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Effect of the Cardioactive Drug AR-L 57 on Free Intracellular Calcium and Ouabain-Insensitive Ion Fluxes in the Squid Giant Axon

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SUMMARY

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The cardioactive drug AR-L 57 [6-(2,4-dimethoxyphenyl)-imidazo-(4,5-β)-pyridine] causes a rapid and reversible reduction of calcium efflux and light output from axons of *Loligo forbesi* which received microinjections of both ^{45}Ca and the calcium-sensitive photoprotein aequorin. The light output from aequorin injected into extruded axoplasm is also reduced by AR-L 57, but the drug does not inhibit the light from an aqueous buffer solution containing aequorin. The effect on calcium efflux is prevented if the axon is injected with enough calcium-ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid to buffer the intracellular ionized calcium close to the physiological level. The large Na_0- and Ca_0-dependent calcium efflux from axons poisoned with cyanide or carbonyl-cyanide *p*-trifluoromethoxyphenyl-hydrazone is insensitive to AR-L 57. These results indicate that AR-L 57 inhibits the calcium efflux from unpoisoned axons by increasing intracellular calcium binding. AR-L 57 has no noticeable direct effect on the axolemmal calcium pump of unpoisoned axons or on the calcium efflux system operating in poisoned axons. AR-L 57 inhibits the calcium influx from lithium-artificial seawater and the Ca_0-dependent sodium efflux from axons injected with ^{22}Na and superfused with sodium-poor (lithium or potassium) seawater. The light response evoked in axons injected with aequorin by a potassium depolarization in the presence of a high external calcium concentration is reversibly reduced by AR-L 57. It is concluded that AR-L 57 inhibits calcium entry both via sodium-calcium exchange and through the voltage-sensitive late calcium channel. AR-L 57 does not affect the ^{86}Rb efflux at 10 mmoles per liter $[K]_0$ but reversibly inhibits the increased rubidium efflux from axons depolarized by 410 mmoles of potassium per liter. The outward (potassium) current is inhibited by the drug both with time and voltage during a voltage-clamp depolarization, whereas the transient inward (sodium) current is unaffected. However, AR-L 57 does inhibit the maintained tetrodotoxin-sensitive ^{22}Na efflux through sodium channels kept open by veratridine. The normal sodium inactivation mechanism appears to protect the sodium channels from blockade by AR-L 57. None of the effects on the axolemma reported here, if applied to the sarcolemma of myocardial cells, provides an obvious explanation for the cardiotonic action of AR-L 57. Although AR-L 57 has been shown previously to inhibit the sodium pump, the present results show that the drug affects a number of ion transport processes that are insensitive to cardiac glycosides.

INTRODUCTION

AR-L 57[3] is a cardiotonic drug (1) that shares with the cardiotonic steroids an inhibitory effect on the sodium pump. We have characterized this effect using the squid giant axon as a model (2). A possible link between sodium pump inhibition and increased cardiac contractility—an Na_i-dependent calcium influx—was demonstrated in this cell type 10 years ago (3). The striking selectivity of action of the cardiac glycosides has led many investigators to suggest that inhibition of the sodium pump of myocardial sarcolemma constitutes the essential first step in a chain of events which ultimately leads to a positive inotropic effect in the presence of these drugs (e.g., ref. 4).

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[3] The abbreviations used are: AR-L 57, 6-(2,4-dimethoxyphenyl)-imidazo-(4,5-β)-pyridine hydrochloride; ASW, artificial seawater (see Table 1); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetra-acetic acid; FCCP, carbonylcyanide *p*-trifluoromethoxyphenyl-hydrazone; TTX, tetrodotoxin.

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Since there are many other conceivable interventions that could augment the intracellular calcium transient responsible for cardiac contraction, it seemed desirable to test the nonsteroidal drug AR-L 57 on cellular functions other than active sodium transport. Part of the present work deals with the effects of AR-L 57 on the level of ionized calcium within the squid axon and on transaxolemmal calcium fluxes. Effects on the voltage-dependent sodium and potassium channels are also reported. Although our previous results (2) suggest that AR-L 57 differs from ouabain in the molecular mode of inhibition of the sodium pump, the present paper shows that the synthetic drug differs in another important respect from the cardiotonic steroids; it affects a number of ion transport processes that are insensitive to ouabain. Some of the results have been communicated to the British Physiological and German Pharmacological Societies (5, 6).

METHODS

Material. Giant axons from *Loligo forbesi* were isolated and cleaned by the usual methods. The axons, 530 to 1160 μm in diameter, were mainly obtained from refrigerated mantles, although live squid were used in a few experiments. The experiments were performed at room temperature (18°–23°).

Tracer efflux. The general procedures which involved loading the axon with radioactive isotope by intracellular microinjection and mounting it in a glass tube have been described in a preceding paper (2). All reported experiments were performed using axons that were excitable at the end of the experiment with the exception of one axon exposed to cyanide and calcium-poor ASW and all axons treated with veratridine. For the measurement of calcium efflux the axon was injected with approximately 5×10^5 cpm of $[^{45}Ca]CaCl_2$; this raised the axoplasmic calcium concentration by approximately 100 μmoles/liter. ^{45}Ca in the effluent was counted by liquid scintillation spectrometry using a mixture of toluene and Triton X-100. At the end of the experiment, the axon was digested with solvent (Nuclear Chicago Corporation, Chicago, Ill.) and counted. Rubidium efflux was measured from axons injected with up to 10^6 cpm of $[^{86}Rb]RbCl$; this resulted in a final axoplasmic rubidium concentration of up to 5 mmoles/liter. ^{86}Rb was counted with a γ spectrometer. Effluxes are expressed in units of fraction of the total activity lost per min.

Calcium influx. Procedures for the measurement of ^{45}Ca influx were similar to those described in the preceding paper (2) for the measurement of ^{86}Rb influx. The period of exposure to ^{45}Ca was 30 min.

