Contribution of Ca^{2+} -Influx to Generation of the Transient Inward Current in Guinea-Pig Ventricular Muscles

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Abstract Contribution of Ca^{2+} -influx via the slow channel to generation of the transient inward current in guinea-pig ventricular muscles was studied using a single sucrose gap voltage clamp technique. The transient inward current (TI) was induced from superfusion of the preparations with the low- K^+ (0 mM), high- Ca^{2+} (3.6 mM) solution. Application of 2 mM- Co^{2+} quickly and reversibly suppressed the TI amplitude to 25% of the control and delayed its peak timing to 153% during 10–20 min. Inhibition developed as quickly as Co^{2+} suppressed the slow inward current (I_s), and its recovery took place without apparent time lag behind its effect on I_s . The block of both TI and I_s by Co^{2+} was antagonized by raising external Ca^{2+} to 7.2 mM. Removal of external Ca^{2+} caused a prompt suppression of both I_s and TI. Application of 2 or 5 mM-procaine HCl produced a complete abolition of TI with a mild depression of I_s . While 1 mM-cafeic caused a suppression of TI after a transient augmentation, 10 mM-cafeine completely eliminated it without abolishing I_s . These results indicate that the Ca^{2+} -influx through the slow channel acts not only to load the cell with those ions, but also to influence somehow the Ca^{2+} -release from the stores under the Ca^{2+} -overloaded conditions.

Key words: delayed afterdepolarizations, Ca^{2+} -overload, Ca^{2+} -release, slow inward current.

When cardiac muscles are overloaded with Ca by means of various interventions, they develop delayed afterdepolarizations (DADs) and aftercontractions (FERRIER and MOE, 1973; FERRIER, 1977; KASS *et al.*, 1978a, b; EISNER and LEDERER, 1979; MATSUDA *et al.*, 1982); DADs are produced by the transient inward current (TI) (LEDERER and TSIEN, 1976). The mechanism of TI was suggested to be phasic changes in the internal calcium concentration, possibly reflecting cyclic release of Ca^{2+} from the intracellular stores (KASS *et al.*, 1978a, b). Oscillations of

the intracellular free Ca concentration ($[Ca^{2+}]_i$) have recently been demonstrated using the aequorin signal during elevated $[Ca^{2+}]_i$ (WIER *et al.*, 1983; ALLEN *et al.*, 1984).

Since FERRIER and MOE (1973) first described the inhibitory action of Mn^{2+} on DADs, various Ca^{2+} -channel blocking agents have been shown to suppress both DADs and TI (KASS *et al.*, 1978a; EISNER and LEDERER, 1979; KARAGUEUZIAN and KATZUNG, 1982; KASS and TSIEN, 1982). KASS *et al.*, (1978a) proposed that the main action of Mn^{2+} and of D-600 was to reduce TI, indirectly, through their effects on the slow inward current (I_s), thus eliminating the Ca^{2+} -overload in the cell. The same idea was also applied to the inhibitory action of verapamil on TI (KARAGUEUZIAN and KATZUNG, 1982). It has been pointed out, however, that Ca^{2+} transported through the slow channel enters into a confined space where, in turn, it triggers the release of Ca from the sarcoplasmic reticulum, a major site of the stores (FABIATO, 1983). Furthermore, DADs and TI were induced in the wake of a train of action potentials or upon repolarization from preceding depolarizing clamp pulses (LEDERER and TSIEN, 1976). These results may indicate that Ca ions entering into the cell via the slow channel upon each depolarization can, somehow, influence the amplitude and the time course of TI following the repolarization. Therefore, the question may be asked as to whether I_s is a direct trigger for TI, or whether it is a sole mechanism for cell loading with Ca^{2+} . To approach this question, the effects of Co^{2+} as an inhibitor of I_s (KÖHLHARDT and HAAP, 1980) and other agents on TI, were examined using a single sucrose gap voltage clamp technique in guinea-pig ventricular muscles. In this study, the TI was induced in the muscle preparations from superfusing with the low- K^+ , high- Ca^{2+} solution, which was shown to be an easy and reproducible method for the study of this current in muscle preparations (EISNER and LEDERER, 1979; HIRAOKA *et al.*, 1979, 1981). A preliminary report of this work appeared elsewhere (KAWANO *et al.*, 1985).

METHODS

Preparations. Guinea-pigs weighing 250–350 g were stunned with a blow on the neck, and their hearts were rapidly removed through a thoracotomy. Papillary muscles of suitable dimensions (length longer than 4 mm, diameter less than 0.7 mm) were dissected from the right ventricle. To handle the preparation, a fine silk thread was tied to the tendinous end of the preparation.

