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MODE OF ACTION OF THE CALCIUM IONOPHORES X-537A AND A23187 ON CARDIAC CONTRACTILITY

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Abstract—The effects of two antibiotics, X-537A and A23817, which transport Ca^{2+} as a lipid-soluble complex through a number of natural and artificial membranes, have been investigated using a perfused working rat heart preparation. When the contractility of the hearts was depressed by either low Ca^{2+} concentration or by addition of ruthenium red to the perfusion medium, X-537A produced a large increase of coronary flow rate, left ventricular pressure development and work output. These effects were suppressed in the presence of propranolol and in the hearts from animals pretreated with reserpine. The other ionophore, A23187, was without significant effect on cardiac contractility but increased the coronary flow rate. These results suggest that the mechanism of action of X-537A in the isolated heart is not via an increased permeability of the plasma membrane to Ca^{2+}. The positive inotropic effect of X-537A is better explained as resulting from the release of endogenous norepinephrine.

COMPOUNDS which affect the ionic permeability of biological membranes offer tools for perturbing cellular functions and provide potentially useful methods for investigating ion transport mechanisms. During the last decade, a number of antibiotics have been found to increase the permeability of natural and artificial membranes to monovalent cations,[1–4] and the use of these molecules has markedly contributed toward understanding mechanisms of monovalent cation transport. X-537A and A23187 are two antibiotics which have recently been shown to form complexes with divalent cations, thereby permitting their transport across biological and model membranes. X-537A is an antibiotic of well-defined structure obtained in crystalline form from an unidentified *Streptomyces*.[5] The molecular structure is that of a monocarboxylic acid containing a number of cyclic ether moieties and an aromatic chromophore group.[6] X-ray crystallographic studies have shown that two molecules of X-537A encircle a molecule of divalent cation together with its water of crystallization to form a complex of spherical shape with a hydrophilic pocket for the hydrated cation and a hydrophobic exterior.[7]

A23187 is another monocarboxylic acid isolated from a different *Streptomyces*.[8] Although detailed structural studies are still lacking, it is thought that A23187 also forms complexes with divalent cations which are similar to those formed by X-537A. The major difference between the two molecules rests in their selectivity toward cations; X-537A forms complexes with both mono- and divalent cations, whereas A23187 is more specific for divalent cations.[9] Facilitated transport of Ca^{2+} induced

by these antibiotics has been reported in sarcoplasmic reticulum,[10–13] mitochondria[9,14,15] and red blood cells.[16]

It has been previously reported that both X-537A and A23187 induced contraction of rabbit aortic ring[17,18] and that X-537A increased left ventricular pressure and cardiac output in anesthetized dogs[19] and isolated rat heart,[20] The pupose of this paper is to examine the mode of action of X-537A and compare it to A23187. Preliminary reports of these experiments have been presented.[20,21]

METHODS

Hearts from male Wistar strain rats (220–250 g) were perfused at 35° using modifications of the working heart apparatus of Neely *et al.*,[22] as described previously.[23] The perfusion fluid was Krebs bicarbonate medium containing variable concentrations of Ca^{2+} which was equilibrated with 95% O_2 and 5% CO_2. Hearts from frogs were perfused at room temperature through the aorta using air-saturated Ringer's fluid and a pressure head of 25 cm.

Left ventricular pressure and its first derivative (dP/dt) were measured with a Statham P23 pressure transducer by inserting a 22-gauge needle through the ventricular wall, and the responses were recorded using a Brush 440 recorder. Aortic pressure was measured similarly by means of a T-tube located above the aortic cannula. Cardiac output was estimated from the sum of the left ventricular output measured with a rotameter, and the coronary flow rate measured with an extra-corporeal electromagnetic flowmeter (Biotronex Laboratory, Inc.). Pressure work was calculated according to Neely *et al.*[22] Oxygen consumption was calculated from the coronary flow rate and the arterial-venous oxygen tension difference. The oxygen tension of the coronary effluent fluid was measured continuously by allowing it to pass through a small (0·3 ml) chamber fitted with a Clark oxygen electrode. The fluid was stirred to minimize the flow dependence of the oxygen electrode. Depletion of endogenous catecholamines was induced by injecting rats with 10 mg/kg of reserpine 6 hr before sacrifice.

