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Suppression of L-type Ca^{2+} current by fluid pressure in rat ventricular myocytes: Possible role of Cl^-–OH^- exchange

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ABSTRACT

The application of fluid pressure (FP) in ventricular myocytes using pressurized fluid flow inhibits L-type Ca^{2+} current (I_{Ca}), with approximately 80% of this effect coming through the enhancement of Ca^{2+} releases from the sarcoplasmic reticulum. In the present study, we explored the remaining mechanisms for the inhibition of I_{Ca} by FP. Since FP significantly increases H^+ concentration and H^+ is known to inhibit I_{Ca}, we examined whether pH regulation plays a role in the inhibitory effect by FP on I_{Ca}. A flow of pressurized (~16.3 dyne/cm^2) fluid, identical to that bathing the myocytes, was applied onto single rat ventricular myocytes for which the I_{Ca} was monitored using whole-cell patch-clamp under HEPES-buffered conditions. Extracellular application of the alkalizing agent, NH_4Cl (20 mM), enhanced I_{Ca} by ~34% in the control conditions while increasing I_{Ca} significantly less (by ~21%) in FP-pretreated myocytes, suggesting an inhibition of the effect of NH_4Cl on I_{Ca} possibly by FP-induced acidosis. Application of DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid, 500 μM), which blocks Cl^-—HCO_3^- exchange but not Cl^-–OH^- exchange, did not alter the inhibitory effect of FP on I_{Ca}. Replacement of external Cl^- with aspartate attenuated the inhibitory effect of FP on I_{Ca}. In highly Ca^{2+}-buffered cells, where Ca^{2+}-dependent inhibition of I_{Ca} was minimized, the external Cl^- removal eliminated the inhibitory effect of FP on I_{Ca}. These results suggest that the decrease of I_{Ca} in the presence of FP is at least partly caused by intracellular acidosis via activation of Cl^-–OH^- exchange in rat ventricular myocytes.

1. Introduction

Activation of the cardiac L-type Ca^{2+} channels during a cardiac action potential leads to a Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum (SR) [1–4]. These channels are subsequently inactivated by the elevation of cytosolic Ca^{2+} concentration and by membrane depolarization [5,6]. This inactivating property is important in regulation of the intracellular Ca^{2+} concentration and the action potential duration of cardiac myocytes.

Pathological conditions, such as valve disease, hypertension, or heart failure, may lead to haemodynamic or mechanical dysfunction of the heart, causing arrhythmia [7–12]. In both whole heart preparations and *in situ* hearts, an increase in ventricular pressure shortens the duration of the action potential and the effective refractory period [9–11]. Moreover, clinical evidence exists for a predisposition to fibrillation caused by regurgitant jets of blood in patients with valve incompetence [12], as well as for the occurrence of ectopic tachycardia in a patient with a catheter in the heart chamber [13]. These findings suggest the possibility that a fluid pressure (FP) and/or irritation may directly alter membrane excitability. It has been previously reported that fluid pressure suppresses L-type Ca^{2+} current (I_{Ca}) and increases systolic and diastolic Ca^{2+} levels in rat ventricular myocytes [14]. The inhibition of I_{Ca} by FP observed in single ventricular cells could be one of the possible mechanisms that explain the shortenings of action potential and of the effective refractory period during increases in ventricular pressure [9–11].

Interestingly, most (≅80%) of the inhibition of L-type Ca^{2+} current by FP was eliminated by dialysis of myocytes with high concentrations of Ca^{2+} buffer [14]. However, even in highly Ca^{2+}-buffered myocytes, Ca^{2+} or Ba^{2+} current through the L-type Ca^{2+} channels continued to be significantly inhibited by FP [14]. These previous findings suggest that, although the main inhibitory mechanism for the Ca^{2+} channel during FP is the increase in intracellular Ca^{2+} transients in rat ventricular myocytes, another inhibitory mechanism may exist for the Ca^{2+} channel under FP stimulus. FP also induces intracellular acidosis under nominally HCO_3^--free, HEPES-buffered conditions in rat ventricular myocytes [15]. As L-type Ca^{2+} channels are inhibited by intracellular acidosis [16–18], intracellular acidosis is another possible mechanism for the inhibition of I_{Ca} with FP. In the present study, we examined this possibility by monitoring I_{Ca} in rat ventricular myocytes using whole-cell patch-clamp in combination with the same micro-jet method previously described by Lee et al.

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[14]. The present data indicate that, in addition to the cytosolic Ca^{2+} increase, FP-induced I_{Ca} inhibition may be partly caused by intracellular acidosis through the activation of Cl^{-}–OH^{-} exchange in rat ventricular myocytes.

