Effects of K^+ Channel Modulators on the Relationship Between Action Potential Duration and Ca^{2+} Transients in Single Ventricular Myocytes of the Guinea Pig

Tomoaki Tokuno¹, Katsuhiko Muraki², Minoru Watanabe² and Yuji Imaizumi^{1,*}

¹Department of Pharmacology & Therapeutics and ²Department of Chemical Pharmacology, Faculty of Pharmaceutical Sciences, Nagoya City University, Nagoya 467–8603, Japan

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ABSTRACT—Effects of K^+ channel modulators, cromakalim and E4031 [1-[2-(6-methyl-2-pyridyl)-ethyl]-4-(4-methylsulfonylamino)benzoyl] piperidine], on the relationship between the action potential duration (APD) and Ca^{2+} transients were examined in single myocytes isolated from guinea pig cardiac left ventricle. Application of cromakalim decreased APD at 90% repolarization (APD₉₀) and Ca^{2+} transient elicited at 0.5 Hz (IC₅₀s = 0.6 and 3 μ M, respectively). Application of 0.3 μ M E4031 increased these parameters by 110% and 45%, respectively. Under voltage-clamp, the relation between the duration of depolarization to 0 mV and Ca^{2+} transients could be described by the sum of two exponential components; the time constants were approximately 5 and 280 msec, respectively. The first component was abolished by 10 μ M ryanodine, suggesting the involvement of Ca^{2+} -induced Ca^{2+} release (CICR). Neither cromakalim nor E4031 directly affected Ca^{2+} current and Ca^{2+} transients under voltage clamp. When APD was changed by K^+ channel modulators, the relation between APD₉₀ and Ca^{2+} -transients was almost similar to that obtained by changing the depolarization duration under voltage-clamp. CICR was changed significantly only when APD₉₀ was markedly shortened by cromakalim. The extensively prolonged AP and Ca^{2+} transient in the presence of E4031 were reduced by an addition of cromakalim. It is concluded that these two K^+ channel modulators can significantly alter the AP-induced Ca^{2+} transient mainly by changing APD, which regulates both Ca^{2+} influx and extrusion.

Keywords: Heart, K^+ channel, Cromakalim, E4031, Ca^{2+} transient

Several categories of drugs, which selectively modulate K^+ channel activities, have been developed for the therapy of cardiovascular disorders (1). Cromakalim, which is a typical opener of ATP-sensitive K^+ channels and has been evaluated as an antihypertensive drug, reduces action potential duration (APD) in cardiac myocytes and shows cardioprotective effects in ischemic heart (2, 3). E4031 is a selective blocker of the rapidly activating delayed rectifier (HERG) K^+ current (4, 5), which contributes to action potential (AP) repolarization in several types of cardiac myocytes (4, 6). E4031 was developed as a class III antiarrhythmic drug that would prolong the AP refractory period (7). The reverse frequency dependence of HERG K^+ channel blockers (5), including E4031, makes the clinical use of these drugs for tachyarrhythmia difficult. For example, HERG K^+ channel block

may result in arrhythmogenic actions and occasionally can induce QT-prolongation and *torsades de pointes* (TdP) (8). Although these two drugs are selective for K^+ channels and do not directly affect voltage-dependent Ca^{2+} channel activities, the drug-induced changes in APD modulate the amount of Ca^{2+} -influx. Significant changes in APD in ventricle can cause either an increase or decrease in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and, thereby, result in positive and negative inotropic action, respectively.

The elevation of $[Ca^{2+}]_i$ corresponding to AP in cardiac myocytes is, however, substantially due to Ca^{2+} -induced Ca^{2+} release (CICR) from sarcoplasmic reticulum (9–11). The relation between the amount of Ca^{2+} influx and the initiation of CICR has been extensively studied in several types of cardiac myocytes (12–14). However, little is known about how drugs which act on K^+ channels alter the Ca^{2+} transients induced by APs.

* To whom correspondence should be addressed.

The relationship between APD and Ca^{2+} transient is of importance with respect to understanding of the overall effects of these K^+ channel modulators on contractility of cardiac myocytes. The present study was undertaken to clarify quantitatively the relation between APD and Ca^{2+} transients in the presence of two K^+ channel modulators. In corresponding voltage clamp experiments, the relation between the duration of the depolarization and Ca^{2+} transient was obtained under control conditions and in the presence of E4031 or cromakalim. The postulated effect of a K_{ATP} channel opener on long QT syndrome (15) which could be, at least in part, mimicked by treatment with E4031 was also examined by the simultaneous measurement of the AP and Ca^{2+} transients.

MATERIALS AND METHODS

Cell isolation

The cell isolation procedure was similar to that described previously (16). In brief, after a male guinea pig (250–350 g) was anesthetized by i.p. administration of thiopentone, the heart was removed and mounted on a Langendorff perfusion system and first infused with normal Krebs' solution for 5 min and then with nominally Ca^{2+} -free Krebs' solution for 10 min. Thereafter, Ca^{2+} -free Krebs' solution containing 0.03–0.04% collagenase (Yakult, Tokyo) was applied for approximately 10 min. The residual collagenase was then washed away by perfusion with Kraftbrühe (KB) solution (17). Strips of tissue from the left ventricular wall were dissected and agitated in the KB solution with a Pasteur pipette having a fire-polished tip. The isolated cells were kept at room temperature ($23 \pm 1^\circ\text{C}$) in a HEPES-buffered solution (see solutions) containing 100 μM Ca^{2+} until use.

