1.8 mM and 10.8 mM $Ca^{2+}$ conditions

| | | | 1.8 mM $Ca^{2+}$ | 10.8 mM $Ca^{2+}$ |
|---|---|---|---|---|
| D20 (msec) | SA | None (n=6) | 147.3 ± 18.3 | 158.1 ± 19.2 |
| | | SEA0400 (n=6) | 144.0 ± 11.4 | 143.2 ± 16.2 |
| | ISO | None (n=6) | 124.7 ± 9.3* | 169.6 ± 35.5† |
| | | SEA0400 (n=6) | 130.9 ± 6.3§ | 153.8 ± 19.9 |
| D20 | SA | None (n=6) | 147.3 ± 18.3 | 158.1 ± 19.2 |
| | | CPA (n=6) | 339.6 ± 70.0# | 536.5 ± 80.7#, † |
| | ISO | None (n=6) | 124.7 ± 9.3* | 169.6 ± 35.5† |
| | | CPA (n=6) | 408.6 ± 81.7# | 554.4 ± 149.6† |
| CaW (dimensionless) | SA | None (n=6) | 0 | 0.084 ± 0.046 |
| | | SEA0400 (n=6) | 0 | 0.073 ± 0.028 |
| | ISO | None (n=6) | 0 | 0.114 ± 0.054 |
| | | SEA0400 (n=6) | 0 | 0.121 ± 0.041‡ |
| CaW | SA | None (n=6) | 0 | 0.084 ± 0.046 |
| | | CPA (n=6) | 0 | 0# |
| | ISO | None (n=6) | 0 | 0.114 ± 0.054 |
| | | CPA (n=6) | 0 | 0# |

*, $P < 0.05$ vs. None in SA; §, $P < 0.05$ vs. SEA0400 in SA; #, $P < 0.05$ vs. None; †, $P < 0.05$ vs. 1.8 mM $Ca^{2+}$; ‡, $P < 0.05$ vs. SEA0400 in SA; n: number of hearts. In each group, data from 20-28 slices were averaged.

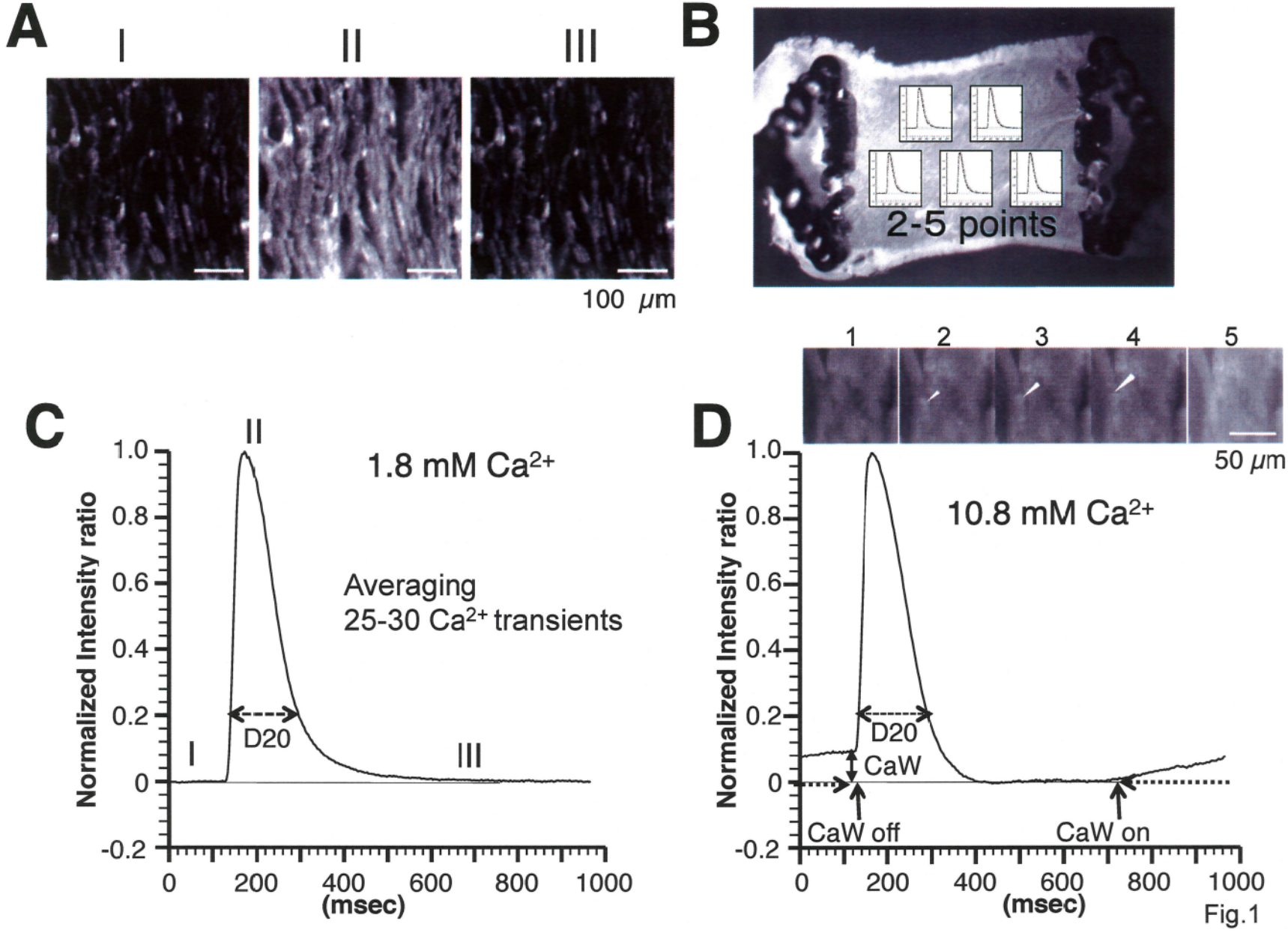
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Fig.1