Aequorin experiments. After the axon had been cleaned and cannulated, 0.18 μl of aequorin solution was injected into the axon over a length of 10 mm. After the injection, an equilibration time of at least 30 min was allowed. As in the efflux experiments the axon was mounted in a glass tube and continuously superfused with ASW. The glass tube containing the axon was positioned in front of a photomultiplier tube (EMI type 9635 B), and the set-up was enclosed in a light-tight box. Further details of the method have been described (7). In some experiments both light output and tracer efflux were measured simultaneously from an axon. For this purpose the aequorin was injected such that it overlapped the patch of previously injected ^{45}Ca. In experiments designed to study calcium entry through the late calcium channel activated by depolarization, the high-potassium ASW was applied by flowing 50 ml through the glass tube at a flow rate of about 2 ml/sec as described previously (8). To test for a possible direct effect of AR-L 57 on the aequorin, a solution of aequorin was sealed inside a porous cellulose acetate tube 800 μm in diameter (9). The tube was filled and superfused with a solution containing (millimoles per liter) KCl, 500; potassium phosphate (pH 7.2), 20; EGTA, 5; $CaCl_2$, 2. AR-L 57 was added to the superfusion solution.

Experiments on extruded axoplasm. Axoplasm was extruded with a glass rod and taken up by suction into a Pyrex glass capillary tube geometrically similar to the axon. The isolated axoplasm was injected with aequorin and the light output was recorded. AR-L 57 was applied by injecting it such that it overlapped the patch of aequorin in the axoplasm.

Electrical measurements. The resting transmembrane potential was recorded in a few experiments by inserting axially a micropipette 50 μm in diameter and filled with 0.6 mole/liter of KCl. In these experiments the axon was mounted horizontally in a chamber that allowed cannulation of both ends of the axon and continuous superfusion of the middle part. In one experiment, intracellular pH was measured by inserting a pH-sensitive glass electrode in addition to the voltage-recording electrode and through the opposite cannula. The pH-sensing electrode was made by melting a capillary of pH-sensitive glass (Corning 0150), 150 μm in outside diameter and 5 mm in length, on to the open tip of a Pyrex glass capillary tube with an outside diameter of 90 μm. The tip of the pH-sensitive glass capillary was sealed with a flame. The pH was measured by using the intracellular potential-recording electrode as reference electrode. The voltage-clamp experiment shown in Fig. 10 was kindly performed by Dr. G. C. Malachowski and Dr. D. van Helden. The axon was cleaned, and most of the axoplasm was removed by carefully coring the axon with a glass capillary tube. The axon was then continuously superfused with ASW and perfused intracellularly with a solution containing (millimoles per liter) KF, 300; sucrose, 400; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10; pH 7.6. The apparatus described in ref. 10 was used. The voltage-clamp response time was less than 1 μsec. Series resistance was not compensated, and the (very small) leakage current was not subtracted. The temperature of the experimental region of the axon was controlled to within 1°.

Solutions and drugs. The main external solutions are listed in Table 1. ASW to which no calcium had been added was contaminated with calcium by approximately 10 μmoles/liter as determined with atomic absorption spectroscopy. AR-L 57 was obtained from Dr. Karl Thomae GmbH, Biberach/Riss, West Germany; FCCP from Boehringer Mannheim, Mannheim, West Germany; lithium chloride from Fisher Scientific Company, Pitts-

TABLE 1

Composition of main external solutions

All concentrations in millimoles per liter. All solutions were pH 7.8.

Reference	Sodium	Potassium	Lithium	Choline	Magnesium	Chlorine	HCO_3	Calcium
Sodium-ASW	402.5	10			100	630	2.5	10
Potassium-ASW	2.5	410			100	630	2.5	10
O-Ca(K)	2.5	410			100	610	2.5	
Lithium-ASW	2.5	10	400		100	630	2.5	10
O-Ca(Li)	2.5	10	400		100	610	2.5	
112-Ca(K)	2.5	410				634	2.5	112
112-Ca(choline)	2.5	10		400		634	2.5	112

burgh, Pa.; TTX from Sankyo, Ltd., Tokyo, Japan; veratridine from EGA Chemie GmbH, Steinheim/Albuch, West Germany; and radiochemicals from the Radiochemical Centre, Amersham, England. The aequorin was a gift of Dr. O. Shimomura.

RESULTS

Effect of AR-L 57 on Calcium Efflux

AR-L 57 produced a prompt and reversible reduction of ^{45}Ca efflux up to approximately 40% of the total calcium efflux. Figure 1 illustrates this effect (*inset*) and shows the concentration-response relationship using data from six axons. The steepness of the log concentration-response relationship appears to be greatest between 10 and 100 μmoles/liter and is similar in this respect to the curve for the inhibition of the sodium pump by AR-L 57, which is also shown in Fig. 1. However, the effect on calcium efflux is not coupled to the reduction of sodium pump activity because it persisted after complete inhibition of the sodium pump with ouabain, 10 μmoles/liter: AR-L 57 (100 μmoles/liter) reduced calcium efflux reversibly by 32% under this condition (one experiment). Additionally, ouabain itself is known to lack an effect on the calcium efflux from squid axons (11, 12).

To determine whether AR-L 57 reduced calcium efflux by promoting intracellular calcium binding, both calcium efflux and the concentration of intracellular ionized calcium were monitored from an axon injected with ^{45}Ca and the calcium-sensitive photoprotein aequorin. As shown in Fig. 2, the reversible reduction of calcium efflux induced by AR-L 57 was accompanied by a reversible decrease in the light output from the axon. Since AR-L