Electrical measurements

A single myocyte was voltage or current clamped using the standard whole-cell clamp technique (18) (amplifier: CEZ-2100; Nihon Kohden, Tokyo). The resistance of microelectrodes when filled with internal solution was less than 3 M Ω . All experiments were carried out at $37 \pm 1^\circ\text{C}$. Action potentials were elicited by current injection of 4–6 nA for 0.7 msec through recording pipettes at 0.5 Hz. Current and voltage signals were stored on a videotape using a PCM system (501ES, modified to allow DC recordings; Sony, Tokyo). Electrical signals stored on the videotape were replayed and digitized on an IBM-AT computer using an analog-digital converter (DT2801A; Data Translation, Marlborough, MA, USA) controlled by acquisition (AQ) and analysis (Cellsoft) program developed by D. Bergman at The University of Calgary, Canada. The command potentials under voltage clamp were corrected for -9 mV to account for junction potential (19).

Measurement of indo-1 signal from single myocytes

To load myocytes with indo-1, 100 μM indo-1 was added to the pipette solution. Since it took several minutes to load myocytes with indo-1, experiments were started at least 5 min after the rupture of the patch membrane. The indo-1 signal was measured in the same way as reported previously (20), using a spectrometer attached to an inverted microscope (TMD; Nikon, Tokyo). In brief, the UV light for excitation was obtained from a xenon lamp (XBO 150W; Osram, Munich, Germany), filtered at 360 ± 5 nm (Nikon filter, EX360IF) and reflected by a dichroic mirror (Nikon DM 380). The emitted fluorescence was filtered (Nikon BA 400) and split into two wavelengths by a dichroic mirror (Nikon DM 450) and filters (Nikon BA 405 and 485). The two ranges of wavelength measured in this system were approximately 405–450 (F_{405}) and 485–520 nm (F_{485}). The F_{405} and F_{485} signals were filtered at cut-off frequency of 300 Hz and the ratio of fluorescent intensity, F_{405}/F_{485} , was obtained by using an analog divider (DIV 100; Burr-Brown, Tucson, AZ, USA). The changes in Ca^{2+} transient during APs by application of cromakalim or E4031 could not be evaluated clearly by the measurement of peak value since the marked change occurred in the sustained component of Ca^{2+} transient level (see Results). Therefore, the evaluation of Ca^{2+} transients was performed by integrating the signal for 1 sec from the start of stimulation rather than by measuring the peak values. The baseline for the integration was the level averaged for 100 msec before the start of stimulation. In this paper the ratio signals shown have been filtered at 30 Hz. Although the calibration of $[\text{Ca}^{2+}]_i$ could not be performed in every cell, it was done in 7 cells using the methods introduced by Ganitkevich and Isenberg (21).

Solutions

Normal Krebs' solution for cell isolation had the following composition: 117 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl_2 , 1.2 mM MgCl_2 , 2.2 mM KH_2PO_4 , 14 mM glucose and 10 mM NaHCO_3 . This solution was gassed with 95% O_2 – 5% CO_2 . The composition of the external solution for electrical recordings was as follows: 139 mM NaCl, 5.9 mM KCl, 2.2 mM CaCl_2 , 1.2 mM MgCl_2 , 14 mM glucose and 10 mM HEPES. The pH of the solution was adjusted to 7.4 with NaOH. The pipette solution contained 100 mM K-aspartate, 50 mM KCl, 2 mM Na_2ATP , 1 mM MgCl_2 , 5 mM HEPES and 0.1 mM indo-1. The pH was adjusted at 7.2 with KOH.

Chemicals

Ryanodine (Wako Junyaku, Osaka), CdCl_2 (Wako Junyaku), adenosine triphosphate disodium salt (ATP; Sigma, St. Louis, MO, USA), cromakalim (Sigma), col-

lagenase (500 unit/mg, Yakult), EGTA (Dojin Kagaku, Kumamoto) and 1-[2-amino-5-(6-carboxy-2-indolyl)phenyl]-2-(2-amino-5-methylphenoxy)ethane-*N,N,N',N'*-tetra-acetic acid dipotassium (indo-1) (Dojin) were obtained from the companies shown in parentheses. E4031 [1-[2-(6-methyl-2-pyridyl)-ethyl]-4-(4-methylsulfonylamino benzoyl) piperidine] was provided by Eisai Pharmaceutical Japan (Tokyo).

Statistics

Pooled data are expressed as the mean $\pm$ S.E.M. Statistical significance between two and multiple groups were examined by Student's *t*-test and Scheffé's test following one-way ANOVA, respectively. In some analyses, the fitness of data to theoretical values was examined using the χ^2 test.

