Use of Calcium Ionophores to Determine the Effects of Intracellular Calcium on the Action Potential of Canine Cardiac Purkinje Fibers

JEREMIAH M. GELLES

SUMMARY. The effects of calcium ionophores X-537A, A23187, and PR-47 on the action potential of canine cardiac Purkinje fibers were studied using standard microelectrode techniques. The ionophores hyperpolarize the membrane potential, eliminate the notch on the action potential plateau, decrease action potential duration, decrease plateau amplitude and duration, decrease spontaneous diastolic depolarization and excitability, and decrease the rate effect on action potential duration. These effects were shown to be independent of catecholamines, membrane-bound calcium, and the patency of the slow inward current channel. The effects of the ionophores depended on the presence of calcium in the external solution. A plausible interpretation of the data is that an ionophore-mediated increase in intracellular calcium shifts the voltage dependence of transmembrane potassium currents to more negative levels.

SEVERAL carboxylic ionophoric antibiotics act as electroneutral transporters of calcium across cell membranes. The ionophores provide a potentially useful tool with which to study the role of perturbations in intracellular calcium on cellular function. One such ionophore, X-537A, can form complexes with mono-, di-, and trivalent cations as well as with catecholamines. A23187 is more specific than X-537A but can form complexes with divalent cations other than calcium. Nevertheless, the effects of X-537A and A23187 on a number of biological properties is best explained by their ability to transport calcium across cell membranes. In addition, in both cardiac muscle and skeletal muscle, the action of the ionophores is best explained by their ability to increase calcium available to the contractile apparatus.

Because the divalent cation ionophores transport calcium into the cell, they may be useful as inotropic agents and as tools with which to study the effects of intracellular free calcium ion concentration, [Ca<sup>2+</sup>], on the electrical and mechanical properties of excitable cells. It has been suggested that [Ca<sup>2+</sup>] modulates potassium permeability in cardiac Purkinje fibers (PF) and ventricular muscle. In other species, increased [Ca<sup>2+</sup>] has been shown to increase outward potassium current. The present study was undertaken to determine the effects of several divalent cation ionophores on the action potential of canine cardiac Purkinje fibers and to determine whether these effects are due to an increase in [Ca<sup>2+</sup>]. Preliminary reports of these findings have appeared.

Methods

Strands of Purkinje fibers (0.5–0.8 mm in diameter and 5–10 mm in length) are removed from the ventricles of mongrel dogs, 15–18 kg, anesthetized with pentobarbital sodium (Nembutal), 30 mg/kg, iv, and mounted in a plexiglass tissue bath with a volume of 1.0 ml described in detail elsewhere. The tissue is superfused at a rate of 15–20 ml/min with modified Tyrode’s solution of the following composition in millimoles per liter: Na, 150.8; K, 2.0–5.4; Cl, 146.1; Ca, 2.7; Mg, 0.5; HCO<sub>3</sub>, 12; PO<sub>4</sub>, 1.8; glucose, 5.5. The solution is equilibrated with 95% O<sub>2</sub>-5% CO<sub>2</sub>, resulting in a pH of 7.4. The temperature of the fluid is maintained at 36–37°C. In the experiments in which lanthanum chloride is added, the buffer system (NaHCO<sub>3</sub>, NaH<sub>2</sub>PO<sub>4</sub>) is replaced by N-2-hydroxyethylperazine-N'-2-ethanesulfonic acid (HEPES)-NaOH buffer. Electrical recording is achieved by means of glass microelectrodes filled by boiling under vacuum in 3 M KCl. The voltage-recording electrode is connected to the remaining circuitry by a plexiglass microelectrode holder containing modified Tyrode’s solution and a sintered Ag-AgCl electrode (Annex Research). The microelectrode holder used for recording transmembrane voltage is connected to a negative capacity bridge amplifier with high input impedance (Rockefeller University). The bath is grounded by means of a sintered Ag-AgCl disk electrode (Annex Research). This electrode is connected to an operational amplifier which maintains the bath at virtual ground.