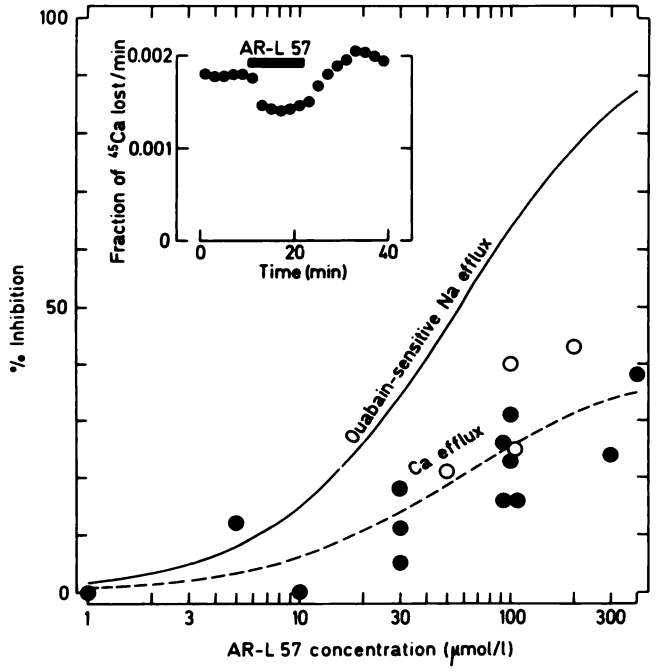

FIG. 1. *Inhibition of calcium efflux by AR-L 57*

The ordinate denotes the percentage inhibition of either ouabain-sensitive sodium efflux (2) or total ^{45}Ca efflux from axons superfused with sodium-ASW (●, data from four axons) or to the same solution with calcium reduced to 50 μmoles/liter (○, data from two axons). Axon diameters 550–830 μm; temperature 18°–23°. – – –, a first-order curve fitted by eye to the calcium efflux points and obtained simply by scaling the sodium pump inhibition curve down to 0.4. It serves to illustrate the similar position of the two concentration-response relationships. *Inset*, the time course of the inhibition of calcium efflux by 100 μmoles/liter of AR-L 57 during a single experiment (axon diameter 700 μm; temperature 22°).

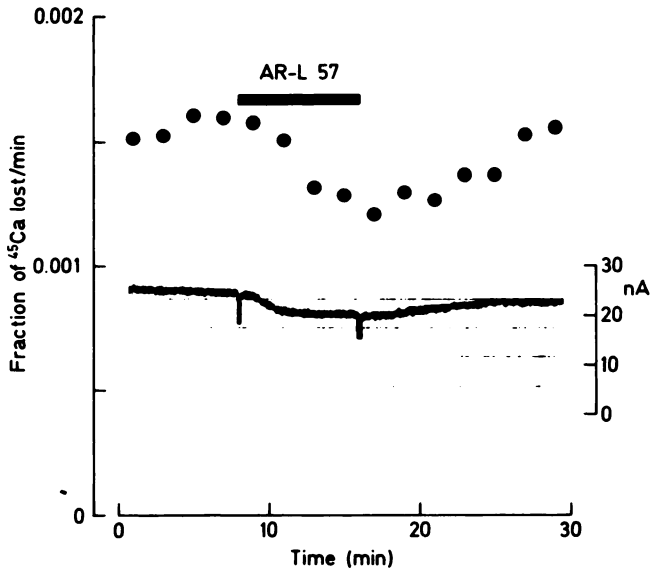

FIG. 2. *Simultaneous inhibition by AR-L 57 of calcium efflux and light emitted by an axon injected with aequorin*

The left ordinate applies to the points and the right ordinate, which gives the output of the photomultiplier tube in nA, applies to the continuous trace. The aequorin was injected such that it overlapped the patch of ^{45}Ca. The axon was continuously superfused with a sodium-ASW containing 50 μmoles/liter of calcium. AR-L 57 was applied at 100 μmoles/liter during the time indicated. The sharp downward deflections of the current trace are artifacts caused by the air bubbles which separated drug-free from drug-containing solutions. Axon diameter 850 μm; temperature 23°.

57 did not inhibit the aequorin reaction directly (Fig. 3A), this observation indicates that the drug reduces the free calcium concentration in the axoplasm. In the presence of a reduced calcium efflux, such an effect could result from a reduction of calcium influx or from increased axoplasmic calcium binding. The former interpretation is unlikely because AR-L 57 reduced the light output at strongly reduced $[Ca]_o$ (Fig. 2), and the latter explanation is further strengthened by the finding that axoplasm which was extruded from an axon into a glass capillary tube and injected with aequorin responded with a decrease in light output to the application of AR-L 57 (Fig. 3B). Similar relative reductions of the light output as shown in Fig. 2 were observed in two other axons superfused with sodium-ASW containing calcium, 10 mmoles/liter.

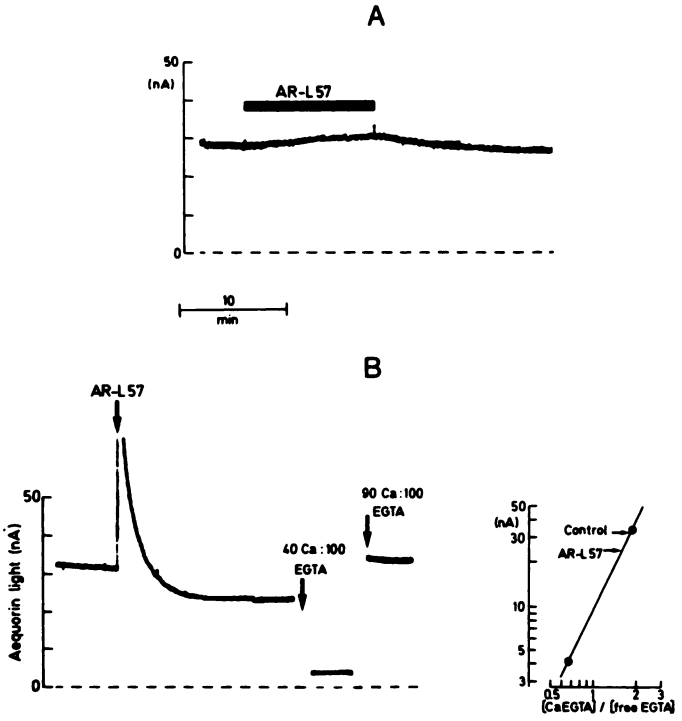

FIG. 3. *Effect of AR-L 57 on the light output from an aequorin solution sealed in a porous tube (A) and from isolated axoplasm injected with aequorin (B)*

A. The porous tube containing aequorin (see Methods) was superfused with a solution containing 100 μmoles/liter of AR-L 57 during the period indicated.

B. The axoplasm was extruded into a Pyrex glass capillary tube 800 μm in diameter. AR-L 57 was injected to a final concentration of 360 μmoles/liter. The initial rise in light output is attributed to calcium contamination of the injectate. It was also observed after injection of 0.5 moles/liter of KCl. Note that the light output stabilized at a lower level. Subsequently the aequorin reaction of this axoplasm sample was calibrated by injecting two different known mixtures of $CaCl_2$ and EGTA (pH 7.2). Final concentrations of EGTA were 7.2 and 14.4 mmoles/liter, respectively. The ordinate of the double logarithmic plot is the photomultiplier output in nA (linearly related to the rate constant for aequorin consumption under the conditions used) and the abscissa the ratio of [calcium-EGTA]/[free EGTA]. The straight line drawn through the points has a slope of 2, confirming the square-law relationship between light production and ionized calcium concentration (7). On the basis of the calibration curve, AR-L 57 reduced the free calcium concentration by 14% in this axoplasm sample.