RESULTS

Effects of cromakalim on APD and Ca²⁺ transients

Figure 1 shows effects of cromakalim and glibenclamide on the AP and Ca²⁺ transients, which were simul-

taneously measured in a single ventricular myocyte of the guinea pig. APs were elicited at 0.5 Hz. Under the control conditions, averaged values of resting membrane potential (RMP) and APD at 90% repolarization (APD₉₀) were -84.7 ± 0.4 mV and 204 ± 8 msec ($n=54$), respectively. Averaged values of the resting [Ca²⁺]_i and the peak [Ca²⁺]_i upon AP were 95 ± 5 and 363 ± 28 nM ($n=54$), respectively. Application of 3 and 10 μ M cromakalim markedly shortened the APD and decreased the indo-1 fluorescence signal (Fig. 1A). To prevent photobleaching, Ca²⁺ transients were measured for 15–30 sec only when stable APs were recorded following each application of cromakalim. The resting [Ca²⁺]_i did not change significantly in the presence of 3 μ M cromakalim (97 ± 25 nM, $n=8$; $P>0.05$ vs control, 88 ± 14 nM, $n=11$). Ca²⁺ transients were evaluated by integrating the ratio signal above the baseline for 1 sec following stimulation. Baseline value was determined as the average for 100 msec just before the stimulation. The effects of cromakalim on the AP and Ca²⁺ transients were strongly antagonized by application of 10 μ M glibenclamide. The time courses of changes in APD₉₀ and the RMP in the

Fig. 1. Effects of cromakalim and glibenclamide on AP and Ca²⁺ transients in single myocytes isolated from cardiac ventricle of the guinea pig. A: APs were elicited by current injection at 0.5 Hz. Ca²⁺ transients elicited by APs were simultaneously recorded using indo-1. The traces from "a" to "d" were obtained at the time indicated in panels B and C. B: Resting membrane potential (squares) and APD₉₀ (circles) were plotted against time. Horizontal bars indicate application of 3, 10 μ M cromakalim and 1 μ M glibenclamide. C: Ca²⁺ transients were integrated for 1 sec from the stimulation. The integrated Ca²⁺ transients were plotted against time. Data shown in panels B and C were obtained simultaneously from a myocyte. Effects of cromakalim on both APD₉₀ and integrated Ca²⁺ transients were completely removed by addition of glibenclamide.

presence of cromakalim and after the addition of glibenclamide are shown in Fig. 1B. The integrated value of Ca^{2+} transient varied substantially from record to record even under the same conditions (Fig. 1C). AP generation was occasionally suppressed in the presence of 3 or 10 μM cromakalim, even when supramaximal stimuli under the control conditions were applied. The averaged RMPs were -83.1 ± 0.81 ($n=5$) and -84.0 ± 0.71 mV ($n=5$) in the absence and presence of 10 μM cromakalim, respectively ($P>0.05$). The averaged APDs at 0 mV and 90% repolarization were 173 ± 8 and 233 ± 10 msec ($n=15$) under the control conditions and 62.3 ± 12.5 ($n=7$, $P<0.01$ vs control) and 93.7 ± 18.8 msec ($P<0.01$ vs control) in the presence of 1 μM cromakalim.

Figure 2 shows summarized results describing the concentration-dependent effects of cromakalim. The APD_{90} (closed squares) was significantly decreased by cromakalim at concentrations of 1, 3 and 10 μM ($P<0.01$, Scheffé's test); the IC_{50} was 0.6 μM . The integrated Ca^{2+} transients (open circles) were also significantly decreased by cromakalim at concentrations of 3 and 10 μM ($P<0.01$). The IC_{50} was approximately 3 μM . The decrease in APD and Ca^{2+} transients induced by cromakalim was removed by addition of 10 μM glibenclamide ($n=10$, $P<0.01$). In the presence of 1 μM

Fig. 2. Concentration-response relationships for cromakalim to reduce APD_{90} and integrated Ca^{2+} transients. Closed squares and open circles indicate mean values of the relative APD_{90} and the relative integrated Ca^{2+} transients, respectively. Vertical bars indicate S.E.M. ($n=5-8$). The values of APD_{90} and integrated Ca^{2+} transients before the application of cromakalim were obtained by averaging at least 10 continuous recordings and taken as unity in each myocyte. The values after application of cromakalim at a certain concentration were also obtained by averaging about 10 recordings after the effects of cromakalim reached a steady level. Approximate IC_{50} s of cromakalim for the decrease in APD_{90} and the integrated Ca^{2+} transients were 0.6 and 3 μM , respectively. The relative APD_{90} in the presence of 1 μM cromakalim was significantly smaller than the corresponding relative integrated Ca^{2+} transients. ** and ##: $P<0.01$ vs control (Scheffé's test).

cromakalim, the relative APD_{90} was significantly smaller than the corresponding relative integrated Ca^{2+} transients ($P<0.01$, t -test). Under these conditions, a 55% decrease in APD_{90} did not significantly affect the Ca^{2+} transients, whereas further decrease in APD_{90} markedly affected Ca^{2+} transients.