External stimuli are isolated from ground and applied to the tissue by small coaxial electrodes (Rhodes Medical Instruments). The timing of stimuli is determined by a series of digital counting modules triggered by a crystal-controlled clock source (Rockefeller University). The counting modules trigger two pulse generators (Rockefeller University).

The ionophore is dissolved in dimethyl sulfoxide at a concentration of 5 X 10<sup>−6</sup> M. A sample of this solution is added to 5 ml of distilled water and this in turn is added to 1 liter of Tyrode’s solution to give a final concentration of the sodium salt of the ionophore in the range 10<sup>−7</sup> to 10<sup>−6</sup> M. The amount of dimethyl sulfoxide required has no electrophysiological effect on cardiac Purkinje fibers (personal observation).

X-537 was obtained from Hoffmann-LaRoche; A23187...
from Eli Lilly; and PR-47 from Dr. Irvin Borowitz of Yeshiva University, New York.

Results

Figure 1 shows the typical effect of X-537A on a canine cardiac Purkinje fiber ([K+], = 4 mM). The records in panel A were taken under control conditions during external stimulation at 0.63 Hz. After exposure to X-537A, 10^-5 m, for 31 minutes the action potential duration (APD) decreased and there was a marked decrease in plateau amplitude and duration. The maximum diastolic potential (MDP) became more negative as did the takeoff potential (Fig. 1B; Table 1). The spontaneous rate of this preparation during the control period was 30/min. During exposure to X-537A spontaneous activity ceased. Another preparation during the control period was 30/min. During exposure to X-537A spontaneous activity ceased. Another effect of X-537A was the loss of the notch at the onset of the plateau (Fig. 1A) during X-537A exposure (Fig. 1B). Decrease in plateau amplitude and duration hyperpolarization of the membrane potential during diastole, decrease of spontaneous activity, and loss of the plateau notch were consistent effects of ionophore exposure in 30 experiments. These effects were not present in the absence of extracellular calcium.

Figure 2 shows the effects of X-537A on spontaneously active PF ([K+], = 2 mM). Two electrodes were used to record action potentials from this preparation, one of which was in the pacemaker of the preparation. Figure 2A shows the control action potentials. Figure 2B shows the action potentials after exposure to X-537A, 10^-5 m, for 7 minutes. Spontaneous activity had ceased and the preparation had to be externally stimulated. The resting potential of the driven fiber and the MDP of the former pacemaker fiber both became more negative. Phase 4 of spontaneous diastolic depolarization (SDD) became almost flat during exposure to X-537A (Fig. 2B; Table 1). Gibson et al.41 have also reported suppression of the slope of diastolic depolarization in canine Purkinje fibers exposed to 10^-5 M X-537A.

It has been suggested that the electrophysiological effects of X-537A on canine Purkinje fibers are catechola-

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**Table 1.** Effect of Calcium Ionophores on Various Action Potential Parameters