2. Materials and methods

2.1. Single cell isolation

Rat ventricular myocytes were enzymatically isolated from male Sprague–Dawley rats (200–300 g) as described previously [14]. Briefly, rats were deeply anesthetized with sodium pentobarbital (150 mg/kg, *i.p.*), the chest cavity was opened and hearts were excised. This surgical procedure was carried out in accordance with the ethical guidelines of the university. The excised hearts were retrogradely perfused at 7 ml/min through the aorta (at 36.5 °C), first for 5 min with Ca^{2+}-free Tyrode solution composed of (in mM) 137 NaCl, 5.4 KCl, 10 HEPES, 1 $MgCl_2$, 10 glucose, pH 7.3, and then with Ca^{2+}-free Tyrode solution containing collagenase (1.4 mg/ml, Type 1, Roche) and protease (0.14 mg/ml, Type XIV, Sigma) for 12 min, and finally with Tyrode solution containing 0.2 mM $CaCl_2$ for 8 min. The ventricles of the digested heart were

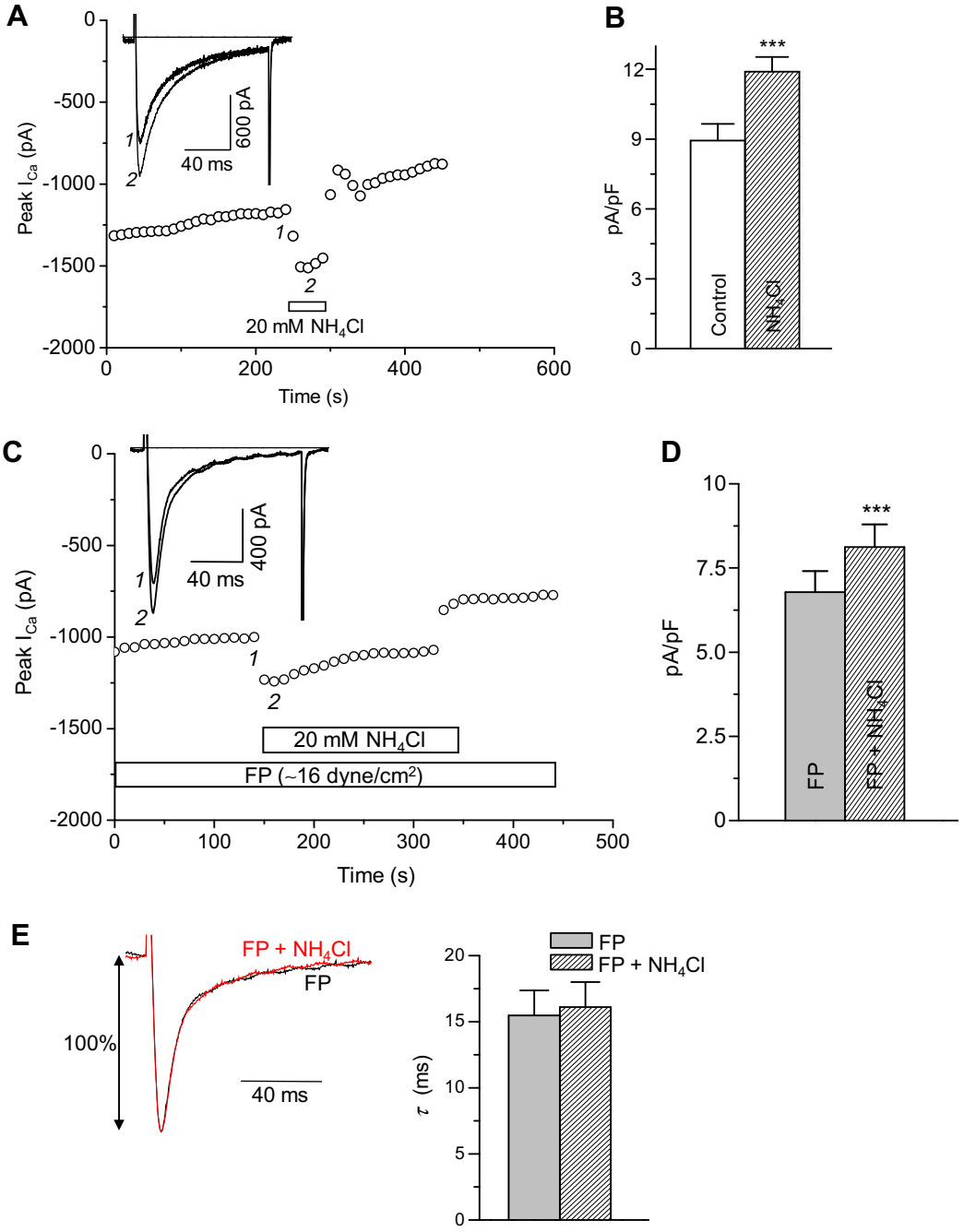

Fig. 1. Reversal of the inhibitory effect of FP on I_{Ca} by alkali loading. (A) Time course of the changes in I_{Ca} by 20 mM NH_4Cl in a representative rat ventricular myocyte. *Inset*, Superimposed current traces recorded at the numbered points. (B) Comparison of mean I_{Ca} between control and NH_4Cl ($n = 12$). $^{***}P < 0.001$ *vs.* control. (C) Time course of the change in I_{Ca} and NH_4Cl in a representative rat ventricular myocyte, which was pre-treated with FP (~16.3 dyne/cm^2). *Inset*, Superimposed current traces recorded at the numbered points. (D) Comparison of mean I_{Ca} between "FP" and "FP + NH_4Cl" ($n = 8$). $^{***}P < 0.001$, *vs.* FP. (E) Left panel: current traces, recorded at "FP" and "FP + NH_4Cl", were superimposed after normalization to the peaks. Right panel: Mean time constants (τ) of I_{Ca} inactivation measured at each intervention ($n = 6$).

then cut into several sections and subjected to gentle agitation to dissociate the cells. The freshly dissociated cells were stored at room temperature in Tyrode solution containing 0.2 mM $CaCl_2$.

2.2. Application of fluid pressure