Effects of E4031 on APD and Ca^{2+} transients

Application of 0.3–3 μM E4031 markedly prolonged APD_{90} (0.3 and 1 μM , Fig. 3: A and B) and increased the Ca^{2+} transients (Fig. 3: A and C). The averaged RMPs in the absence and presence of 1 μM E4031 were -85.8 ± 0.6 ($n=9$) and -85.5 ± 0.6 mV ($n=9$), respectively ($P>0.05$). Effects of E4031 were removed by washout. In four out of thirteen myocytes that were treated with 1 or 3 μM E4031, APD_{90} became longer than the interpulse duration (2 sec) (not shown, see also Fig. 7). Figure 4 shows summarized results describing the effects of E4031 on APD_{90} (closed squares) in 9 myocytes, where APD_{90} was not longer than 2 sec. APD_{90} was significantly prolonged by E4031 at concentrations higher than 0.1 μM ; the APD at 0 mV and APD_{90} in the presence of 0.3 μM E4031 were 185 ± 23 ($n=7$, 1.41 ± 0.07 of the control; $P<0.01$ vs control) and 403 ± 58 msec (2.14 ± 0.21 of the control, $P<0.01$ vs control), respectively. The resting $[\text{Ca}^{2+}]_i$ did not change significantly in the presence of 1 μM E4031 (96 ± 16 nM, $n=9$; $P>0.05$ vs control, 74 ± 10 nM, $n=14$). The integrated Ca^{2+} transients (open circles) were increased by E4031; the Ca^{2+} transient in the presence of 0.3 μM E4031 was 1.45 ± 0.18 of the control ($n=7$, $P<0.01$ vs control). The E4031-induced increase in APD_{90} was, however, was significantly larger than that in the integrated Ca^{2+} transients ($P<0.01$, at 1 μM E4031).

Effects of cromakalim and E4031 on Ca^{2+} transient under voltage-clamp

Effects of cromakalim on membrane currents and Ca^{2+} transients elicited by two step depolarization from -80 to -40 mV for 50 msec and to 0 mV for 200 msec were examined in detail (Fig. 5). $[\text{Ca}^{2+}]_i$ under voltage-clamp was 92 ± 10 and 352 ± 46 nM ($n=37$) at the holding potential of -80 mV and at the peak during depolarization, respectively. Application of 1 and 3 μM cromakalim shifted the holding current at -80 mV slightly in the outward direction and markedly increased the outward currents at -40 and 0 mV. The current at the end of the second pulse was measured from the zero current level and plotted as steady state current (I_{ss}) against time in Fig. 5C. Cromakalim-induced I_{ss} was completely blocked by addition of 10 μM glibenclamide. Note that the Ca^{2+} transients were not affected by either cromakalim or glibenclamide (Fig. 5D). The relative integrated Ca^{2+}

Fig. 3. Effects of E4031 on APs and Ca²⁺ transients. **A:** APs were elicited by current injection at 0.5 Hz. The traces from "a" to "d" were obtained at the time indicated in "B" and "C". **B:** Resting membrane potential (squares) and APD₉₀ (circles) were plotted against time. Horizontal bars indicate application of 0.3 and 1 μM E4031. **C:** The integrated Ca²⁺ transients were plotted against time. Data shown in panels B and C were obtained simultaneously from a myocyte.

Fig. 4. Concentration-response relationships of E4031 for the increase in APD₉₀ and integrated Ca²⁺ transients. Closed squares and open circles indicate mean values of relative APD₉₀ and the relative integrated Ca²⁺ transients, respectively. Vertical bars indicate S.E.M. (n=5-9). The values were obtained in the same manner as those shown in Fig. 2. *: P < 0.05 vs control. ** and #: P < 0.01 vs control (Scheffé's test).

transients in the presence of 1 and 3 μM cromakalim were 1.00 ± 0.05 and 0.90 ± 0.19 of the control (n=4, P > 0.05 vs control), respectively. Subsequent addition of 10 μM glibenclamide in the presence of 3 μM cromakalim also failed to significantly affect the Ca²⁺ transients (1.12 ± 0.24 , n=4; P > 0.05 vs control). When membrane currents were measured by ramp depolarizations from -80 to +40 mV for 500 msec in the absence of cromakalim, then in the presence of 3 μM cromakalim, and after the further addition of 10 μM glibenclamide, cromakalim-induced current was completely blocked by glibenclamide (not shown). The current-voltage relationship of the glibenclamide-sensitive current showed outward rectification and had a reversal potential of -85 mV (n=3), indicating that the charge carrier of the current is K⁺ (22).