<table>
<thead>
<tr>
<th></th>
<th>RMP (mV)</th>
<th>MDP (mV)</th>
<th>TO (mV)</th>
<th>OS (mV)</th>
<th>Plateau (mV)</th>
<th>APD (50%) (msec)</th>
<th>APD (100%) (msec)</th>
<th>Time (min)</th>
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<tr>
<td>A (control)</td>
<td>Spont</td>
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<td>293</td>
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<td>-93</td>
<td>+28</td>
<td>-2 (50 msec)</td>
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<td>Figure 2</td>
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<tr>
<td>A (control)</td>
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<td>-101</td>
<td>-84/-98</td>
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<tr>
<td>B (X-537A)</td>
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<td>-22 (100 msec)</td>
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<td>Figure 4</td>
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<td>A (control)</td>
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<td>Figure 6</td>
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<td>-96</td>
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<td>250</td>
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<td>101</td>
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<td>Figure 7</td>
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<td>A (control)</td>
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<td>-85/-92</td>
<td>+37/+39</td>
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<td>370/307</td>
<td>533/437</td>
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<td>B (PR-47)</td>
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<td>+38/+39</td>
<td>-7/-10</td>
<td>258/248</td>
<td>413/395</td>
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RMP = resting membrane potential (nonspontaneous preparations); MDP = maximum diastolic potential; TO = takeoff potential (membrane potential at onset of upstroke of action potential); OS = overshoot (peak potential achieved by upstroke of action potential); plateau = voltage of plateau measured 50 or 100 msec after TO; APD = action potential duration (at 50% and at 100% repolarization); Time = time of recording after onset of indicated experimental maneuver; Spont = spontaneous. In Figure 7, the initial notation of each pair refers to the lower set of action potentials. In Figure 7, the initial notation of each pair refers to the action potentials driven at a cycle length of 1,600 msec. Measurements of action potential parameters were made with a Nicolet model 1090 digital oscilloscope.
mine-mediated.45-46 Because of evidence that calcium ionophores release catecholamines from storage sites and that X-537A transports catecholamines across membranes,3 several experiments were performed to exclude the possibility that effects of X-537A observed were catecholamine-mediated as has been reported for the inotropic effects of X-537A.47 The effect of epinephrine on a PF action potential that had developed the triangular appearance seen after prolonged exposure to X-537A, $10^{-5} \text{M}$, was observed in one such experiment. The membrane potential became more negative by 5 mV and the APD (50% repolarization) increased by 50 msec. Epinephrine did not induce automaticity in the ionophore-treated preparation. Epinephrine exposure did not mimic the ionophore effect. The effects of both $\alpha$- and $\beta$-adrenergic compounds on the electrical activity of caridac PF are substantially different from the ionophore effects herein described.44

Further experiments were performed on a propranolol-treated preparation and a practolol-treated preparation. An experiment was performed on a practolol-treated PF from a dog pretreated with reserpine, 1.2 mg/kg, in divided intramuscular doses over 3 days prior to the experiment. The results of these experiments were similar as regards the ionophore effects. Figure 3 shows the effects of X-537A on a PF exposed to practolol, $10^{-4} \text{M}$. The control action potentials ($[K^+]_e = 4 \text{ mm}$) during external stimulation at 1.25 Hz are shown in Figure 3A. Figure 3B shows the effects of exposure to practolol for 137 minutes (Table 1). Exposure of the preparation at this point to epinephrine confirmed the completeness of $\beta$-blockade: plateau amplitude decreased and APD increased ($\alpha$ effects of epinephrine).47 Figure 3C shows the effects of 50 minutes of exposure to X-537A ($10^{-5} \text{M}$). Amplitude and duration of the plateau are decreased; APD is decreased and the resting membrane potential (RMP) is more negative (Table 1). These results indicate that the ionophore effect on the action potential is not due to release or transport of endogenous catecholamines.

Calcium entry into the cardiac PF is mediated by the slow inward current (SIC) that underlies the plateau of the cardiac action potential.48 To show that the mode of action of the ionophore is not to modify the SIC, perhaps by affecting the SIC channel, a PF was exposed to X-537A after exposure to verapamil (1 mg/liter) for 45 minutes. Figure 4A shows the control action potential during external stimulation at 1.25 Hz. Figure 4B shows the action potential after exposure to verapamil (Table 1). The plateau is decreased in amplitude and shortened and the terminal phase of repolarization is delayed, the usual effects of verapamil on the cardiac PF action potential.49 Figure 4C shows the effects of 50 minutes of exposure to X-537A, $10^{-5} \text{M}$, and verapamil, 1 mg/liter, on the action potential shown in Figure 4B. The membrane potential is hyperpolarized and the APD is decreased. Plateau amplitude and duration are also decreased (Table 1). Thus ionophore effects do not depend on the integrity of the SIC channel.