Experimental animals were purchased from West Jersey Supply, Wenonah, N.J., and ruthenium red, $[Ru(NH_3)_4OHCl]Cl.2\,H_2O$, from Alfa Inorganics Inc., Beverly, Mass. X-537A and A23187 were generous gifts of Dr. J. Berger of Hoffman-LaRoche, Nutley, N.J., and of Dr. R. Hamill of Eli Lilly & Co., Indianapolis, Ind. respectively. The ionophores in the free acid form were dissolved in ethanol to give concentrations of 1–6 mg/ml.

RESULTS

Figure 1 shows the effect of a single addition of 150 μg X-537A on left ventricular pressure changes induced in rat hearts perfused continuously with Krebs–Henseleit buffer containing 10 mM glucose. The ionophore, dissolved in 10μl ethanol, was injected into tubing above the aortic cannula. Control experiments with the same amount of ethanol alone showed no effects on contractility. The heart was initially perfused with buffer containing 0·5 mM Ca^{2+}. When the Ca^{2+} concentration in the perfusate was decreased to 0·1 mM, contractility diminished by over 90 per cent, but was restored to a value greater than the initial upon addition of X-537A. The effect was transient, and the heart was unresponsive to a second similar addition of X-537A. From Fig. 1 it is evident that X-537A is not only a powerful inotropic agent,

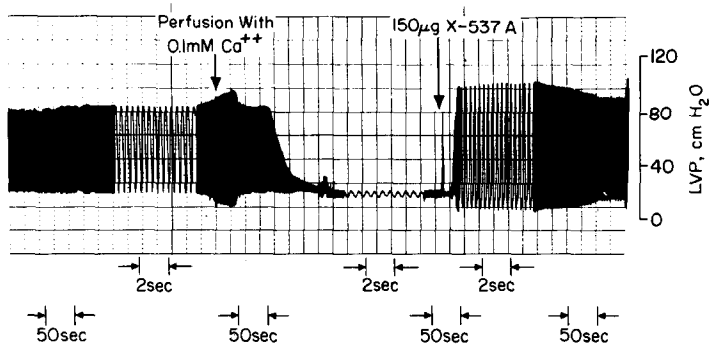

FIG. 1. Effect of X-537A on left ventricular pressure (LVP) of rat heart perfused with 0·1 mM Ca^{2+}. The heart was first perfused with Krebs bicarbonate medium containing 0·5 mM Ca^{2+}, followed by a change of perfusion medium to 0·1 mM Ca^{2+} and subsequent addition of 150 μg X-537A in 25 μl ethanol to the fluid circulation immediately above the aorta.

but also has a marked chronotropic effect since it increased the heart rate from 120 to 185 beats/min. Similar responses were seen after addition of X-537A to perfused hearts of guinea pigs and pigeons; however, no effects could be demonstrated in perfused hearts from frogs.

In Fig. 2, using a flow-through perfusion system with medium containing 10 mM glucose, the rate of oxygen uptake and the cardiac work is shown as a function of increasing concentrations of Ca^{2+}. At Ca^{2+} concentrations below 1·5 mM, the presence of 100 μg X-537A in 150 ml recirculating medium increased the sensitivity of the heart toward external Ca^{2+}. This is seen from the shift of curves relating the dependence of oxygen consumption and work output with Ca^{2+} concentration toward lower values of Ca^{2+}.