Effects of 3 μM E4031 on membrane currents and Ca²⁺ transients were also examined using the same voltage-clamp protocol. Under these experimental conditions, the block of rapidly activating component of delayed rectifier K⁺ current (I_{Kr}) could not be clearly observed since I_{Kr} overlapped Ca²⁺ current inactivation. Ca²⁺ transients were not affected by E4031. However, in

Fig. 5. Effects of cromakalim and glibenclamide on membrane currents and Ca^{2+} transients under voltage-clamp. **A:** A myocyte was depolarized from a holding potential of -80 mV to -40 mV for 50 msec and then to 0 mV for 200 msec once every 2 sec. The traces from "a" to "c" were obtained correspondingly at the time indicated in panels C and D. **B:** Ca^{2+} transients elicited by depolarization were simultaneously recorded using indo-1. **C:** The amplitude of steady state outward current (I_{ss}) was measured from the zero current level at the end of 200-msec depolarization to 0 mV in panel A and plotted by squares against time. Horizontal bars indicate application of 1 , 3 μM cromakalim and 1 μM glibenclamide. **D:** A Ca^{2+} transient induced by the two-step depolarization was integrated for 1 sec from the start of depolarization. The integrated Ca^{2+} transient was plotted by closed circles against time. Data shown in C and D were obtained simultaneously from a myocyte. The integrated Ca^{2+} transients were not affected by cromakalim and glibenclamide. The increase in I_{ss} by cromakalim was completely removed by addition of glibenclamide.

the presence of 50 μM Cd^{2+} to block Ca^{2+} current, the outward current activated by depolarization from -40 to 0 mV for 150 msec was significantly reduced by 1 μM E4031 to $72 \pm 6\%$ ($n=4$, $P < 0.01$) of the control. As expected, the outward tail current corresponding to I_{Kr} after repolarization to -40 mV was completely blocked by 1 μM E4031 ($n=4$). When the ramp depolarization from -80 to $+40$ mV for 500 msec was applied, application of 1 μM E4031 reduced a part of the elicited outward current. The E4031-sensitive current reversed at approximately -85 mV and showed strong inward rectification.

The relative value of the integrated Ca^{2+} transients elicited by two-step depolarization in the presence of 3 μM E4031 was 1.08 ± 0.23 ($n=4$, $P > 0.05$ vs control).

In the next series of experiments, the effects of 1 μM cromakalim and 3 μM E4031 on voltage-dependent L-type Ca^{2+} channel current (I_{Ca}) were examined. The current-voltage relationship of I_{Ca} was obtained from the peak inward current activated by depolarization from a holding potential of -40 mV to selected potentials in the range of -30 and $+30$ mV. It was not changed by application of 1 μM cromakalim or 3 μM E4031 (not shown); the averaged peak amplitude of I_{Ca} at 0 mV was -939 ± 307 and -756 ± 263 pA ($n=3$, $P > 0.05$) in the absence and presence of 1 μM cromakalim, respectively, and -1163 ± 230 and -927 ± 207 pA ($n=3$, $P > 0.05$) in the absence and presence of 3 μM E4031, respectively.

The relationship between depolarization duration and Ca^{2+} transient

Figure 6A(a) shows membrane currents and Ca^{2+} transients that were measured by changing the duration of the depolarizing pulse at 0 mV in the two-step voltage-clamp pulse protocol in the presence of 30 μM tetrodotoxin (TTX). The duration of the first step at -40 mV was fixed at 50 msec and the waveform was applied at 0.5 Hz. The peak amplitude of residual I_{Na} at -40 mV was less than 500 pA. Thus, acceptable space clamp conditions were obtained. The magnitude of Ca^{2+} transients increased progressively with the increases in the duration of depolarization at 0 mV (12, 23). When both I_{Na} and I_{Ca} were blocked by 30 μM TTX and 50 μM Cd^{2+} , respectively, no Ca^{2+} transient upon depolarization could be detected (not shown). The integrated value of Ca^{2+} transient, which was elicited by APs at 0.5 Hz under current clamp, was taken as unity in each myocyte before the start of voltage-clamp experiments. The averaged APD_{90} was 204 ± 8 msec at 0.5 Hz ($n=54$) (open diamond in Fig. 6B). In the same myocytes, the averaged value of the relative Ca^{2+} transients elicited by depolarization to 0 mV for 200 msec under voltage-clamp was 0.95 ± 0.05 of that elicited by AP ($n=7$, $P > 0.05$ vs unity). The closed squares in Fig. 6B show the relationship between the duration of depolarization to 0 mV and the integrated Ca^{2+} transients. These data were well fitted by the sum of two exponential components as follows:

[Relative integrated value]

$$= A_0 - A_1 \exp(-t/\tau_1) - A_2 \exp(-t/\tau_2),$$

where A_1 and A_2 are the constants, and τ_1 and τ_2 are the time constants of the first and the second components, respectively. The values obtained by the fitting of the solid line are as follows: $A_0 = 1.51 \pm 0.07$, $A_1 = 0.24 \pm 0.02$, $A_2 = 1.15 \pm 0.07$, $\tau_1 = 4.5 \pm 0.3$ msec and $\tau_2 = 276 \pm 25$ msec ($n=7$).