Because of the role of membrane-bound calcium in electromechanical coupling, an experiment was performed to determine whether the trivalent cation, lanthanum, which displaces calcium from its sarcolemmal binding sites, would interfere with the ionophore effect. A PF preparation was exposed to LaCl$_3$, 50 $\mu\text{M}$, for 34 minutes, a dose which displaces most "membrane-bound" calcium.46 Figure 5A shows the control preparation during external stimulation at 1.25 Hz. Figure 5B shows the action potential after exposure to LaCl$_3$ (Table 1). The main alteration is prolongation of total APD due to this concentration of La, as previously described.49 The effects of X-537A on the La-treated preparation are shown in Figure 5C. The typical ionophore-mediated effects are evident (Table 1). Hyperpolarization was not seen in this preparation. Thus, membrane-bound calcium is probably not necessary to the ionophore effect.

X-537A is a relatively nonspecific ionophore.1,3 In order to be sure that the effects of X-537A heretofore described are indeed the effects of transmembrane calcium movement, experiments were performed with two additional ionophores, A23187, specific for Ca and Mg,4 and PR-47, a synthetic ionophore highly selective for Ca.50 Figure 6 shows the effects of A23187 on a PF preparation ($[K^+]_e = 4 \text{ mm}$). Figure 6A shows the control action potentials during external stimulation at 1 Hz. Figure 6B shows the action potentials after exposure to A23187, $2 \times$
Figure 5 Effect of X-537A on Purkinje fiber exposed to lanthanum chloride. A: control. B: during exposure to LaCl₃. C: during exposure to LaCl₃ and X-537A. 0 on calibration indicates membrane potential. Horizontal dots mark off 100-msec intervals.

Figure 6 Effect of A23187 on Purkinje fiber action potential. A: control. B: during exposure to A23187. C: during wash-out of A23187. 0 on calibration indicates membrane potential.

Figure 7 Effect of PR-47 on Purkinje fiber action potential and on rate-related changes in action potential duration. A: control; first action potential at cycle length of 1,600 msec; remainder at cycle length of 600 msec. B: during exposure to PR-47; same stimulus program as in A. 0 on calibration indicates membrane potential.

Discussion

The results of this study show that calcium ionophores shorten plateau amplitude and duration, eliminate the notch at the beginning of the plateau, shorten APD, hyperpolarize the membrane, and suppress automaticity and excitability in cardiac Purkinje fibers. These effects seem to be independent of "membrane-bound" calcium but dependent on the presence of external calcium. The ionophore effects do not depend on the presence of catecholamines nor do they depend on the patency of the slow inward current channel. Various interventions in cardiac Purkinje fibers which (among other effects) increase [Ca²⁺]ᵢ have effects in common with the ionophores:

1. Metabolic inhibition causes a marked decrease in amplitude and duration of the plateau of the action potential.⁵⁵
2. Increased [Ca²⁺]₀ causes a decrease in APD but increases SDD and plateau amplitude.⁶⁰⁻⁶⁶
3. Epinephrine exposure causes the APD to shorten but the plateau amplitude increases and SDD is enhanced.⁴⁴
4. Increasing [Ca²⁺]ᵢ by an iontophoretic technique shortens the PF action potential.³¹
5. Rapid stimulus rates shorten the action potential.

Other interventions abbreviate and lower the plateau of the cardiac action potential by mechanisms which may be independent of [Ca²⁺]ᵢ. Valinomycin,⁵⁷ increased [K⁺]₀, and slow channel blockers such as verapamil⁵⁸ are examples. Interventions that increase [Ca²⁺]ᵢ without a change of [Ca²⁺]₀ and/or the SIC appear to mimic the ionophore effect. Interventions that increase [Ca²⁺]ᵢ by increasing [Ca²⁺]₀ or increasing SIC (e.g., epinephrine) have mixed effects on the PF action potential.⁵⁸

During exposure to X-537A, it was observed that contractile activity became more vigorous and then ceased, at which point it became quite difficult to maintain an electrode implemment, suggesting that the fiber was in contracture. This observation supports the idea that the ionophore effects are mediated by an increase in [Ca²⁺]ᵢ. Although the ionophore effects were not present in the absence of external Ca, this result cannot be taken as proof that the ionophore effect depends on the transsarcolemmal transport of Ca. Zero [Ca²⁺]₀ is quite deleterious to the fiber and rapidly results in irreversibly abnormal electrical activity. If the ionophore effects are mediated by
the release of Ca from intracellular stores, or by a mechanism not involving [Ca^{2+}]_i, these effects might still not be detectable in the fibers exposed to zero [Ca^{2+}].