Myocytes were continuously superfused with the Tyrode solution composed of (in mM) 137 NaCl, 5.4 KCl, 10 HEPES, 1 $MgCl_2$, 10 Glucose, 2 $CaCl_2$, pH 7.4. Pressurized flows of solutions were applied onto the single myocytes through a microbarrel (internal diameter = 250 μm) as previously described [14,19]. The tip of microbarrel was placed at ≈150 μm from the cell and was connected to a fluid reservoir with 400-mm height. This height produced FP of about 16 dyne/cm^2 [14]. Electronic solenoid valve was installed in the middle of tubing connecting the fluid reservoir and the microbarrel, the tip of which, touching the chamber bottom, was tilted to one side with an angle of 45°. The positioning of the microbarrel was performed under microscope (TS2000, Nikon) using a micromanipulator (Prior England 48260).

The experimental cells were attached to the bottom of the chamber without a coating material. Using a microscope and video monitor, it was confirmed that no movement of the cell occurred during the fluid puffing before the start of the patch clamp experiments. The cells used for the recordings did not float or move at a FP of approximately 16 dyne/cm^2.

2.3. Current measurements and analysis

I_{Ca} was recorded using the whole-cell configuration of the patch-clamp technique [20] using an EPC7 amplifier (HEKA Electronik, Lambrecht/Pfalz, Germany). The patch pipettes were made of glass capillaries (Kimble Glass Inc., Vineland, NJ, USA) to have resistance of 2–3 MΩ when filled with the internal solution containing (in mM) 110 CsCl, 20 TEA-Cl, 20 HEPES, 5 MgATP, and 2 EGTA, with the pH adjusted to 7.2 with CsOH; in some experiments 10 mM BAPTA was also included in the pipette solution (see Fig. 4). Outward K^+ currents were suppressed by replacing internal K^+ with Cs^+ and TEA^+, and inward rectifier K^+ current was suppressed by replacing external K^+ with Cs^+. Na^+ current was inactivated by holding the membrane potential at −40 mV. Trains of test pulses were to 0 mV for 120 ms with 0.1 Hz. The I_{Ca} was fully sensitive to 20 μM of nifedipine (*data not shown*) and to 200 μM Cd^{2+} [14]. Measurement of I_{Ca} was carried out between 5 and 6 min after rupture of the membrane with the patch pipette, at the time when the rundown of Ca^{2+} channels was slowed and stabilized. Generation of voltage protocols and acquisition of data were carried out using pCLAMP (9.0, Axon Instruments, CA, USA) combined with an A/D converter (Digidata 1322, Axon Instruments). The series resistance was 1.5–3 times the pipette resistance and was electronically compensated through the amplifier. The current signals were digitized at 10 kHz and low-pass filtered at 1 kHz.