If the reduction of Ca efflux induced by AR-L 57 is strictly related to the decrease of free axoplasmic calcium, buffering the latter should make calcium efflux resistant to this action of the drug. Figure 4 shows the calcium efflux from an axon which had been injected with a solution containing 200 mmoles/liter of EGTA and 80 mmoles/liter of $CaCl_2$, a procedure that was expected to stabilize the ionized calcium concentration close to that which normally exists inside squid axons (7). Although this axon showed the typical sensitivity to removal of external sodium (13) and to poisoning with cyanide (13, 14), the calcium efflux did not respond to a high concentration of AR-L 57. The same lack of effect of AR-L 57, up to 200 μmoles/liter, was observed in a second axon. Thus both the Na_o-dependent and the uncoupled component of calcium efflux which constitute the calcium efflux from unpoisoned squid axons (15) appear not to be directly affected by AR-L 57.

Depleting squid axons of ATP leads to a marked increase of calcium efflux, and this flux is almost entirely dependent on either Ca_o or Na_o (12). Figure 5 shows an experiment in which the axon was poisoned with cyanide. First, in the absence of cyanide, AR-L 57 reduced calcium efflux as expected. During poisoning with cyanide, calcium efflux increased approximately 10-fold. Although it was typically inhibited by the withdrawal of external cations, it was unaffected by AR-L 57 in concentrations up to 400 μmoles/liter. In another axon (not illustrated) the calcium efflux increased 47-fold following a 1-hr exposure to FCCP (8 μmoles/liter). In the continued presence of FCCP, the addition of 200 μmoles/liter of AR-L 57 had no effect, while exposure to *O*-Ca(Li)-ASW reduced the calcium efflux by 83%. Thus, ATP depletion apparently prevents the ability of AR-L 57 to modulate calcium efflux by affecting axoplasmic calcium binding, and the cation-dependent calcium efflux itself, which operates under this condition, is insensitive to the drug.

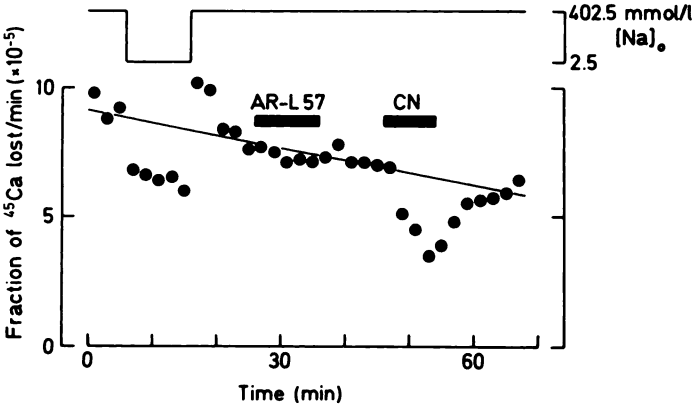

FIG. 4. *Lack of effect of AR-L 57 on calcium efflux under conditions of constant free intracellular calcium concentration*

The axon was injected over a length of 3 cm with a mixture of 200 mmoles/liter of EGTA and 80 mmoles/liter of $CaCl_2$ to a final axoplasmic concentration of 13 mmoles/liter of EGTA; this column overlapped the subsequently introduced patch of ^{45}Ca by approximately 1 cm at each end. The external solution was sodium-ASW or choline-ASW with calcium reduced to 50 μmoles/liter throughout in order to avoid changes of the specific activity of intracellular calcium. AR-L 57 was applied at 100 μmoles/liter, NaCN at 2 mmoles/liter. Axon diameter 600 μm; temperature 17°.

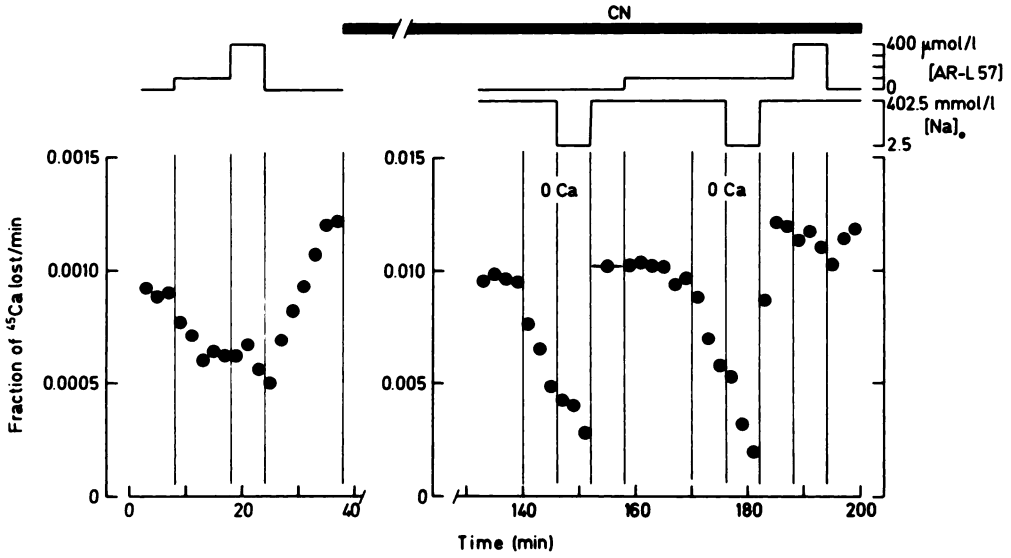

FIG. 5. *Lack of effect of AR-L 57 on calcium efflux after poisoning with cyanide*

The axon was superfused with sodium-ASW, *O*-Ca(Na)-ASW or *O*-Ca(Li)-ASW as indicated. Before the addition of cyanide, AR-L 57 produced a clear inhibition of calcium efflux. After 100 min of exposure to NaCN (2 mmoles/liter), the calcium efflux had increased to approximately 10 times its original value (see the corresponding ordinate) and was sensitive to removal of external calcium and sodium AR-L 57 (up to 400 µmoles/liter) did not inhibit the calcium efflux in the presence of cyanide and did not alter the Ca_o- and Na_o-dependent fractions of calcium efflux. Axon diameter 550 µm; temperature 21°.