Solutions. The modified Tyrode's solution had the following composition (mm): NaCl 150; KCl 4.0; $CaCl_2$ 1.8; $MgCl_2$ 1.0; Tris-HCl 10.0; glucose 5.5; pH adjusted to 7.3 by titration with NaOH. The low- K^+ , high- Ca^{2+} solution had 0 mm KCl and 3.6 mm $CaCl_2$, and other compositions were the same as the modified Tyrode's solution. The Co^{2+} -containing solution was prepared by adding 2 mm $CoCl_2$ in the low- K^+ , high- Ca^{2+} solution. To vary Ca^{2+} concentration of the solutions, $CaCl_2$ was added or removed from the low- K^+ , high- Ca^{2+} solution with or without $CoCl_2$. No osmolarity correction was made with these solutions. In some

experiments, caffeine (Wako Pure Chemical Ind., 1 or 10 mM) and procaine HCl (Maruishi Pharm. Co., 2 or 5 mM) were added to the low-K⁺, high-Ca²⁺ solution. The pH of all these solutions was 7.2–7.4 by continuous bubbling with 100% O₂. The isotonic KCl solution was made by substituting KCl for NaCl in the Tyrode's solution. The isotonic sucrose solution contained 292 sucrose, 5.5 glucose, and 0.05 CaCl₂. All these solutions were gassed with 100% O₂ throughout the experiments. The temperature of the superfusates was maintained at 36–37°C.

Experimental setup. In the single sucrose-gap voltage clamp experiments, the ventricular preparations were mounted into a three-compartment chamber similar to that described previously (HIRANO and HIRAOKA, 1986). After mounting the preparation in the chamber, all of the three compartments were superfused with the Tyrode's solution for an equilibration period of 30 min. At the end of this period, the superfusing fluid in the sucrose gap and in the current injection pool was replaced by the isotonic sucrose and by the KCl solution, respectively. The effectiveness of the sucrose gap as a seal was judged from comparing the amplitude of the action potential recorded intracellularly (AP_i) and the one extracellularly (AP_o). The ratio AP_i/AP_o below 1.15 was decided to be a good sealing and selected to be used in further experiments. Experimental procedures and check of the adequacy of the voltage control were, similar to those described in the previous report (HIRANO and HIRAOKA, 1986). Records of membrane potentials and currents were displayed on an oscilloscope (VC-10, Nihon Kohden, Tokyo) and photographed with a camera (PC 2B, Nihon Kohden, Tokyo) or inscribed with a direct-writing recorder (Recti-Horiz 8K, San-ei Sokki, Tokyo). The preparation was stimulated at a frequency of 0.2 Hz. The stimuli were supplied from the pulse generator (SEN-7103, Nihon Kohden, Tokyo).

Induction of the transient inward current. To induce TI, the superfusing fluid in the test compartment was changed from the modified Tyrode's solution to the low-K⁺, high-Ca²⁺ solution, as we used in the induction of the delayed afterdepolarizations (HIRAOKA *et al.*, 1979, 1981, see also EISNER and LEDERER, 1979). The muscle fibers were stimulated with rectangular pulses of supra-threshold intensity at a frequency of 0.2 Hz, and they were placed under the voltage clamp condition every 5 min in this superfusate. The preparations usually began to develop TI during the voltage clamp condition after 10–15 min in the low-K⁺, high-Ca²⁺ solution. The amplitude and time courses of TI became large enough to be measurable with repeated applications of clamp pulses after 30 min of the low-K⁺, high-Ca²⁺ superfusion. Under this condition, the current measurements were done in the control and in the Co²⁺- or drug-containing solutions.

The measurement of TI in the present experiment was done after repolarization from depolarizing pulses to the holding potential of –45 to –35 mV. A low level of the holding potential was chosen so as to inactivate the fast Na⁺ current and to avoid the non-homogeneous space clamp during the flow of this current. The regular clamp pulses of a 1-s duration were given at a frequency of 0.2 Hz. The amplitude of TI was measured with respect to an up-sloping or a down-sloping

baseline drawn tangent to two positive peaks which flanked the trough (see, Fig. 2B, inset). The peak timing of TI was an interval between the end of the depolarizing pulse and the peak of the largest TI. The amplitude of I_s was measured according to the method proposed by McDONALD and TRAUTWEIN (1978) in cat ventricular muscles. The data collection was made when the stable microelectrode impalement was maintained during the application of Co^{2+} and other agents.