Fig. 6. Relationships between Ca²⁺ transients and depolarization duration or APD₉₀. **A:** "a" Membrane current (middle traces) and Ca²⁺ transients (lower traces) elicited by two-step depolarization (top traces) in a similar manner as shown in Fig. 5 at 0.5 Hz were measured under voltage clamp in the presence of 30 μ M TTX. The duration of the depolarization to -40 mV was fixed as 50 msec. The duration of the depolarization to 0 mV was changed stepwise of 10, 100 and 200 msec. "b" after treatment with 10 μ M ryanodine. **B:** Similar experiments were performed under voltage clamp by changing the second pulse duration of 0, 1, 5, 10, 20, 50, 70, 100, 150, 200 and 400 msec. Ca²⁺ transient was integrated for 1 sec from the start of depolarization. Ca²⁺ transients, which were elicited by APs at 0.5 Hz under current clamp, were integrated and the averaged value was taken as the control (unity) in each myocyte. The averaged APD₉₀ was 204 ± 8 msec ($n=54$) as indicated by an open diamond. The relative values of integrated Ca²⁺ transients at various duration under voltage-clamp were plotted as closed squares against the duration of the second pulse ($n=6-13$). The solid line indicates a fitted curve that consists of the sum of two exponential components. [Relative integrated value] = $A_0 - A_1 \exp(-t/\tau_1) - A_2 \exp(-t/\tau_2)$, where A_0 , A_1 and A_2 are the constants, and τ_1 and τ_2 are the time constants of the first and the second components, respectively. The values obtained by the fitting of the solid line are as follows: $A_0=1.51$, $A_1=0.24$, $A_2=1.15$, $\tau_1=4.5$ msec and $\tau_2=276$ msec. Open squares indicate the relation obtained after treatment with 10 μ M ryanodine. The dotted line indicates a fitted curve of a single exponential component. The A_0 , A_1 and τ_1 are 1.15, 1.03 and 285 msec, respectively. **C:** The relation between APD₉₀ and the relative integrated Ca²⁺ transient in the presence of cromakalim or E4031. Closed circles indicate the relation in the presence of cromakalim ($n=5-8$). Closed triangles indicate the relation in the presence of E4031 ($n=5-9$). An open reverse triangle indicates the relation in the absence of these drugs after treatment with ryanodine. An open triangle indicates the relation in the presence 0.3 μ M E4031 after treatment with ryanodine. The solid and dotted lines and diamond are the same as those shown in panel B.

Although the application of 10 μ M ryanodine slightly but not significantly increased the resting $[Ca^{2+}]_i$ (125 ± 23 nM, $n=6$, $P>0.05$ vs control, 92 ± 21 nM, $n=8$), Ca²⁺ transients under voltage-clamp at 0 mV were markedly reduced (Fig. 6Ab). The peak $[Ca^{2+}]_i$ during 200-msec depolarization was 178 ± 36 nM ($P<0.01$ vs control, $n=7$). However, even after application of ryanodine, the Ca²⁺ transients increased with the increase in the pulse duration at 0 mV. The first component in the control conditions disappeared after treatment with ryanodine, indicating that it is due to CICR (11). The time-course relationship after the treatment with 10 μ M

ryanodine (Fig. 6B: open squares) could be fitted by a single exponential component ($A_0=1.15 \pm 0.13$, $A_1=1.03 \pm 0.13$, $\tau_1=285 \pm 33$ msec, $n=6$) (Fig. 6B: dotted line).

Closed circles and triangles in Fig. 6C show the relationship between APD₉₀ in the presence of 0.01–3.0 μ M cromakalim or 0.01–1.0 μ M E4031 and the integrated Ca²⁺ transients. The integrated Ca²⁺ transient elicited by AP before the application of drugs was taken as unity in each myocyte. It is important to note that the relation between APD₉₀ and Ca²⁺ transients fits approximately to that obtained under voltage clamp ($P>0.05$ vs solid line, χ^2 test). The data obtained from the experiments were

also well fitted by two exponential components with the following parameters, which are close to those obtained under voltage-clamp, respectively: $A_0=1.69$, $A_1=0.27$, $A_2=1.45$, $\tau_1=7.3$ msec and $\tau_2=245$ msec. It is also clear that CICR was markedly diminished when APD_{90} was reduced to less than approximately 40 msec by cromakalim. After treatment with $10 \mu\text{M}$ ryanodine, APD_{90} was not affected significantly (ryanodine: 234 ± 35 msec vs control: 246 ± 41 msec, $P > 0.05$, $n=6$). The peak $[Ca^{2+}]_i$ elicited by AP was markedly reduced by $10 \mu\text{M}$ ryanodine (159 ± 39 nM, $n=6$; $P < 0.05$ vs the control). Correspond-

Fig. 7. Effects of cromakalim on E4031-induced prolongation of APs. A: APs were elicited by current injection at 0.5 Hz. APs and Ca^{2+} transient were simultaneously recorded under the control conditions (a), in the presence of $1 \mu\text{M}$ E4031 (b), after the addition of $1 \mu\text{M}$ cromakalim (c), and after the further addition of $10 \mu\text{M}$ glibenclamide (d). B: Similar experiments were performed in a separate myocyte, where APD was prolonged from the control (a) to over 2 sec in the presence of $1 \mu\text{M}$ E4031 (b). Such fused APs were recorded in four myocytes out of 13. Addition of $1 \mu\text{M}$ cromakalim removed the effects of E4031 (c). C: Summarized results obtained from experiments typically shown in panel A. Data of fused APs such as shown in panel B were not included. Open and closed columns indicate the relative values of APD_{90} and the integrated Ca^{2+} transients, respectively. *, \$ and % indicate $P < 0.05$ vs control, E4031 plus cromakalim and plus glibenclamide, respectively. **, ## and \$\$ indicate $P < 0.01$ vs control, E4031 and plus cromakalim, respectively (Scheffé's test).