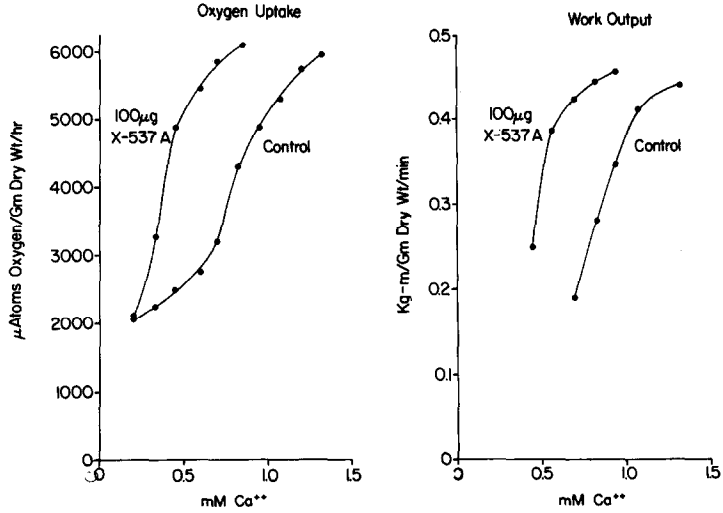

FIG. 2. Effect of X-537A on oxygen uptake and work output at varying concentrations of Ca^{2+}. Hearts were perfused in a recirculation apparatus with 150 ml Krebs bicarbonate medium containing 100 μg X-537A.

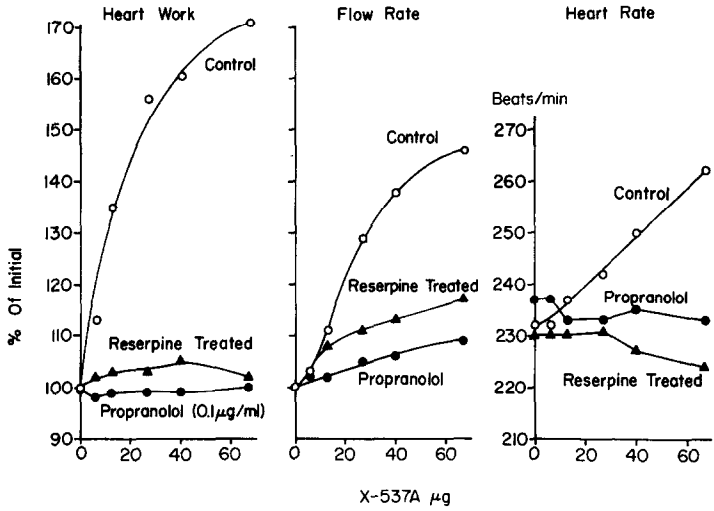

FIG. 3. Effect of increasing concentrations of X-537A on heart work, coronary flow rate and heart rate in hearts perfused in a recirculation apparatus with 0·5 mM Ca^{2+} in the presence or absence of 0·1 μg/ml of propranolol, or with hearts from rats pretreated with reserpine. The volume of perfusion fluid was 150 ml.

The effect of X-537A on cardiac work, flow rate and heart rate in a series of rat hearts perfused with a recirculating buffer containing 0·5 mM Ca^{2+} is shown in Fig. 3. The maximal response was obtained with 75 μg X-537A/150 ml perfusate, which produced a 70 per cent increase in cardiac work, a 45 per cent increase in coronary flow, and a small but significant increase of heart rate. Pretreatment of the heart *in vitro* with propranolol, a β-adrenergic blocking agent, or treatment of the rat *in vivo* with reserpine to deplete endogenous norepinephrine stores caused an almost complete inhibition of the effects induced by X-537A. These data suggest a clear link between the positive inotropic effect of X-537A and the release of norepinephrine from endogenous stores.

In Fig. 4, the effects of X-537A on cardiac contractility are compared with those of epinephrine and Ca^{2+} in different hearts. The ionophore, dissolved in ethanol, was added to 500 ml perfusion medium containing 10 mM glucose and 0·3 mM Ca^{2+} to produce a final concentration of 0·27 μM. At the low Ca^{2+} concentration of 0·3 mM, the heart was unable to perform external work against the aortic pressure head of 80 cm H_2O. Immediately after addition of the ionophore, left ventricular pressure increased slightly while ventricular output and external work increased (cf. Fig. 2). X-537A also increased both +dP/dt and −dP/dt (Fig. 4A), indicating that both the speed of contraction and the speed of relaxation were increased. Similar results were obtained by the addition of epinephrine (Fig. 4B). On the other hand, when contractility was augmented by an increase of the Ca^{2+} concentration from 0·3 to 0·5 mM (Fig. 4C), the speed of contraction was characteristically increased more than the speed of relaxation as evidenced by +dP/dt being greater than −dP/dt.