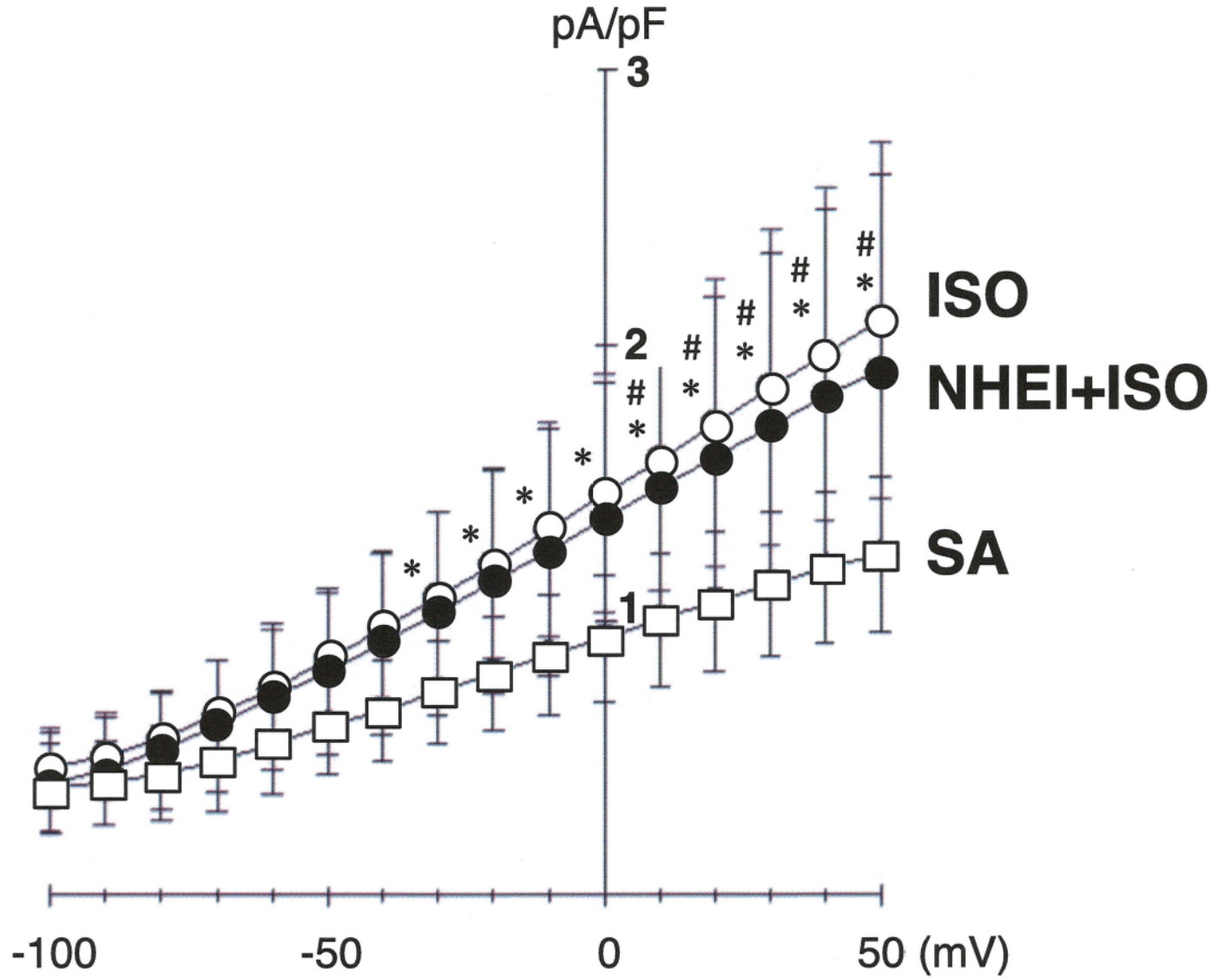


Fig. 2