Statistical analysis. The data were expressed as a mean $\pm$ standard error of mean (M.S.E.). The statistical analysis was performed using the paired *t*-test. A *p* value less than 0.05 was considered to be significant.

RESULTS

When depolarizing pulses of a 1-s duration were applied from the holding potential about -40 mV, several inward deflections of the current trace, the TIs, superimposing on the tail current were observed. In the present experiment, TI usually developed on the decaying outward tail current (see, Fig. 1) but it was also seen on the inward tail current on some occasions. When 2 mM-Co^{2+} was applied to the preparations, the current was rapidly suppressed. The amplitude of TI became less than 30% of the control value at 10–20 min in the Co^{2+} -solution. Figure 1 shows voltage clamp records demonstrating the depressive action of Co^{2+} on TI. The Co^{2+} not only depressed the TI amplitude but also delayed its peak timing (Fig. 1B). These changes in TI recovered to the control level after the wash-out of Co^{2+} (Fig. 1C). Table 1 summarizes the Co^{2+} effect during the 10–20-min application obtained from 7 preparations. The 2 mM-Co^{2+} decreased the TI amplitude to $25 \pm 5\%$ (mean $\pm$ M.S.E.) of the control and prolonged the peak timing to

Fig. 1. Effect of 2 mM-Co^{2+} on the membrane current of the guinea-pig ventricular muscle during the low- K^+ , high- Ca^{2+} superfusion. In each picture, the upper trace represents the voltage and the lower is the membrane current. A 1-s pulse was applied from the holding potential of -42 to $+13$ mV at the upper and to 0 mV at the bottom row. In the control before application of Co^{2+} (A), the TIs of several fluctuations were observed upon repolarization to -42 mV from the depolarizing pulses. They were largely suppressed by the application of 2 mM-Co^{2+} (B). The wash-out of Co^{2+} caused a reappearance of the TI (C). The records in C were obtained at 15 min in the Co^{2+} -free solution.

Table 1. Effects of Co²⁺ on the TI.

	Amplitude of TI				Peak timing of TI		
	Before Co ²⁺	Co ²⁺ 2mM	Wash-out Co ²⁺		Before Co ²⁺	Co ²⁺ 2mM	Wash-out Co ²⁺
$\times 10^{-7}$ A	22.2 $\pm$ 5.1	3.9 $\pm$ 1.4	25.8 $\pm$ 7.4	ms	187 $\pm$ 9.3	281.2 $\pm$ 29.7	209.4 $\pm$ 11.5
%	100	25 $\pm$ 5	114 $\pm$ 8	%	100	153 $\pm$ 13	116 $\pm$ 6
	* *				* *		
<i>p</i> value	NS			<i>p</i> value	NS		

n = 7. **p* < 0.05. NS, not significant.

Fig. 2. Effects of Co²⁺ on the current-voltage relationships of the guinea-pig ventricular muscle in the low-K⁺, high-Ca²⁺ solution. A: shows the current-voltage relationships of the late current (○, ●) and of I_s (△, ▲). B: represents the TI-activation voltage relationship obtained before (□) and during (■) the application of 2 mM-Co²⁺. The inset indicates how to measure the amplitude of TI.

$153 \pm 13\%$. These changes recovered completely upon the wash-out of Co^{2+} . In 3 fibers, the exposure to the Co^{2+} solution was extended to 30 min. The amplitude of TI decreased to 23% (mean) of the control at 10 min and it was 27% at 20 min, and 31% at 30 min during the Co^{2+} application. Thus, no further depression of TI with the exposure to Co^{2+} longer than 20 min was noted but it showed a tendency to recover at a later exposure time. This is partly due to a non-steady condition of the 0 mM-K^+ solution used in this experiment.