Further evidence in favor of the cardiotropic effects of X-537A being mediated through release of catecholamines rather than by a direct effect of increased Ca^{2+} entry across the plasma membrane induced by the ionophore is provided by the data shown in Fig. 5. The left ventricular pressure development of perfused rat heart can

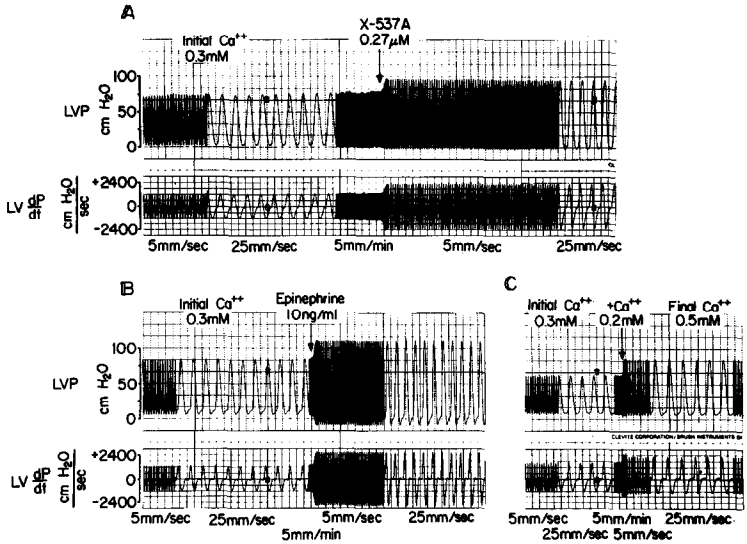

FIG. 4. Effects of X-537A, norepinephrine and Ca^{2+} on rat hearts perfused initially with Krebs bicarbonate medium containing 0·3 mM Ca^{2+}. The changes in left ventricular pressure (LVP) and dP/dt are shown after the addition of (A) 0·27 μM X-537A, (B) 10 ng/ml of epinephrine and (C) 0·2 mM Ca^{2+}. Hearts were perfused without recirculation of the fluid, which was alternately passed to the heart from three separate reservoirs.

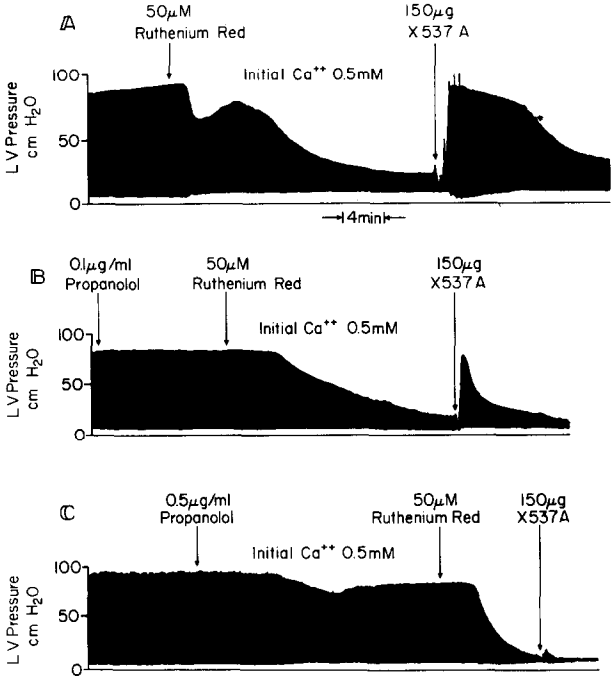

FIG. 5. Effect of X-537A after prior addition of ruthenium red on left ventricular pressure of perfused rat hearts in the absence (A) and in the presence of 0·1 μg/ml of propranolol (B), or 0·5 μg/ml of propranolol (C). Hearts were perfused without recirculation of the fluid. Ruthenium red and propranolol were added to the fluid reservoir above the heart to give the concentrations indicated, while X-537A was added as a single pulse immediately above the aortic cannula.