We usually monitored raw currents as well as currents leak-subtracted by the P/N method ($N = 5$). Data in the figures are shown without leak subtraction to demonstrate that the cells had low, stable leak current. The% suppression of I_{Ca} by various interventions was evaluated after a gradual decrease in I_{Ca} by subtracting the rundown from the raw current [21]. Briefly, the time course of changes in peak I_{Ca} measured in the control conditions was fitted using double exponential curve fitting; the fitted curve was then considered to be a rundown component. The difference between the original time course and the fitted curve resulted in pure changes in I_{Ca}, which were used to measure percentage changes during the interventions.

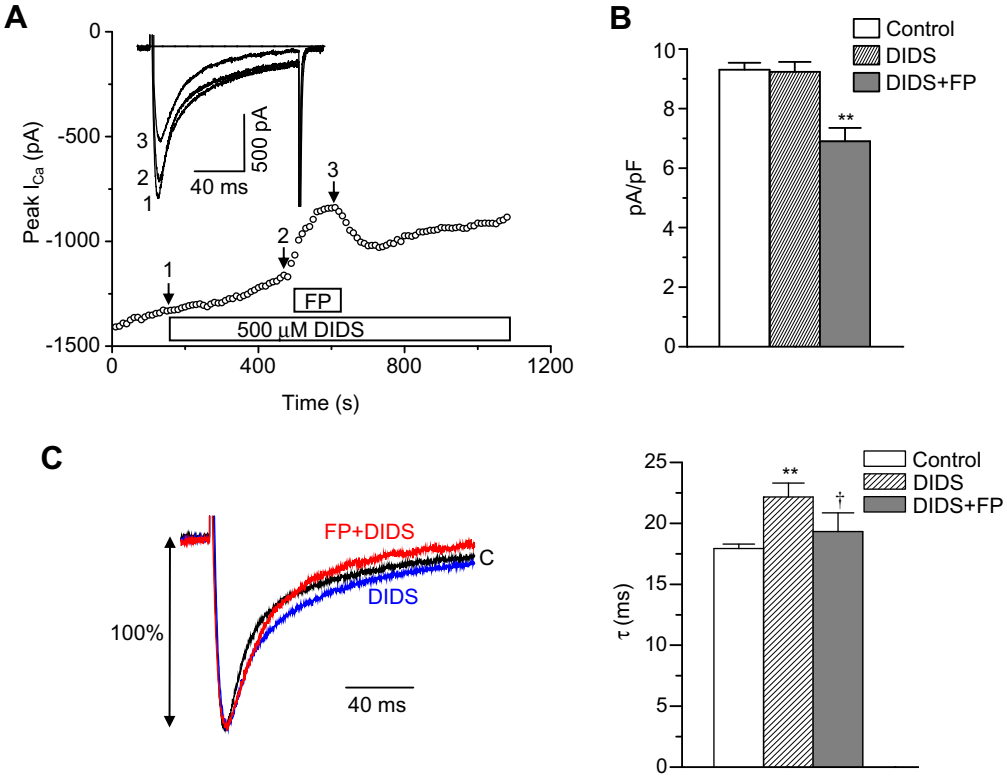

Fig. 2. Effects of DIDS on FP-induced I_{Ca} suppression. (A) Time courses of the changes in I_{Ca} by FP (~16.3 dyne/cm^2) in a representative myocyte pre-incubated with DIDS (500 μM). *Insets* in the panel A shows superimposed currents recorded at the numbered points. (B) Comparisons of mean peak I_{Ca} among the "Control", "DIDS", and "DIDS + FP" ($n = 7$). $^{**}P < 0.01$ *vs.* "DIDS". (C) Left panel: Current traces recorded at control ("C"), "DIDS" and "DIDS + FP" were superimposed after normalization to the peaks. Right panel: Mean time constants (τ) of I_{Ca} inactivation measured at each intervention ($n = 7$). $^{**}P < 0.01$ *vs.* control. $^{\dagger}P < 0.05$ *vs.* DIDS.

Peak detection was performed with Clampfit (9.0, Axon Instruments), and the time constant (τ) of inactivation of I_{Ca} was obtained with single exponential curve fitting using the equation:

$$y = (A_i - A_f) \cdot \exp(-t/\tau) + A_f$$

where A_i and A_f are, respectively, the intitial ($t = 0$) and final (t = infinity) values of the parameter, and τ is a time constant of exponential decay.

All the experiments were performed at (22–25 °C).

2.4. Statistics

Numerical results are presented as mean ± standard error of the mean (SEM) (n = number of cells). A paired Student's t test was used to evaluate the statistical significance of differences between means, and differences at $P < 0.05$ were considered to be significant.