ingly, the integrated Ca^{2+} transient was also markedly reduced (0.61 ± 0.04 of the control, $n=6$; $P < 0.01$ vs the control), when that under control conditions is taken as unity (Fig. 6C: open reverse triangle). Further addition of $0.3 \mu\text{M}$ E4031 prolonged APD_{90} and increased the integrated Ca^{2+} transient (387 ± 34 msec, 0.89 ± 0.23 of the control, $n=3$) (Fig. 6C: open triangle). Note that the relation between APD_{90} and Ca^{2+} transient after treatment with ryanodine appears to be close to that obtained under voltage-clamp.

Effects of cromakalim on E4031-induced AP prolongation and arrhythmia

Figure 7 shows the changes in APD and Ca^{2+} transients following the addition of $1 \mu\text{M}$ cromakalim in the presence of $1 \mu\text{M}$ E4031. Both the APD_{90} prolongation and the increase in Ca^{2+} transients in the presence of $1 \mu\text{M}$ E4031 were completely compensated by $1 \mu\text{M}$ cromakalim (Fig. 7: Ab and Ac). The effects of cromakalim were removed by subsequent addition of $10 \mu\text{M}$ glibenclamide (Fig. 7Ad).

It is well known that class III antiarrhythmic drugs, including E4031, can induce early afterdepolarization (EAD) and TdP (24). Figure 7B shows long APs in the presence of $1 \mu\text{M}$ E4031 that had a plateau phase exceeding the normal interpulse duration (2 sec). Such APs, exceeding 2 sec in APD, were observed in 4 out of 13 myocytes in the presence of 1 or $3 \mu\text{M}$ E4031. During the long plateau phase, the membrane potential and $[Ca^{2+}]_i$ were in the range of -10 and -20 mV and 200 – 250 nM, respectively. Both the extreme AP prolongation and the elevated and maintained $[Ca^{2+}]_i$ were removed by addition of $1 \mu\text{M}$ cromakalim.

Figure 7C summarizes results describing the effects of combination of $1 \mu\text{M}$ E4031 and cromakalim and subsequent addition of $10 \mu\text{M}$ glibenclamide on APD_{90} (open columns) and the integrated Ca^{2+} transients (closed columns), typical examples of which are shown in Fig. 7A. The data from myocytes where 'fused' APs were recorded were not included in the results. The values of APD_{90} and the integrated Ca^{2+} transients obtained under control conditions were each taken as unity in each myocyte. The APD_{90} and integrated Ca^{2+} transients, which were increased by E4031, were completely reversed and even slightly decreased by the addition of cromakalim (APD_{90} : $73.7 \pm 11.2\%$ of the control, $P < 0.05$ vs control; Ca^{2+} transients: $83.6 \pm 9.1\%$, $P > 0.05$ vs the control; $n=7$). The effects of cromakalim were blocked by subsequent addition of glibenclamide.

DISCUSSION

The present study provides new information concern-

ing the relation between APD₉₀, which we changed by treatment with selective K⁺ channel modulators, cromakalim and E4031, and simultaneously measured Ca²⁺ transients in cardiac ventricular myocytes.

The relation between the duration of depolarization at 0 mV under voltage-clamp, and the integrated Ca²⁺ transient can be described by sum of two exponential functions under our experimental conditions. The fast component, which had a time constant of approx. 5 msec, was abolished by ryanodine (Fig. 6), indicating that it is mainly due to CICR from SR (10). CICR can be triggered by an initial Ca²⁺-influx for less than approx. 10 msec mainly through voltage-dependent Ca²⁺ channels (VDCC) but also Na⁺-Ca²⁺ exchange (10, 25). Although not examined further in this study, VDCC responsible for the Ca²⁺ influx triggering CICR is mainly the L-type Ca²⁺ channel (12, 26), since the Ca²⁺ transient induced by the depolarization to 0 mV was almost completely abolished by D600 (not shown). A small component at time 0 (0.12) in Fig. 6B may suggest that the activation of a small part of the L-type Ca²⁺ channels by the depolarization from -80 to -40 mV also resulted in some CICR. Alternatively, T-type Ca²⁺ channels may contribute some Ca²⁺ influx to trigger CICR. In the present study, the Ca²⁺ transient induced by depolarization was completely blocked by the addition of TTX and Cd²⁺, and therefore, the possibility of voltage-gated Ca²⁺ release (27) was not further examined.

The second component had a time constant of 280 msec and appeared to be mainly attributable to Ca²⁺-influx through VDCC. Of importance is that the time constant of the second component is close to that obtained after treatment with ryanodine. When APD₉₀ was in a physiological range (approx. 200 msec) at 0.5 Hz, we can calculate from the present results that approximately 80% of the elevation of [Ca²⁺]_i induced by APs at 0.5 Hz is due to CICR. These roughly agree qualitatively with the previous findings (11). In the present study, however, the integrated Ca²⁺ transient rather than the peak [Ca²⁺]_i was used as a parameter since changes in AP-induced Ca²⁺ transients by E4031 or cromakalim were more clearly resolved by the procedure.