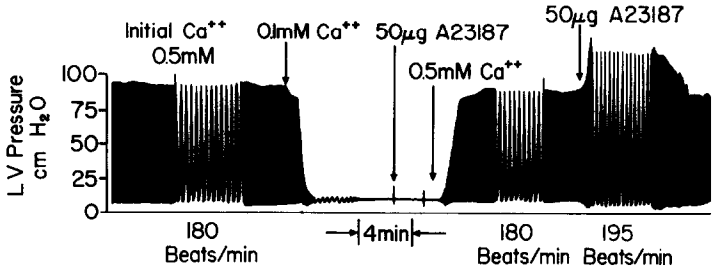

FIG. 6. Effect of A23187 on contractility of perfused rat heart. Flow-through perfusion of fluid containing either 0·5 or 0·1 mM Ca^{2+} was used. A23187 was added immediately above the aortic cannula.

be decreased by addition of ruthenium red, which effectively competes with Ca^{2+} for transport and/or binding at the level of the plasma membrane.[20,21] Figure 5 shows that the negative inotropic effect of ruthenium red was reversed by a single addition of 150 μg X-537A. In similar experiments, when contractility of perfused rat heart was decreased by ruthenium red, pressure development was fully restored by the addition of norepinephrine.[21] The response to X-537A was transient and was further diminished in magnitude by addition of 0·1 μg/ml of propranolol (Fig. 5B). Figure 5C shows that the addition of 0·5 μg/ml of propranolol, which produced a slight negative inotropic effect, completely prevented restoration of contractility upon addition of X-537A after ruthenium red treatment.

A23187, a second antibiotic which has been shown to be at least as effective as X-537A in transporting Ca^{2+} across biological membranes,[16,21] was found to have little effect on the contractility of perfused hearts. In an experiment similar to that of Fig. 1, a single addition of 50 μg A23187 was ineffective in restoring pressure development of rat hearts perfused with 0·1 mM Ca^{2+} (Fig. 6). At 0·5 mM Ca^{2+}, 50 μg A23187 produced a small and transient increase of left ventricular pressure and no effect on heart rate (Fig. 6). The dose–response curve of A23187 versus cardiac work, coronary flow rate and heart rate is shown in Fig. 7. Cardiac work increased by 25

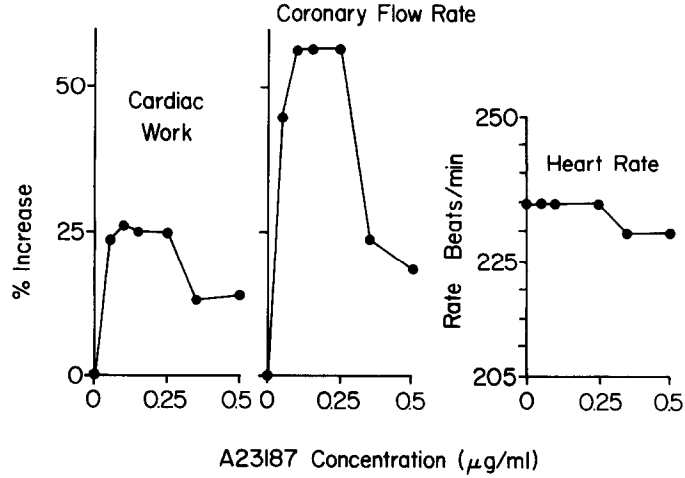

FIG. 7. Effect of increasing concentrations of A23187 on cardiac work, coronary flow rate and rate of rat hearts perfused with 150 ml Krebs bicarbonate medium containing 0·5 mM Ca^{2+}.

per cent at A23187 concentrations up to 0·25 μg/ml, while the coronary flow rate increased by 55 per cent. Little change in heart rate was observed up to 0·5 μg/ml of A23187. Therefore, of the two Ca^{2+} ionophores, X-537A clearly shows a more striking effect on the contractility of perfused hearts. However, this effect is only observed when endogenous stores of catecholamines are available and when β-receptor sites are not blocked by propranolol.