3. Results

3.1. Reversal of fluid pressure-induced I_{Ca} suppression by alkali loading

It is well known that intracellular acidosis inhibits L-type Ca^{2+} current [16–18]. To determine the remaining mechanism (except intracellular Ca^{2+} increase) for the inhibition of I_{Ca} during FP, we examined whether a part of the inhibition of I_{Ca} by FP was caused by FP-induced intracellular acidosis [15]. We applied alkalizing agent NH_4Cl from the external side to inhibit FP-induced acidosis. Extracellular NH_4Cl produces transient intracellular alkalinization during application and transient acidification upon wash-out [22]. I_{Ca} transiently increased during the application of external NH_4Cl, and the current decreased again after removal of external NH_4Cl (Fig. 1A). This is consistent with the previous observations [22,23]. In the absence of FP, the external application of 20 mM NH_4Cl increased I_{Ca} by 34 ± 5.2% (n = 12, $P < 0.001$; Fig. 1B). When the cells were exposed to FP, I_{Ca} was reduced by 26 ± 4.3% (n = 24). In the continued presence of FP, additional treatment of the same concentration (20 mM) of NH_4Cl increased the peak I_{Ca} by 21 ± 3.1% (n = 8, $P < 0.001$; Fig. 1D). The level of increase in I_{Ca} by NH_4Cl was significantly smaller in the presence of FP than that observed in the absence of FP (~34% without FP *vs.* ~21% with FP). The inactivation time constant (τ) of I_{Ca} was not altered by NH_4Cl in the presence of FP (Fig. 1E; "FP", 15.5 ± 1.9 ms *vs.* "FP + NH_4Cl", 16.1 ± 1.9 ms, n = 6, $P > 0.05$). FP-induced acidosis may oppose NH_4Cl-mediated alkalosis, thereby reducing the effect of NH_4Cl on I_{Ca}. The results suggest that FP-induced I_{Ca} suppression may be partly mediated by intracellular acidosis.

3.2. No effect of DIDS on fluid pressure-dependent suppression of I_{Ca}

In the present study, we used HCO_3^--free, HEPES-buffered solutions. Under these conditions, the acid loading mechanism is most likely to be activation of Cl^-–OH^- exchange (or alternatively a Cl^-–H^+ symport) or inhibition of Na^+–H^+ exchange [24]. We tested the effect of the stilbene drug DIDS, which blocks HCO_3^- transport, but not Cl^-–OH^- exchange [24–26]. DIDS (500 μM) itself did not change I_{Ca} (Fig. 2A and B), but significantly slowed inactivation of I_{Ca} (Fig. 2C; τ: "Control", 17.9 ± 0.37 ms *vs.* "DIDS", 22.2 ± 1.14 ms, n = 7, $P < 0.01$). In DIDS pre-treated ventricular myocytes, FP (~16 dyne/cm^2) suppressed I_{Ca} by ≅25% (compare "2" and "3" in