Figure 1 also demonstrates effects of Co^{2+} on currents other than TI under this condition. I_s showed a decreased amplitude as well as its peak value during the Co^{2+} application (Fig. 1 B). Its amplitude became about 50% of the control. There was no complete inhibition of I_s during 10–20 min in the Co^{2+} solution with 7 examined preparations. This is probably due to a high- Ca^{2+} concentration used in the present experiment, since the block of I_s by Co^{2+} was dependent on $[\text{Ca}^{2+}]_o$ (KOHLHARDT and HAAP, 1980). The wash-out of Co^{2+} during 15 min produced an incomplete recovery of I_s in this preparation. Another feature of the Co^{2+} effect was a depression of late outward current and of outward tail current upon repolarization. The changes in the outward current showed a tendency to recover after the wash-

Fig. 3. Time course of changes in I_s and TI during and after the application of Co^{2+} . In the ordinate, the upper row represents the amplitude of I_s and the lower is that of TI. Depolarizing pulses to about 0 mV with a 1-s duration were applied from the holding potential of about -40 mV in each experiment. The I_s induced by the depolarizing pulses and the TI upon repolarization to the holding potential were measured. Each point represents the mean of 7 experiments and vertical bars indicate $\pm$ M.S.E. The abscissa shows the time and 0 time corresponds to the start of the Co^{2+} application. WO means the wash-out of Co^{2+} and its value was measured at 10–20 min in the Co^{2+} -free solution. $*p < 0.05$, $**p < 0.02$, $***p < 0.01$. NS, not significant.

out of Co²⁺ (Fig. 1C). Depression of the outward current by Co²⁺ and its recovery were confirmed in 6 other preparations.

Figure 2 demonstrates the current-voltage relationships in this condition and the effects of Co²⁺ on them. While the late current, which was expressed as the amplitude at the end of 1-s pulse, was slightly depressed, I_s was markedly inhibited by Co²⁺ at all potentials (Fig. 2A); Fig. 2B shows the dependence of TI on the activation voltage and the effect of Co²⁺ on it. During the control, the TI amplitude increased with depolarization of activating pulses up to about +20–+30 mV. Further depolarizing steps caused a depression of the amplitude of TI, which confirmed the previous observation in ventricular muscles (KARAGUEUZIAN and KATZUNG, 1982). The peak value of TI was obtained at +29 mV of the activation voltage in average from 7 preparations. This value was positive by 25 mV compared to that of I_s (about +3 mV, see also Fig. 2A). The application of Co²⁺ depressed TI at all voltages and flattened the relationship between the activation voltage and TI.

Since Co²⁺ exhibited a quick depressive action on TI as it blocked I_s (KOHLEHARDT and HAAP, 1980), its effects on both I_s and TI were examined with another 7 preparations during short exposure (Fig. 3). At 2 min in the Co²⁺ solution, the amplitude of I_s had already significantly decreased to $71 \pm 5\%$ of the control and that of TI did to $48 \pm 11\%$. Thus, inhibitory action of Co²⁺ on TI

Fig. 4. Time course of changes in I_s and TI during and after the application of 2 mM- Mn^{2+} . The result was obtained from a single preparation. The fiber was voltage-clamped at the holding potential of -45 mV and a depolarizing pulse to -5 mV with a 1-s duration was applied. The I_s (upper) was measured from the response to the depolarizing pulse and the TI (lower) upon repolarization from the preceding depolarization at indicated time. During the rest of the time, the fiber was off-clamped and stimulated with a rectangular pulse at 0.2 Hz.

became manifest and developed with a time course similar to those on I_s . The amplitude of I_s decreased to $42 \pm 9\%$ and that of TI to $14 \pm 7\%$ at 10 min in the Co^{2+} solution. These changes recovered to the control level after the wash-out of Co^{2+} for 10–20 min. No time lag as to the recovery of TI as compared to that of I_s was noted. This result was somewhat different from the inhibitory actions of Mn^{2+} on both currents in digitalized Purkinje fibers as reported by KASS *et al.* (1978a). They found that the development and removal of the TI inhibition by Mn^{2+} lagged far behind its effect on the I_s . Therefore, the action of Mn^{2+} on both currents was compared in our preparations. Figure 4 demonstrates the time course of changes in

Fig. 5. Time course of changes in I_s and TI during the elevation of external Ca^{2+} in the presence of Co^{2+} . The upper half represents the voltage clamp records obtained at the indicated time in the lower graph. In each picture, the top trace shows the voltage, the middle is the current with low magnification and the bottom that with high magnification. The lower half is graphs of I_s and TI with application of Co^{2+} and of high Ca^{2+} . The concentrations of $[\text{Ca}^{2+}]_o$ and of $[\text{Co}^{2+}]_o$ are shown by the two top lines. The experimental protocol was similar to those shown in Figs. 3 and 4.