Effect of AR-L 57 on Components of Calcium Influx

Apart from the intermittent inward leak of calcium through voltage-sensitive calcium channels (see below), there is also a component of calcium inflow that is coupled to sodium efflux and particularly large in axons immersed in sodium-poor media (3). To determine whether AR-L 57 affects this system, two series of experiments were carried out. In one series, a pair of axons was dissected from the same squid and the ^{45}Ca influx from lithium-ASW, measured in one fiber, was compared with that from lithium-ASW containing 200 µmoles/liter of AR-L 57 in the second fiber. As shown in Fig. 6A, AR-L 57 significantly reduced calcium influx. This reduction amounted to 57% ± 8% (SE) of the value in the paired control axon. In the second series, AR-L 57 was applied to axons in which the Ca_o-dependent sodium efflux had been activated. An example is illustrated in Fig. 6B. The increase in sodium efflux following the addition of 10

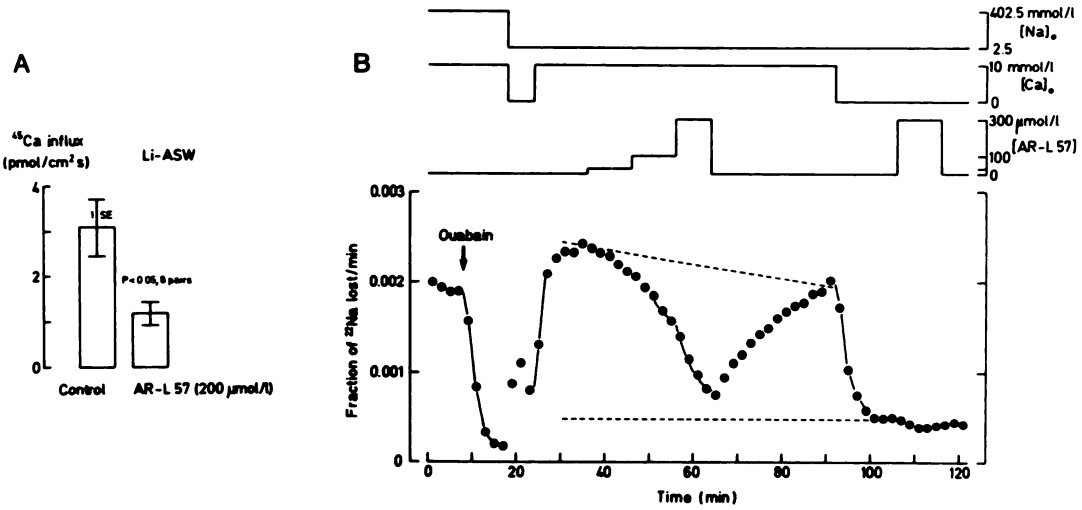

FIG. 6. *Inhibition of sodium-calcium exchange by AR-L 57*

A. Ca influx: ^{45}Ca was added to lithium-ASW in tracer amounts. In each of the eight pairs of axons, one member served as control and the other member of the pair was incubated in lithium-ASW containing 200 µmoles/liter of AR-L 57. Mean values ± standard error are shown. Temperature 20°.

B. Na efflux: the axon was superfused at first with sodium-ASW and later with calcium-free or calcium-containing potassium-ASW as indicated. At the *arrow* ouabain was added at 10 µmoles/liter and applied throughout the remainder of the experiment. - - - encloses the fraction of sodium efflux activated by 10 mmoles/liter of Ca_o under control conditions. The concentrations of AR-L 57 are shown. Axon diameter, 875 µm; temperature 19°.

mmoles/liter of calcium to the calcium-free potassium-ASW containing ouabain represents the sodium efflux component of sodium-calcium exchange. AR-L 57 inhibited this flux in a concentration-dependent and reversible manner. Although AR-L 57 appears to be less effective in reducing Ca_o-dependent sodium efflux as compared with ouabain-sensitive sodium efflux at 30 and 100 μmoles/liter (cf. Fig. 1), this difference may not be real, because the effect had not reached a steady state during the 10-min exposure periods chosen for these concentrations in the experiment of Fig. 6. The inhibitory effect on Ca_o-dependent sodium efflux was confirmed in two other axons immersed in lithium-ASW. In these experiments 200 and 300 μmoles/liter of AR-L 57 reduced this flux by 82% and 92%, respectively.

Since the Ca_o-dependent sodium efflux is known to be sensitive to intracellular pH in squid axons (16), it appeared possible that AR-L 57 acted by acidifying the cell interior. However, the measurement of pH_i in one axon exposed to 300 μmoles/liter of the drug for 10 min revealed no change, whereas application of CO_2 (ASW equilibrated with a mixture of 5% CO_2 and 95% O_2) resulted in a reduction of pH_i both before and after application of AR-L 57 by 0.29 and 0.25 pH unit, respectively, from a control value of 7.55.

Calcium entry through the late calcium channel (7) was investigated by depolarizing an aequorin-injected axon with potassium in the presence of high external calcium and recording the light output from the axon. As is evident from Fig. 7, AR-L 57 at 300 μmoles/liter reduced nearly completely the aequorin response to depolarization, and the effect was reversible.

Effect of AR-L 57 on Voltage-Sensitive Sodium and Potassium Channels

Resting membrane potential. In three axons the resting membrane potential remained unchanged to within 1 mV when AR-L 57 (200–300 μmoles/liter) was applied in sodium-ASW for 10–20 min.