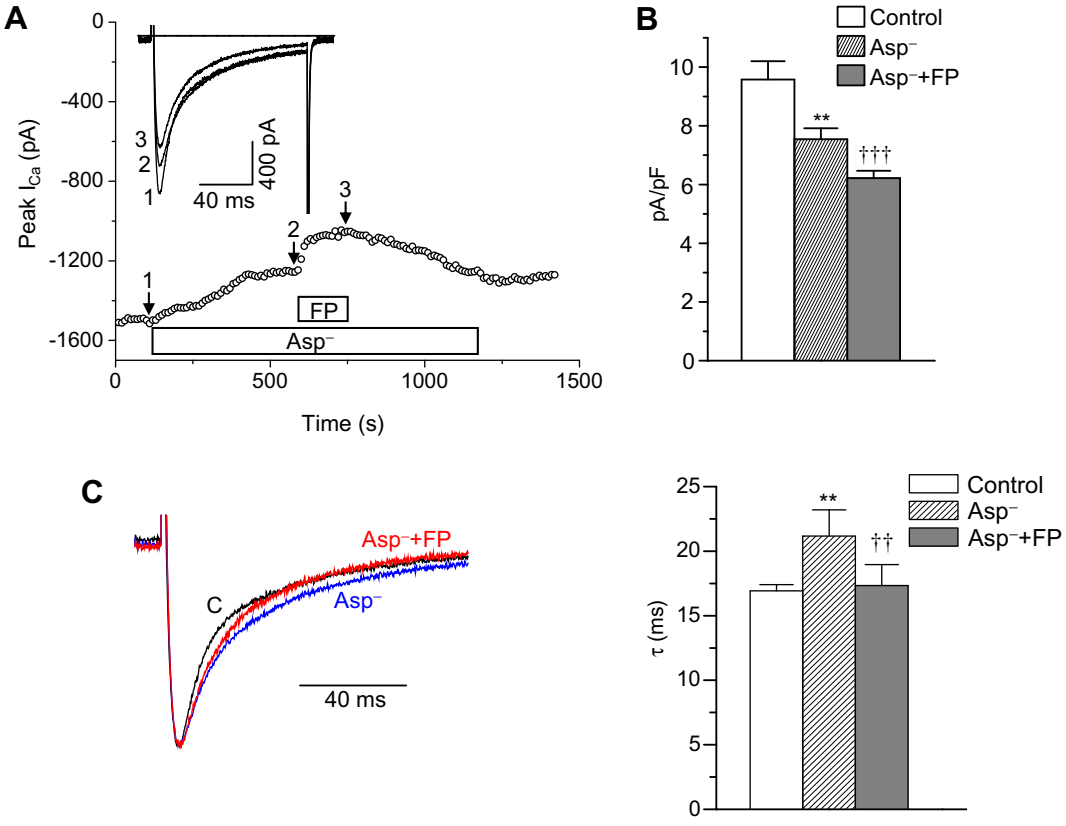

Fig. 3. Effects of external Cl^- removal on FP-induced I_{Ca} supression. (A) Time courses of the changes in I_{Ca} by FP (~16.3 dyne/cm^2) in a representative myocyte pre-incubated with low Cl^- external solution ("Asp^-"). *Insets* shows superimposed currents recorded at the numbered points. (B) Comparisons of mean peak I_{Ca} among the control, "Asp^-", and "Asp^- + FP" (n = 8). $^{**}P < 0.01$ *vs.* Control. $^{\dagger\dagger\dagger}P < 0.001$ *vs.* "Asp^-". (C) Left panel: Current traces recorded at control, "Asp^-", and "Asp^- + FP" were superimposed after normalization to the peaks. Right panel: Mean time constants (τ) of I_{Ca} inactivation measured at each intervention (n = 8). $^{**}P < 0.01$ *vs.* control. $^{\dagger\dagger}P < 0.01$ *vs.* "Asp^-".

Fig. 2A and B), which is comparable with the level of inhibition of I_{Ca} by FP in control conditions (26 ± 4.3%, n = 24; [14]). In the presence of DIDS, FP significantly accelerated I_{Ca} inactivation (τ: "DIDS", 22.2 ± 1.14 ms *vs.* "DIDS + FP", 19.3 ± 1.53 ms, n = 7, P < 0.05). However, the level of acceleration was smaller in DIDS-treated cells (12.9 ± 1.4%) compared with untreated cells (τ: control, 17.8 ± 2.0 ms *vs.* FP, 14.2 ± 1.1 ms; 20.5 ± 1.6%, n = 45, P < 0.01; Fig. 3 in Ref. [14]). This result supports the idea that suppression of peak I_{Ca} by FP is not mediated by HCO_3^- transport.

3.3. External Cl^- removal diminishes the inhibitory effect of FP on I_{Ca}

In the next series of experiments, we further examined the possible role of Cl^-–OH^- exchange on the inhibitory effect of FP on I_{Ca} by removing external Cl^-. 137 mM extracellular Cl^- was replaced with equimolar aspartate (final external [Cl^-] = 11.4 mM). We observed a significant decrease in I_{Ca} when external Cl^- was replaced with aspartate. Peak I_{Ca} density recorded in the presence and absence of external Cl^- was 9.58 ± 0.64 pA/pF and 7.55 ± 0.37 pA/pF, respectively (n = 8, P < 0.01; Fig. 3B). When the I_{Ca} became stable after removal of external Cl^-, additional treatment of FP (~16 dyne/cm^2) further suppressed I_{Ca} by approximately 17% (6.22 ± 0.25 pA/pF, n = 8, P < 0.001; Fig. 3A and B), indicating that the inhibitory effect of FP on I_{Ca} was attenuated by external Cl^- removal. The inactivation of I_{Ca} was retarded by removal of external Cl^- (τ: "Control", 16.9 ± 0.48 ms *vs.* "Asp^-", 21.2 ± 2.04 ms, n = 8, P < 0.01; Fig. 3C). FP accelerated I_{Ca} inactivation in low Cl^- extracellular solution (τ: "Asp^- + FP", 17.3 ± 1.63 ms, n = 8, P < 0.01 *vs.* "Asp^-"; Fig. 3C). The extent of I_{Ca} acceleration by FP measured at low external Cl^- solution (18.6 ± 2.1%) was similar to that measured in the control condition (P > 0.05).