DISCUSSION

Interest in the growing series of compounds termed ionophores, which have the ability to transport ions across lipid barriers, arises partly because of their potential usefulness as tools in probing basic molecular events associated with transport and partly because their pharmacological effects on more complex systems may provide a basis for their use as therapeutic agents.[17] In order to realize the second goal, it becomes important not only to demonstrate that suitable effects can be obtained with isolated preparations and animals *in vivo*, but also to determine as far as possible the mechanism of the effects observed in a complex physiological environment. Recent studies from a number of laboratories have shown that the ionophore X-537A induced contraction of aortic strips, increased tension development and rate of contraction of perfused rabbit heart,[17,18] and increased left ventricular pressure, peak dP/dt, heart rate and cardiac output in normal anesthetized dogs,[19] while studies with isolated strips of rat diaphragm and isolated rabbit atrium showed that high concentrations of X-537A (5–20 μM) caused an increased resting tension and contraction in quiescent or contracting preparations.[24] Using lower concentrations of ionophore, the latter authors observed a positive inotropic effect of X-537A on electrically driven rabbit heart atria and guinea pig ventricular strips. On the other hand, the ionophore A23187 has only been reported to sensitize rabbit aorta to contraction induced by 10 mM Ca^{2+}.[17,18] The present studies confirm our earlier observations[20] that X-537A has a powerful positive inotropic and chronotropic effect on the isolated working rat heart when added at low concentrations, and show that this preparation responds very differently to the two divalent cation ionophores.

The principal reason for the fact that only X-537A behaves as a cardiotropic agent probably relates to the finding that X-537A, besides facilitating transport of divalent cations across biological membranes, also has a selective affinity for complex formation with norepinephrine, thereby aiding its transport through nonpolar solvents.[17,18,21] The ionophore A23187 does not have this additional property.[21] Our results are consistent with the proposal that the effects of X-537A observed with the isolated perfused heart preparation are mediated through release of norepinephrine from endogenous stores. This is most clearly shown by the suppression or marked attenuation of the effects of X-537A on cardiac work, coronary flow rate and heart rate in rats pretreated with reserpine. It is also of interest that no effects of X-537A could be observed in frog hearts, which naturally lack catecholamine stores. Furthermore, the effects of X-537A in the working rat heart or in hearts pretreated with ruthenium red to inhibit Ca^{2+} entry through the sarcolemma[21] were essentially abolished in the presence of the β-blocking agent propranolol. A characteristic of epinephrine or norepinephrine effects on cardiac muscle is that they increase not only the speed of contraction but also decrease the duration of the active state and increase the speed of relaxation.[25] The similarity of the effects of X-537A and catecholamines

on cardiac contractility is shown in the present experiments by observed increases of both $+dP/dt$ and $-dP/dt$ with these agents, while the increased left ventricular pressure development associated with increased cardiac work induced by an increase of Ca^{2+} concentration had a more pronounced effect on $+dP/dt$ than on $-dP/dt$. On the other hand, the main effect observed upon addition of A23187 to the perfused rat heart was a 50 per cent increase of coronary flow and a small increase of cardiac work which could be secondary to the vasodilator effect. No effects of A23187 were observed in hearts pretreated with ruthenium red. It may be concluded, in agreement with the report by Levy *et al.*,[24] that the cardiotropic properties of X-537A *in vitro* and probably also *in vivo* are secondary to release of endogenous catecholamines, and do not represent a direct effect of the ionophore on increased permeability of the plasma membrane or sarcoplasmic reticulum membrane to Ca^{2+}. However, it is not justified to conclude that all effects of the divalent cation ionophores on different types of muscle are mediated by catecholamine release. At higher concentrations of X-537A, contracture of isolated muscle preparations has been reported,[24] which would indicate an abnormally high intracellular Ca^{2+} concentration caused possibly by increased Ca^{2+} entry across the plasma membrane or release of Ca^{2+} from sarcoplasmic reticulum. In the present experiments, no evidence for impaired relaxation of the working rat heart could be obtained by an increase of X-537A concentration, although diminished positive inotropic effects were observed possibly due to the depressant effect of ethanol on the contractile proteins.[26]

A discussion of the mechanism by which X-537A releases norepinephrine from storage vesicles is limited by the present lack of detailed knowledge of the regulation of catecholamine uptake and release.[27] X-537A may induce norepinephrine release either indirectly by increasing the permeability of the catecholamine storage granule membrane to Ca^{2+}[28] or directly by facilitating norepinephrine transport across the lipid phase of the membrane. Preliminary studies with isolated chromaphin granules have indicated that X-537A stimulates the release of norepinephrine, suggesting that its effect is by facilitation of norepinephrine transport, but a possible primary or secondary role of Ca^{2+} has not been eliminated.

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