The amount of Ca²⁺ supplied by CICR strongly depends upon conditions for Ca²⁺ loading in the SR (10, 14, 26). Therefore, the relationship between Ca²⁺ transients and the duration of depolarization or APD₉₀ was determined carefully at a steady state in the limitation of 0.5 Hz stimuli. It has been also established that activities of Na⁺-Ca²⁺ exchanger and SR/sarcolemmal Ca²⁺ pumps substantially modify the Ca²⁺ transients elicited by AP. Under the experimental conditions in this study, it is unlikely that the Na⁺-Ca²⁺ exchange worked in a reverse mode in both normal (25) and modified APs in the

presence of K⁺-channel modulators. The contribution of inward current carried by Na⁺-Ca²⁺ exchange to the AP plateau phase is not completely clear in guinea pig ventricular myocytes, but may be a significant component to prolong the plateau (25). It has been suggested that the force development following a prolonged depolarization is derived from calcium entry into myocardial cells by reversed Na⁺-Ca²⁺ exchange in ferret papillary muscle (28). In the present study, the contribution of Na⁺-Ca²⁺ exchange to APD and Ca²⁺ transients was not examined systematically.

The Ca²⁺ transient induced by depolarization to 0 mV for 200 msec in the two-step pulse protocol under voltage-clamp was not changed by E4031 and cromakalim (Fig. 5). Note, however, that the K⁺ currents were affected as expected; E4031 selectively blocked the fast component of delayed rectifier K⁺ current presumably through HERG K⁺ channels (4, 5); cromakalim activated ATP sensitive K⁺ channels that were blocked by glibenclamide (22). Importantly, the current through VDCC was not affected directly. These results indicate that the changes in AP-induced Ca²⁺ transients by K⁺ channel modulators are simply due to the changes in APD₉₀. The longer the APD, the larger the amount of Ca²⁺-influx in the slow component was. The prolonged Ca²⁺ transients in the presence of E4031 may be, at least in part, responsible for its positive inotropic action (29). The negative inotropic effect of cromakalim is antagonized by glibenclamide in canine atrial muscle (30). It was, however, found that the E4031-induced increase in Ca²⁺ transient was smaller than that in APD₉₀ (Fig. 4). Accordingly, the concentration-response relationship for the decrease in APD₉₀ by cromakalim also markedly dissociated from that for the decrease in Ca²⁺ transient (Fig. 2). The reason for the dissociation is that a substantial fraction of the Ca²⁺ transients was due to CICR which was not directly affected by E4031 and cromakalim. When APD₉₀ was decreased to less than approx. 40 msec by cromakalim, the corresponding Ca²⁺ transient was markedly decreased, indicating that the Ca²⁺ influx induced by the AP was not sufficient to trigger CICR under the conditions. Alternatively, the calcium in SR primed by preceding short APs in the presence of cromakalim might be almost depleted.

The prolonged plateau of AP and the corresponding increase in [Ca²⁺]_i in the presence of E4031 were blocked/counteracted by an addition of cromakalim. The prolongation of APD by E4031 was in some cases accompanied by EAD (not shown) and/or the 'fusion' of APs (Fig. 7) and, thereby, resulted in the sustained intermediate level of [Ca²⁺]_i, which was presumably due to continuous Ca²⁺ influx. The extensively long APs were similar to TdP which has been reported as arrhythmo-

genic effects of a class III antiarrhythmic agent by use of HERG K^+ channel blockers in vivo (24). The sustained Ca^{2+} influx may result in Ca^{2+} overload of the cell and lead to more pathophysiological conditions. The addition of $1\ \mu\text{M}$ cromakalim prevented the marked prolongation of APD in the presence of $1\ \mu\text{M}$ E4031. It has been reported that K_{ATP} channel openers can prevent EAD, DAD and abnormal automaticity induced by the treatment with various agents, in vitro (31, 32), in vivo (32) and the long QT syndrome (15).

K_{ATP} channel openers are thought to contribute to protection under ischemic conditions, and also can limit the injury during reperfusion (2, 3, 33). However, K_{ATP} channel openers may also be arrhythmogenic during the reperfusion since the AP refractory period is shortened (34). Because glibenclamide shows class III antiarrhythmic effects (i.e., prolongation of APD) in the setting of reperfusion-induced fibrillation, the clinical use of K_{ATP} channel opener is contraindicated (35) in spite of its cardio-protective effects (2, 3). Interestingly, an efficacy of E4031 on ischemia- and reperfusion-induced arrhythmias has been reported (36). It may therefore be meaningful to examine the effectiveness of the combination of E4031 and cromakalim in vivo for the intervention of some types of arrhythmias.

In conclusion, we have shown that changes in APD_{90} by E4031 or cromakalim can regulate the component of AP-induced Ca^{2+} transient that mainly depends upon the amount of Ca^{2+} influx. The marked increase in AP plateau duration and $[Ca^{2+}]_i$ caused by E4031 as in long QT syndrome was antagonized by addition of cromakalim.

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