both currents after the application of 2 mM-Mn²⁺ in a single preparation. The development and removal of the inhibition on both I_s and TI appeared with similar time courses. This result was confirmed in 4 preparations. The TI decreased from $0.73 \pm 0.11 \mu A$ in the control to $0.45 \pm 0.06 \mu A$ at 1–2 min in the Mn²⁺ solution and it became $0.38 \pm 0.06 \mu A$ at 10 min. Compared to the control value, the level of TI was $64 \pm 7\%$ at 1–2 min and $57 \pm 13\%$ at 10 min. The latter two values were significantly smaller than the former ($p < 0.02$ and $p < 0.05$, respectively). The I_s decreased to 72 ± 8 and $65 \pm 11\%$ of the control at 2 and 10 min, respectively. The wash-out of Mn²⁺ caused a prompt recovery of TI and its amplitude restored to $92 \pm 8\%$ of the control at 15 min in the Mn²⁺-free solution. The I_s recovered to $102 \pm 9\%$ during the same wash-out period. Therefore, the development and removal of the TI inhibition after a short exposure to Mn²⁺ appeared without a delay compared to those of the I_s inhibition.

Since a close correlation between the inhibition of I_s and that of TI seemed to be evident, effects of conditions which presumably increased or decreased the former were examined. For this purpose, first the extracellular Ca concentration was raised during the presence of Co²⁺, since the inhibitory effect of Co²⁺ on I_s could be antagonized by excess Ca²⁺ (KOHLHARDT and HAAP, 1980). Figure 5 demonstrates time course of changes in both I_s and TI, and their analysis, when $[Ca^{2+}]_o$ was raised from 3.6 to 7.2 mM in the presence of 2 mM-Co²⁺. Inhibition of both I_s and TI by 2 mM-Co²⁺ was removed by raising $[Ca^{2+}]_o$ to 7.2 mM with similar time courses. This result was confirmed in 4 fibers examined with

Table 2. Effects of raising $[Ca^{2+}]_o$ on I_s and TI in the presence of Co²⁺.

		3.6 mM-Ca ²⁺	3.6 mM-Ca ²⁺ + 2.0 mM-Co ²⁺	7.2 mM-Ca ²⁺ + 2.0 mM-Co ²⁺
I_s			***	*
	μA	6.3 ± 1.5	4.8 ± 1.3	7.6 ± 0.9
		NS		
			***	*
	%	100	76 ± 11	112 ± 12
		NS		
TI			*	*
	μA	1.26 ± 0.38	0.29 ± 0.17	0.06 ± 0.15
		*		
			***	*
	%	100	20 ± 8	51 ± 4

$n = 4$. * $p < 0.05$, ** $p < 0.02$, *** $p < 0.01$. NS, not significant.

Fig. 6. Time course of changes in I_s and T_I upon removal and readmission of $[Ca^{2+}]_o$. $[Ca^{2+}]_o$ is indicated at the top in mM. Experimental protocol, and measuring of I_s and of T_I were similar to those in the previous figures.

the same protocol as that shown in Fig. 5. Table 2 shows the results of 4 experiments. In the control condition, the amplitude of I_s was $6.3 \pm 1.5 \mu A$ and that of T_I was $1.26 \pm 0.38 \mu A$. The I_s and T_I decreased to $76 \pm 11\%$ and $20 \pm 8\%$, respectively, in the Co^{2+} solution. Upon raising external $[Ca^{2+}]$ to 7.2 mM, the former recovered to $112 \pm 12\%$ and the latter to $51 \pm 4\%$. Therefore, both currents responded to high $[Ca^{2+}]_o$ showing their recovery from the Co^{2+} effects, but a partial inhibition of T_I continued despite a complete restoration of I_s .

The second approach was to remove extracellular Ca^{2+} and to examine its effect on both I_s and T_I . Figure 6 shows time course of changes in both currents upon removing $[Ca^{2+}]_o$. The 0- $[Ca^{2+}]_o$ quickly inhibited both I_s and T_I with similar time courses. After 6 min in the Ca^{2+} -free solution, readmission of 3.6 mM- Ca^{2+} restored both currents to the control level. However, 0- $[Ca^{2+}]_o$ condition was prolonged to the period longer than 10 min, the recovery of these currents was not complete and the trans-gap potential drastically decreased in these conditions. Therefore, the exposure to 0 mM- $[Ca^{2+}]_o$ was limited to 6–8 min and the results obtained from 6 preparations are shown in Table 3. The 0- $[Ca^{2+}]_o$ depressed I_s to $64 \pm 9\%$ and T_I to $50 \pm 11\%$ during 1–2 min. The former became $37 \pm 7\%$ and the latter was $18 \pm 6\%$, during 6–8 min in the Ca^{2+} -free solution.