3.4. Elimination of the inhibitory effect of FP on I_{Ca} by external Cl^- removal in highly Ca^{2+}-buffered myocytes

We have previously observed that in the myocytes dialyzed with high concentrations of Ca^{2+} buffer (15 mM EGTA or 10 mM BAPTA), FP continues to decrease I_{Ca} although the level of inhibition is small (by about 5–7%; [14]). In addition, Ba^{2+} current was also slightly suppressed by FP (by ~5%) [14]. Therefore, we performed the same series of experiments in highly Ca^{2+}-buffered myocytes to exclude the Ca^{2+}-dependent I_{Ca} inhibition during FP. In 10 mM BAPTA-dialyzed myocytes, the pre-treatment of the low-level Cl^- external solution abolished the inhibition of I_{Ca} by FP (pA/pF: "Asp^-", 10.3 ± 0.56 *vs.* "Asp^- + FP", 10.5 ± 0.45, n = 7, P > 0.05; Fig. 4A and B). In BAPTA-dialyzed cells, external Cl^- removal decreased I_{Ca} and slightly accelerated I_{Ca} inactivation (τ: Control, 30.4 ± 2.05 ms *vs.* "Asp^-", 33.5 ± 2.27 ms, n = 7, P < 0.05; Fig. 4C). In contrast, the application of FP in a low external Cl^- solution did not affect I_{Ca} inactivation (τ:"Asp^- + FP", 33.0 ± 2.12 ms, n = 7, P > 0.05 *vs.* "Asp^-"; Fig. 4C).

4. Discussion

This study showed that enhancement of I_{Ca} by NH_4Cl-mediated alkalosis is attenuated by fluid pressure, and that inhibitory effect of fluid pressure on L-type Ca^{2+} channels is partially inhibited by the removal of external Cl^- in minimally Ca^{2+}-buffered cells, and fully suppressed by this removal in highly Ca^{2+}-buffered cells. The effect of FP was not changed by DIDS, which blocks Cl^-—HCO_3^- exchange, but not Cl^-–OH^- exchange. Based on these findings we propose that a part (~20%) of the inhibitory effect of FP on I_{Ca} is mediated by external Cl^--dependent acid loading via