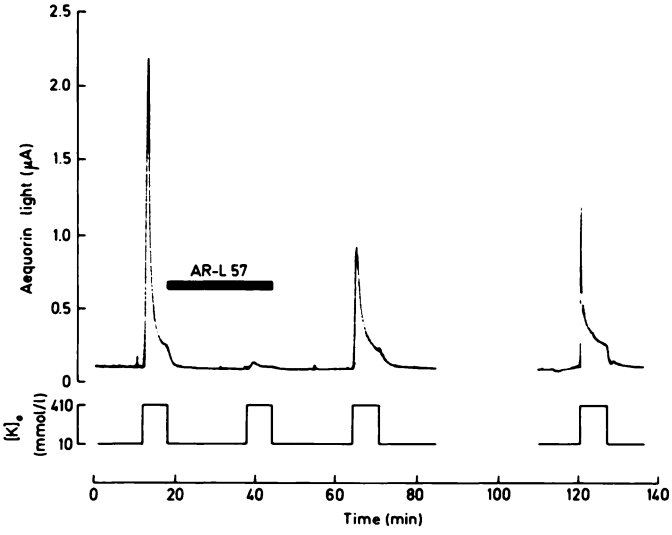

FIG. 7. *Effect of AR-L 57 on the aequorin response to potassium depolarization*

The axon was superfused with 112-Ca(choline)-ASW or 112-Ca(K)-ASW as indicated. AR-L 57 was applied at 300 μmoles/liter. Axon diameter, 1160 μm; temperature 22°.

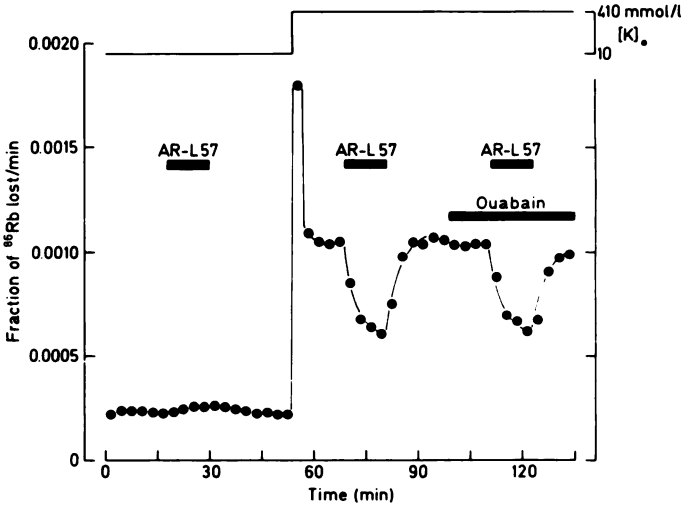

FIG. 8. *Inhibition of rubidium efflux by AR-L 57*

The axon was initially superfused with sodium-ASW and later with potassium-ASW in order to activate potassium channels by depolarization. AR-L 57 was applied at 300 μmoles/liter, ouabain at 10 μmoles/liter. Axon diameter, 750 μm; temperature 22°.

Inhibition of potassium channels. The effect of AR-L 57 on potassium channels was studied in two ways, by measuring ^{86}Rb efflux from depolarized axons and by the voltage-clamp technique. Rubidium ions may be used as a potassium analogue in the study of potassium channels (17). Axons injected with ^{86}Rb showed an efflux that increased progressively when the external potassium concentration was raised to 100 mmoles/liter (two axons, not illustrated) or 410 mmoles/liter (Fig. 8). The increased rubidium efflux was insensitive to a concentration of ouabain that is known to block the sodium pump in potassium-ASW (18), and the increase also occurred if the ouabain treatment had been started prior to the replacement of sodium-ASW with potassium-ASW (the latter protocol was followed in the rubidium efflux experiment underlying Fig. 9). The K_o-dependent and ouabain-insensitive rubidium efflux is therefore interpreted as a flux through potassium channels opened by depolar-

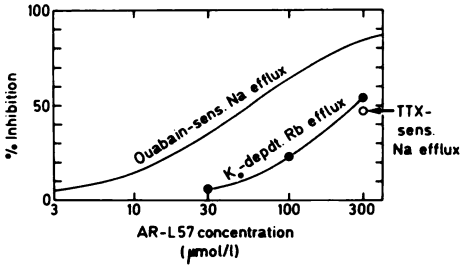

FIG. 9. *Concentration-response relationship for the inhibition of the K_o-dependent rubidium efflux by AR-L 57*

The K_o-dependent rubidium efflux was obtained by subtracting the rubidium efflux into sodium-ASW from the total rubidium efflux into potassium-ASW. ●, steady-state effects determined in an ouabain-treated axon (diameter 820 μm; temp. 20°). For comparison, the inhibition of sodium efflux through sodium channels opened by veratridine (Fig. 11) is also included (○). The curve for the inhibition of the ouabain-sensitive sodium efflux is taken from a preceding paper (2).

ization. As shown in Fig. 8, AR-L 57 had no effect on the rubidium efflux into sodium-ASW, but reversibly inhibited the increased rubidium efflux into potassium-ASW in both the absence and presence of ouabain. This finding indicates that the drug blocks potassium channels. The concentration-response relationship obtained under steady-state conditions for this inhibitory effect from another axon is depicted in Fig. 9. In comparison with the effect on the sodium pump, the curve is shifted to the right by a factor of 4.5.

Voltage-clamp experiments (kindly performed by Dr. G. C. Malachowski and Dr. D. van Helden) gave further insight into the behavior of potassium channels under the influence of AR-L 57. As is evident from the membrane currents shown in Fig. 10A, the internal application of 300 μmoles/liter of AR-L 57 had virtually no effect on the transient inward (sodium) current or on the small outward (potassium) current elicited by the depolarization from −60 to −10 mV. The sodium current was unchanged up to the highest tested depolarization (+50 mV). When the outward current was increased by imposing a depolarization to +50 mV (Fig. 10B), AR-L 57 reversibly reduced the maximal outward current by 20% (here, TTX was added to the ASW to avoid any interference from the sodium current). Depolarizing pulses of 20 msec duration were used in this experiment, and Fig. 10C shows the complete time course of those membrane currents whose initial part is shown in Fig. 10B on an expanded time scale. In the absence of AR-L 57, the outward currents declined slightly during the depolarizing pulse. This decline was much more pronounced in the presence of AR-L 57, indicating a block of potassium channels that increases gradually after the potassium channels have been opened by the step depolarization.