If the mechanism of action of the ionophores is an increase in [Ca^{2+}], then the driving force on Ca current during the SIC would decrease. This would tend to decrease the amplitude of the plateau. However, elimination of the SIC alone does not completely eliminate the plateau nor does it decrease APD when measured from 90-100% repolarization. An increase in [Ca^{2+}] may cause a shift in the kinetic properties of the i_k current to a more negative voltage range. This would result in earlier and more rapid activation of the i_k current during depolarization and would explain the observation (Fig. 7) that cycle length-related changes in APD seem to be diminished during ionophore exposure.

The effects of the ionophores on resting potential and SDD can be explained similarly by a negative shift in the voltage dependence of the slow outward potassium current, i_k, whose decay underlies SDD and which contributes about one-third to the resting membrane conductance. At any level of potential negative to approximately -60 mV (at which point i_k is normally fully activated) the outward current would be greater during ionophore exposure than during the control period, thus hyperpolarizing the membrane. SDD would be slowed and unlikely to bring the membrane to threshold potential.

The shift in the voltage-dependent properties of the potassium currents underlying the cardiac action potential as a result of an ionophore-induced increase in [Ca^{2+}] would not be unexpected. Frankenhaus and Hodgkin suggested that the effects of alterations in [Ca^{2+}] on the electrical properties of squid axons were due to changes in membrane surface charge. They found that increasing [Ca^{2+}] caused a positive shift in the voltage-dependence of the sodium conductance. Reducing [Ca^{2+}] caused the voltage-dependence of the potassium conductance to shift to more negative levels of potential. Chandler et al. observed a depolarizing shift in the h variable controlling sodium influx when they decreased internal ionic strength in the internally perfused squid axon. McNaughton and Noble have suggested that the epinephrine-induced positive shift of the voltage dependence of the s variable controlling the pacemaker current, i_k, may be due to decreased [Ca^{2+}], thus reducing the positive charge density on the inside of the membrane. It is plausible to suggest that the calcium ionophores increase [Ca^{2+}]. Increases in [Ca^{2+}] may alter the density of charge on the inner surface of the PF membrane, thereby causing a negative shift in the voltage-dependent parameters of the potassium currents.

Acknowledgments

I thank Dr. N. Krasnow for his valuable advice in carrying out this work and Marcia Cabo for her valuable technical assistance. I am grateful to Dr. Julius Berger of Hoffmann-LaRoche, Dr. Robert Hamill of Eli Lilly & Co., and Dr. Irvin Borowitz of Yeshiva University, New York, for their gifts of X-537A, A23187, and PR-47, respectively.

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Hemodynamics of Arterial Stenoses at Elevated Flow Rates

DONALD F. YOUNG, NEAL R. CHOLVIN, RICHARD L. KIRKEEIDE, AND ALLAN C. ROTH

SUMMARY This study is concerned with the pressure drop that develops across an arterial stenosis, with particular emphasis on the effect of the stenosis at high blood flow rates induced by a locally administered vasodilator drug. Stenoses, ranging in severity from 55.7% to 91.0% reduction in lumen area were artificially induced in the femoral arteries of rabbits. Results show that blood flow through a particular artery can increase by a large factor, in the range of 4-5, under conditions of vasodilation with a corresponding large decrease in pressure distal to the stenosis. The pressure drop increases in a nonlinear manner with velocity and thereby accentuates the importance of the stenosis at elevated flow rates. We suggest that a critical stenosis be defined in terms of its effect on maximal flow rather than resting flow.

THE DEVELOPMENT of a stenosis in a major artery may significantly alter the blood supply to the peripheral vascular beds supplied by the artery. Since the early work of Mann et al.,' much attention has been given to this problem, with special consideration given to the concept of the "critical stenosis," which generally has been defined as one for which a small, further increase in the severity of the stenosis will cause a significant reduction in blood...
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