There have been several lines of evidence that an increase in intracellular Ca^{2+} plays a key role for the activation of T_I . (KASS *et al.*, 1978a, b; EISNER and LEDERER, 1979; KASS and TSIEN, 1982; MATSUDA *et al.*, 1982). So far, our results indicate that the development and removal of T_I are in good accord with those of I_s in their

Table 3. Effects of Ca²⁺-removal on *I_s* and TI.

		3.6 mM-[Ca ²⁺] _o	0 mM-[Ca ²⁺] _o 1-2 min	0 mM-[Ca ²⁺] _o 6-8 min
<i>I_s</i>	μA	7.8 ± 1.5	4.6 ± 0.8	2.5 ± 0.4
	%	100	64 ± 9	37 ± 7
TI	μA	2.45 ± 0.98	1.29 ± 0.60	0.42 ± 0.15
	%	100	50 ± 11	18 ± 6

n = 6. **p* < 0.05, ***p* < 0.02, ****p* < 0.01.

timings. This may indicate that Ca²⁺ transported through the slow channel plays a role not only for the cell-loading with Ca²⁺, but also has some additional action in regulating the intracellular movement of those ions. As the latter possibility, it may be suggested that Ca²⁺-influx via *I_s*, somehow affects the triggering mechanism of Ca-release from the sarcoplasmic reticulum. In order to examine the latter possibility, pharmacologic interventions which are believed to affect the Ca²⁺-release and -uptake mechanism from the sarcoplasmic reticulum were tested as to their effects on TI. Local anesthetics have been used to block Ca²⁺-release from the sarcoplasmic reticulum in skeletal muscle and they have been shown to exert similar actions on heart muscles as well (ENDO, 1977; CHAPMAN, 1979). Two mM-procaine HCl was found to eliminate TI completely with a mild reduction of *I_s* in 5 among 7 preparations (Fig. 7). The TI in the other 2 preparations was depressed to less than 25% of the control level. The *I_s* was suppressed to 68 ± 12% of the control during the 10 min of the application in 7 fibers. Five mM-procaine was found to abolish the TI completely within 5 min of the application and also to suppress *I_s* to 48 ± 9% of control. Thus, procaine at the concentration used in the present experiments was shown to depress *I_s* of ventricular muscles, in addition to its known action on the Na channel.

Caffeine is known to enhance the force of contraction in heart muscle as it does in skeletal muscle. This effect is attributed in part to increased Ca²⁺-release from the sarcoplasmic reticulum (ENDO, 1977). It also produces a prolongation of relaxation

Fig. 7. Effect of 2 mM-procaine on TI. Application of 2 mM-procaine HCl completely eliminated TI with a mild depression of I_s (B). These changes were reversible upon the wash-out of procaine in C.

and contracture, especially at high concentrations. The latter effect is postulated mainly to inhibit Ca^{2+} -uptake by the sarcoplasmic reticulum, thus depleting its stores (CHAPMAN, 1979; FABIATO, 1983). The application of 1 mM-caffeine was found to cause increased I_s and TI transiently, and to suppress both currents at later time of the application (10 min). Figure 8 shows time course of changes of the caffeine effects on both currents' systems in a single preparation. The TI increased from $0.53 \mu\text{A}$ in the control to $0.71 \mu\text{A}$ at 1 min of the application (Fig. 8B), while I_s increased from 1.53 to $2.0 \mu\text{A}$. At 10 min of the caffeine treatment (Fig. 8C), TI decreased to $0.29 \mu\text{A}$ and I_s to $1.41 \mu\text{A}$. Transient increases in both I_s and TI were observed in 5 among 7 preparations within 2 min of the application while the other 2 were found to have increased I_s with decreased TI from the beginning of the caffeine-treatment. Upon the wash-out of 1 mM-caffeine, TI quickly restored its amplitude beyond the control level (Fig. 8D) in the continued depression of I_s . Later, the former showed a tendency to recover to the pre-treatment level (Fig. 8E).

Fig. 8. Effects of 1 mM-caffeine on membrane currents of the guinea-pig ventricular muscle in the low-K⁺, high-Ca²⁺ solution. The arrangements of the traces are the same as in the previous figures.

Fig. 9. Abolition of TI with application of 10 mM-caffeine. During the application of 10 mM-caffeine (B), TI was completely abolished, while I_s was mildly suppressed. The depression of the late outward current was also noted in B.