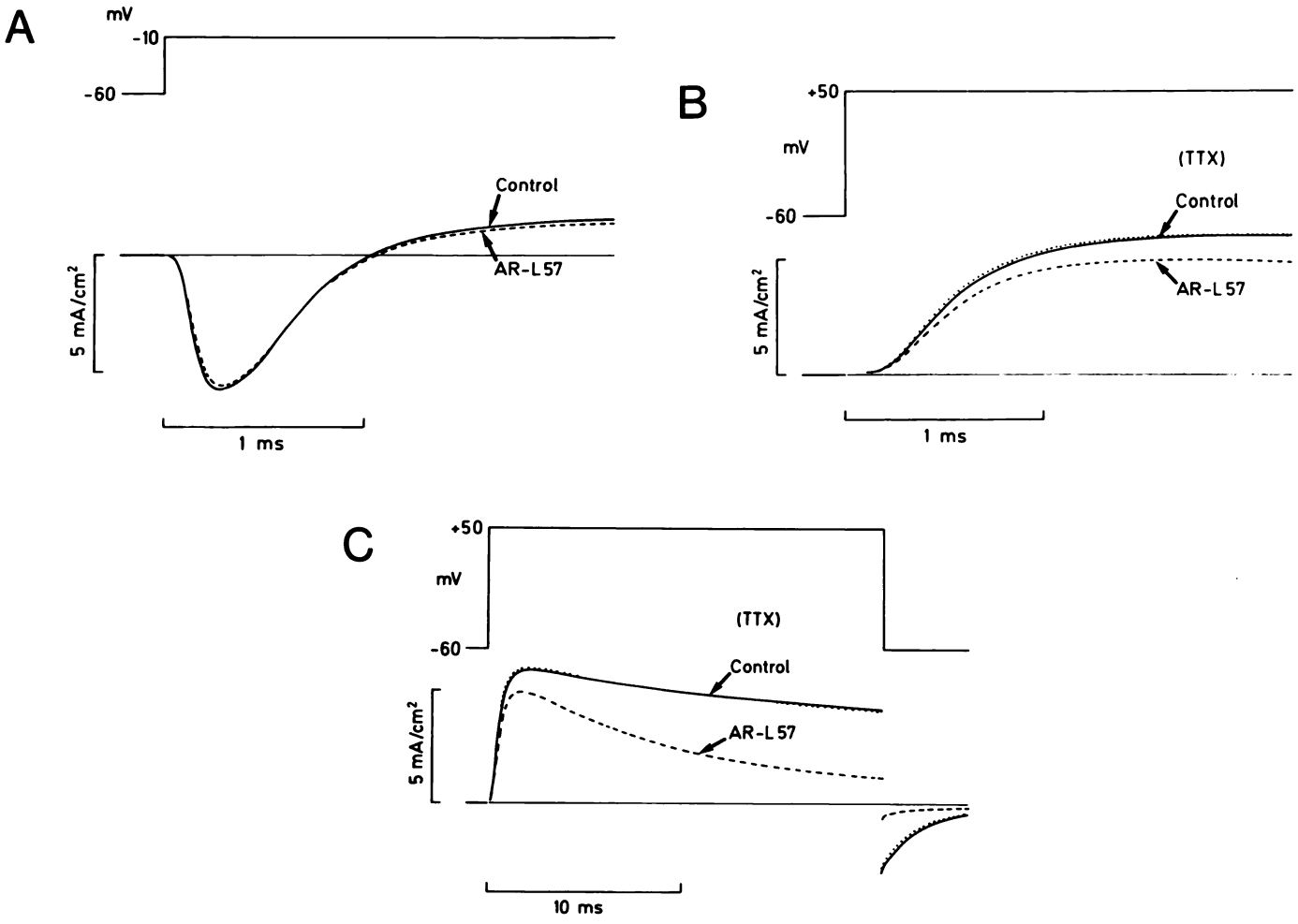

FIG. 10. *Effect of AR-L 57 on voltage-clamp currents*

Superimposed tracings of membrane current from an axon superfused with sodium-ASW and perfused intracellularly (see Methods). Axon diameter 650 μm; temperature 16°. Tracings were taken from computer printouts with a time resolution of 25 μsec. ——, membrane current under control conditions; – – –, records obtained during internal application of 300 μmoles/liter of AR-L 57 and after stabilization of the effect; · · · ·, the membrane current after washout of AR-L 57. Inward current is downward.

A. This depolarization elicited the maximal inward current. Control membrane current and that obtained after washout of AR-L 57 were superimposable.

B and C. Membrane currents in the presence of 1 μmole/liter TTX externally. B represents the initial part of the record shown in C on a compressed time scale. The axon was pulsed at the low rate of 0.67 Hz in order to avoid cumulative effects of AR-L 57 on the outward current (see Results).

As evident from Fig. 10C the degree of reduction of the outward current increased from 20% at the time of the maximal outward current to 72% at the end of the 20-msec pulse. At the end of a 20-msec depolarization to −20 mV (not illustrated), the outward current was reduced by only 57%, indicating a voltage-dependence of the blocking action of AR-L 57. The recovery from block was studied in the experiment of Fig. 10 by applying test depolarizations to +50 mV at various intervals after a conditioning 20-msec pulse to the same potential (not shown). The maximal outward current elicited 10 msec after the end of the conditioning pulse was depressed by 74% as compared with the maximal outward current evoked by the conditioning pulse. The subsequent recovery of this current at a holding potential of −60 mV between pulses occurred monoexponentially with a time constant of 126 msec. This value was obtained after correction for a small depression of the outward currents which occurred in the absence of AR-L 57 after an identical conditioning depolarization in this fiber.

In the experiment shown in Fig. 10, intracellular application of 300 μmoles/liter of AR-L 57 was much more effective than extracellular application of the same concentration, and the addition of the drug to the ASW during internal application of AR-L 57 had no further effect. In a second experiment we observed the reverse situation, i.e., externally applied AR-L 57 was more effective than internally applied drug. In this axon, a thick layer of axoplasm had remained, effecting a relatively large unstirred layer at the inner face of the axolemma. These observations indicate that loss of AR-L 57 across the membrane may prevent the establishment of an effective concentration at the axolemma in internally perfused axons.