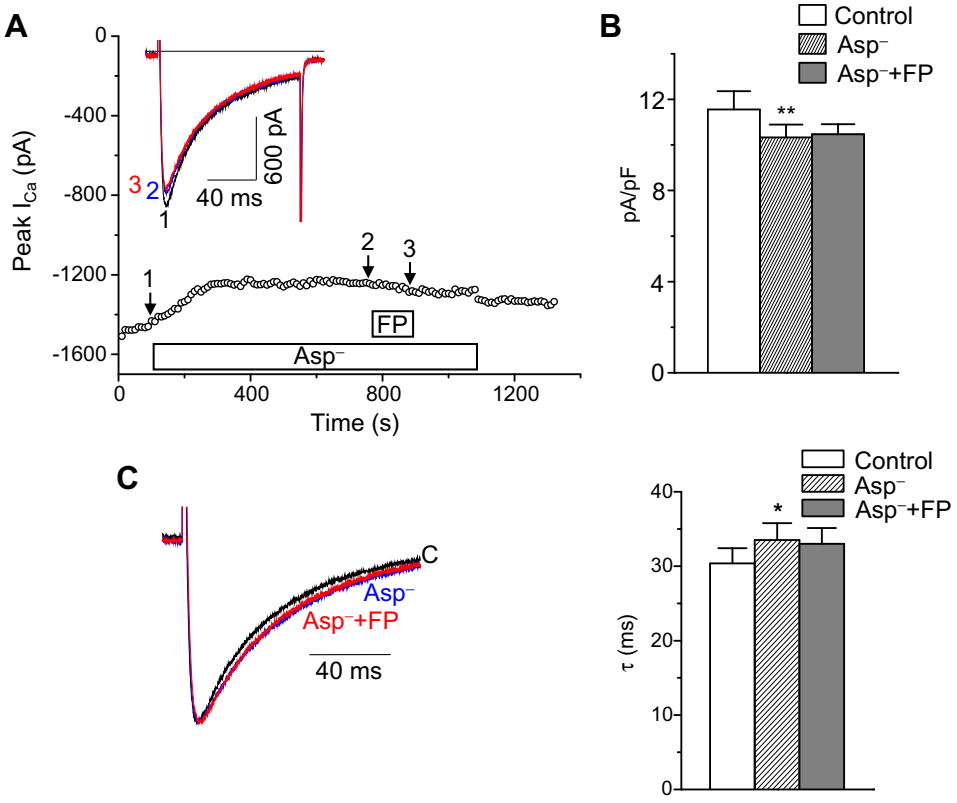

Fig. 4. Inhibitory effect of FP on I_{Ca} was eliminated by external Cl^- removal and intracellular high Ca^{2+} buffering. (A) Time courses of the changes in I_{Ca} by FP (~16.3 dyne/cm^2) in a representative myocyte pre-incubated with low Cl^- (11.4 mM) external solution ("Asp^-"). Cells were dialyzed with 10 mM BAPTA-containing internal solution. *Insets* in the panel A shows superimposed currents recorded at the numbered points. (B) Comparisons of mean peak I_{Ca} among the control, "Asp^-", and "Asp^- + FP" (n = 7). $^{**}P$ < 0.01 *vs.* Control. (C) Left panel: Current traces recorded at control ("C"), "Asp^-", and "Asp^- + FP" were superimposed after normalization to the peaks. Right panel: Mean time constants (τ) of I_{Ca} inactivation measured at each intervention (n = 7). $^{*}P$ < 0.05 *vs.* Control.

the activation of the Cl^-–OH^- exchanger. In HEPES-buffered conditions, it is also possible that the inhibition of Na^+–H^+ exchange causes intracellular acidosis to affect I_{Ca}. This possibility, however, is not likely, because the blockade of Na^+–H^+ exchange using HOE 642 (cariporide) enhanced FP-induced intracellular acidosis ([15]: Data supplement 1). Instead, the previous data suggest that FP may activate Na^+–H^+ exchange.

The mechanism by which the Cl^--dependent OH^- exchange is activated during FP stimulus remains unclear. Nevertheless, our data provide a novel regulatory mechanism of the Cl^-–OH^- exchange in cardiac cells. The activation of Cl^-–OH^- exchange during FP may cause intracellular acidosis, resulting in increase of both $[Na^+]_i$ and cell volume via the activation of Na^+–H^+ exchange and Na^+–K^+–$2Cl^-$ cotransport. Such mechanism is well known as the process of regulatory volume increase (RVI) [27]. We have previously observed that FP induces acidosis and such acidosis is increased by the inhibition of Na^+–H^+ exchange [15]. Fluid pressures of ~16 dyne/cm^2, in fact, induces slight shrinkage in rat ventricular myocytes (Data supplement 2). Possible volume increases through the process of activation of RVI may help cells recover from shrinkage induced by FP. It should be also noted that such shrinkage or local deformation of the surface membrane in the form of concave shape by FP may also induce increase in local Ca^{2+} concentration in the dyadic junctions sufficient to enhance Ca^{2+}-induced Ca^{2+} release. This possibility may explain the Ca^{2+}-dependent I_{Ca} inhibition during FP stimulus [14].

DIDS has been used in the present study to inhibit Cl^-—HCO_3^- exchange except Cl^-–OH^- exchange (Fig. 2; [25,26]). At the concentration of 500 μM, DIDS did not dramatically inhibit I_{Ca}. However, rundown of the I_{Ca} was faster in the presence of DIDS than with the control conditions. This observation is somewhat distinguished from the previous report on significant inhibition of L-type Ca^{2+} current in colonic myocytes by micromolar (10–100 μM) ranges of DIDS [28]. We observed a significant decrease in I_{Ca} when external Cl^- was replaced with aspartate. This appears to be consistent with the previous reports that reducing extracellular Cl^- suppresses L-type Ca^{2+} current independently of changes in extracellular or intracellular pH and induces a hyperpolarizing shift in the voltage-dependence of I_{Ca} [29,30]. The reduction of I_{Ca} by removal of external Cl^- was attenuated in highly Ca^{2+} buffered cells (compare Fig. 3B and Fig. 4B). This finding suggests the reduction of I_{Ca} by Cl^- removal in ventricular cells may be related to change in intracellular Ca^{2+} concentration.

In whole heart preparations and in the *in situ* hearts of human and dogs, a rise in ventricular pressure leads to shorter durations for the cardiac action potential and effective refractory period [9–11]. However, in a ventricle tissue preparation and in single ventricular myocytes, membrane stretch did not consistently decrease the action potential duration; rather, this duration showed both an increase and a decrease, depending on the measurement taken [31,32]. We propose that, although there are other components involved in the action potential repolarization, the FP-induced decrease in the I_{Ca} can contribute to a shortening of the refractory period and/or to arrhythmia in a ventricular chamber wall exposed to the haemodynamic forces of fluid (e.g., a blood jet to the ventricular wall during aortic regurgitation, or the excessive pressure produced during aortic valve stenosis). Additional studies revealing the signaling mechanism by which the fluid pressure regulates Cl^-–OH^- exchange in cardiac myocytes remain to be undertaken.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.08.026.

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