These changes upon the wash-out of the drug were always observed in 7 preparations. A high concentration (10 mM) of caffeine was found to eliminate TI quickly without a transient augmentation at an early period of the application. Within 10 min of the application, 10 mM-caffeine completely abolished TI despite a mild inhibition of I_s ($88 \pm 4\%$ of the control at 10 min. $n=4$) (Fig. 9).

DISCUSSION

The present study disclosed that the manipulations which suppressed the slow channel produced inhibition of the transient inward current as rapidly as they did on the slow inward current in guinea-pig ventricular muscles under the low- K^+ , high- Ca^{2+} condition. Removal of their inhibition upon the wash-out of Co^{2+} or by raising external Ca^{2+} also developed as close a time course as the recovery of I_s . Thus, there was no time lag between the action of these agents on I_s and those on TI. Further, procaine and caffeine, which are known to affect the Ca^{2+} -uptake and -release from the sarcoplasmic reticulum (ENDO, 1977; CHAPMAN, 1979; FABIATO, 1983), completely eliminated the TI despite a mild depression of I_s . Since various experimental results suggest that the increase in internal Ca^{2+} is a key step in the generation of TI (FERRIER, 1977; KASS *et al.*, 1978b; EISNER and LEDERER, 1979; KASS and TSIEN, 1982; MATSUDA *et al.*, 1982), our observation may be compatible with the idea that Ca^{2+} -influx through the slow channel upon each depolarization acts not only to load the cell with Ca ions, but also to produce somehow a phasic increase in $[Ca^{2+}]_i$ upon repolarization. Although the linking mechanism between the Ca^{2+} -influx and the phasic increase in $[Ca^{2+}]_i$ is not known, the final step of the latter seems to trigger the Ca release from the sarcoplasmic reticulum.

The present result that the application of Co^{2+} or of Mn^{2+} rapidly suppressed the TI with a close correlation of the time course of the I_s inhibition is somewhat different from the observation made by KASS *et al.* (1978a) in digitalized Purkinje fibers. They noted that the development and removal of the TI inhibition lagged far behind its effect on the I_s . Therefore, they concluded that the main action of Mn^{2+} was to reduce TI, indirectly, through its effect on I_s , thus causing an elimination of the Ca^{2+} -overload in the cell. It is not certain, however, why two studies gave different results. But there may be a possible factor which accounts for the above discrepancies—a different condition used in both experiments in order to load the cell with Ca. While we exposed the preparations to the low- K^+ , high- Ca^{2+} solution for about 15–30 min, they applied the drug, acetylthiocholine, to Purkinje fibers for more than 60 min for this end. It is, therefore, probable that the degree of the Ca^{2+} -overload in our preparations is not equal to that of their preparations. The binding of the cardiac glycoside to the membrane may also induce a continued accumulation of Ca into the cells, despite a presence of Mn^{2+} in the latter condition. It has been pointed out that differences of Ca^{2+} -overloading in the cells might account for discrepant results when inhibitory effects of the Ca^{2+} -channel blocking agents on TI were examined. ALLEN *et al.* (1984) reported that the

application of D-600 to the preparations, which were loaded with Ca²⁺ through the Na⁺-Ca²⁺ exchange mechanism, did not produce the decrease in the [Ca²⁺]_i oscillations detected by the aequorin signals and in the aftercontractions, while KASS and TSIEN (1982) observed the decreased current fluctuations driven by the intracellular Ca oscillations in the Ca²⁺-overloaded preparations by exposure to digitalis or K⁺-free solutions in the presence of D-600. In spite of the above discrepancies, removal of external Ca²⁺ quickly inhibited both *I_s* and TI in the previous studies (KASS *et al.*, 1978a; EISNER and LEDERER, 1979; KASS and TSIEN, 1982) as well as the present one (Fig. 6). Our result also agrees with the observations that Mn²⁺ suppressed DADs in other preparations including digitalized Purkinje fibers within a few min after the application (FERRIER and MOE, 1973; ROSEN *et al.*, 1974; HIRAOKA *et al.*, 1981). In the generation of TI, therefore, the Ca²⁺-influx through the slow channel acts as a major source of the cell-loading with Ca²⁺ as suggested by KASS *et al.* (1978a), and it also has some additional role in the increase in [Ca²⁺]_i, depending on cellular conditions.

As to the generation of TI, the phasic release of Ca²⁺ from the intracellular stores is implicated (KASS *et al.*, 1978b). If the role of *I_s* is applied to this schema, the Ca²⁺-influx via the sarcolemma must regulate a certain linking step to influence the Ca²⁺-release process from the sarcoplasmic reticulum. One possible link between the two processes is that Ca ions entering via the slow channel are rapidly taken up into the sarcoplasmic reticulum and transported to their release sites during the depolarization. Therefore, the more releasable Ca²⁺ is available upon repolarization or subsequent stimulations, when the amount of Ca²⁺ carried by *I_s* becomes larger, or vice versa. FABIATO (1983) suggested that Ca²⁺ transported through the slow channel could be a triggering mechanism of the Ca²⁺-release from the sarcoplasmic reticulum, although the quantitative relation of these ions between the two processes as well as the detailed mechanism have not yet been clarified.

Even if we admit that *I_s* has some influence on the Ca²⁺-release process which produces TI, our results pose another problem. The TI in our preparations showed a bell-shaped dependence on the activation voltage with a maximum at about +29 mV. Similarly, the voltage dependence of *I_s* was also bell-shaped but with 25 mV shift to more negative values as compared to those of TI. The bell-shaped voltage dependence of two currents as well as that of tension agreed with the previous reports (MORAD and GOLDMAN, 1973; CHAPMAN, 1979). Further, a negative shift of the *I_s*-voltage relation compared to the TI-voltage relation was also described in other tissues (KARAGUEUZIAN and KATZUNG, 1982). Recently, MAYLIE and MORAD (1984) described a transient outward current related to Ca release and development of tension in elephant seal atrial fibers. This current had a bell-shaped voltage dependence similar to those of tension and of *I_s* with 20 mV shift to more positive values compared to the latter two.

As the final step to produce TI, the role of the Ca²⁺-release from the stores was also supported by the present experiments. The applications of procaine and caffeine, which are known to affect the Ca²⁺-uptake and release from the

sarcoplasmic reticulum (ENDO, 1977; CHAPMAN, 1979; FABIATO, 1983), were shown to eliminate TI completely in spite of a mild depression of I_s . The action of procaine on TI of the present experiment confirmed our previous observation of its inhibition on DADs (HIRAOKA and KAWANO, 1984), and agreed with the effects of tetracaine on them in Purkinje fibers (BHATTACHARYYA and VASSALLE, 1981).

Recently, NOBLE (1984) indicated that I_s had two components with different time courses. The slower activating component is produced by the Na^+ - Ca^{2+} exchange mechanism and is therefore related to the development of TI. If this explanation is correct, the inhibition of I_s directly correlates with the suppression of TI, and at least part of the present results can be explained by this hypothesis. However, the quantitative relation between I_s and TI, as well as the actual proof of Noble's explanation, is to be clarified by future studies.

Caffeine has been shown to increase Ca^{2+} -release from the sarcoplasmic reticulum (ENDO, 1977) and to decrease the rate of uptake by causing Ca release, thus depleting the stores (CHAPMAN, 1979; FABIATO, 1983). In addition to these effects, it also demonstrates multiple actions on membrane currents including I_s (EISNER *et al.*, 1979; GOTO *et al.*, 1979). In the present experiment, 1 mM-caffeine produced a transient increase in TI together with an increased I_s . This result was also in accord with our previous observation of the caffeine effect on DADs (HIRAOKA *et al.*, 1979). Augmentation of I_s by caffeine, however, has recently been found to be due to a decrease of the overlapping outward current and the actual changes were a progressive decrease of I_s after its application (HESS and WIER, 1984). If this was taken into account, initial increases in TI may be caused by increased Ca^{2+} -release. This finding was also recently confirmed by the aequorin signal in Purkinje fibers (EISNER and VALDEOLMILLOS, 1986). Later suppression or complete elimination by high concentration of caffeine can be reasonably explained by the inhibition of Ca^{2+} -uptake and the depletion of the stores. This result confirmed the observation reported by EISNER and LEDERER (1982). Therefore, the above results are in line with the hypothesis that the generation of TI is largely dependent on the Ca^{2+} -release from the sarcoplasmic reticulum.

The present result that the slow channel blocking agents could suppress the TI by somehow influencing both the cellular Ca overload and the Ca^{2+} release process, and the effects of these agents developed as rapidly as they inhibit I_s , may have a clinical implication. DADs and triggered-activity are assumed to play a significant role in contributing to the genesis of arrhythmias (CRANFIELD, 1977; FERRIER, 1977). The Ca^{2+} antagonists are known to block the arrhythmias based on the slow response activity. In addition to this effect, they can also exert anti-arrhythmic action directly on the triggered-arrhythmias based on DADs because of their inhibitory effect on the TI as demonstrated in this study.

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