Inhibition of sodium channels opened by veratridine. The insensitivity of the transient inward current to AR-L 57 (Fig. 10 A) might be related to the sodium inactivation mechanism which is known to hinder the access of certain potassium channel blocking compounds to sodium channels (19). To check this point we applied veratridine which is known to slow the activation and remove the inactivation (on sustained depolarization) of sodium channels (20) and measured ^{22}Na efflux through these modified channels. One experiment is illustrated in Fig. 11. To avoid contamination by other sodium efflux components known to be inhibited by AR-L 57, sodium-potassium exchange was blocked by ouabain and Ca_o-dependent sodium efflux by using calcium-free external medium. In order to obtain a high and constant degree of activation of the sodium channels in the presence of veratridine, the axon was depolarized with potassium and the external sodium concentration was adjusted close to the presumed intracellular value. The latter procedure was expected to minimize membrane potential changes due to an eventual blockade of sodium channels. Under these conditions the ^{22}Na efflux was expected to be linearly related to sodium permeability. As shown in Fig. 11, most of the sodium efflux in the presence of veratridine was sensitive to TTX, i.e., it represents sodium flux through sodium channels. AR-L 57 was found to inhibit the TTX-sensitive part of sodium efflux reversibly by nearly 50%. A similar effect (inhibition by 47%) was observed in a second axon in the presence of external calcium and after correction for the superimposed inhibition of Ca_o-dependent sodium efflux.

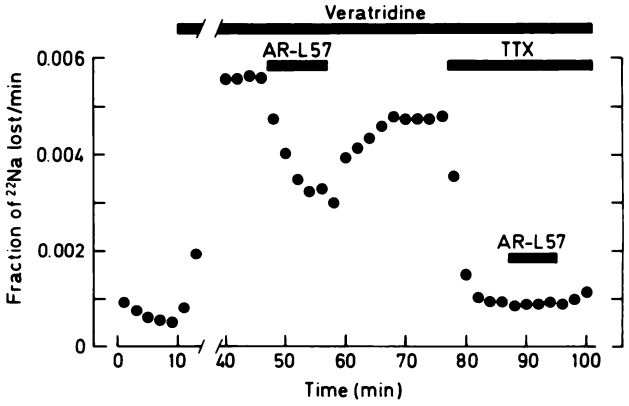

FIG. 11. *Inhibition of veratridine-induced sodium efflux by AR-L 57*

The axon was superfused with a solution containing (in millimoles per liter) NaCl, 70; KCl, 330; $MgCl_2$, 100; calcium, not added; $NaHCO_3$, 2.5; ouabain, 0.01. Veratridine was applied at 100 μmoles/liter, AR-L 57 at 300 μmoles/liter, and TTX at 0.3 μmole/liter. Axon diameter, 770 μm; temperature 23°.

DISCUSSION

One aim of this study was to determine whether the positive inotropic effect which AR-L 57 exerts on myocardial cells might have its counterpart in some action on transmembrane ion fluxes present in squid axons. One such effect is the inhibition of the sodium pump described in a preceding paper (2), an effect that had been observed previously in erythrocytes and which was also suggested by the inhibitory effect on isolated $(Na^+\text{-}K^+)$-ATPase.[4] Impaired sodium pumping will lead to an intracellular accumulation of sodium ions, followed by an increased influx of calcium in exchange for intracellular sodium (3) and, presumably, an increase in the amount of calcium in cellular stores that release calcium to initiate contraction.

Another intervention that would conceivably raise intracellular calcium and cause a positive inotropic effect is the inhibition of the plasmalemmal calcium pump. Although AR-L 57 did clearly inhibit the calcium efflux from unpoisoned squid axons, our evidence points against this effect's being due to direct inhibition of active calcium extrusion. Rather, it seems to reflect the response of the calcium pump to an AR-L 57-induced decrease of the free intracellular calcium concentration. This conclusion is based on the direct and simultaneous measurement of the free calcium concentration within the axon, using the aequorin technique, and on the finding that AR-L 57 failed to alter calcium efflux under conditions of constant free intracellular calcium. Thus we may conclude that AR-L 57 does not directly affect the axolemmal calcium pump of *L. forbesi*. This conclusion applied also to the mainly Na_o-dependent calcium extrusion mechanism that operates in ATP-depleted axons.

Another way to increase intracellular calcium would

[4] W. Kobinger, A. Czongrady, and C. Lillie, personal communication.

be to augment calcium influx. However, we found AR-L 57 to inhibit rather than enhance calcium influx both via sodium-calcium exchange and through the voltage-dependent late calcium channel. The latter conclusion is based on the reduction of the aequorin light response to depolarization in the presence of high extracellular calcium. This reduction of the light response probably cannot be attributed to the effect of AR-L 57 on intracellular calcium binding because AR-L 57 caused a comparatively small reduction of the resting glow. Nevertheless, this point should be re-examined with electrophysiological methods. Internal EGTA or mixtures of calcium and EGTA that buffer the ionized calcium in the physiological range are known to inhibit Ca_o-dependent sodium efflux (21) and the associated calcium influx (13). The inhibitory effect of EGTA may reflect a requirement of this sodium-calcium exchange for some critical intracellular free calcium concentration. DiPolo (22) has recently shown that $[Ca^{2+}]_i$ activates the Na_i-dependent calcium influx in squid axons. Since AR-L 57 lowers $[Ca^{2+}]_i$, it may inhibit sodium-calcium exchange via an action on internal calcium rather than directly on the exchange mechanism.

With regard to the potassium channel blocking activity of AR-L 57, it is of interest that another pyridine derivative, 4-aminopyridine, is also known to block potassium channels (23). However, the two compounds differ in the mode of block: after the start of a voltage-clamp depolarization, the degree of blockade increases gradually in the case of AR-L 57, but decreases progressively in the case of 4-aminopyridine (23). Like AR-L 57, but unlike the classic potassium channel blocker tetraethylammonium, 4-aminopyridine is effective from both inside and outside the axolemma (23). A block of potassium channels that increases with the duration of depolarization has also been observed during intracellular application of tetraethylammonium derivatives in which one of the ethyl groups is substituted by a hydrophobic alkyl chain (24). Furthermore, it has been shown that these compounds, which normally have little effect on sodium channels, induce a time-dependent block of sodium current if sodium inactivation has been destroyed by proteolytic enzymes (25). We have shown that the channel-blocking action of AR-L 57 is similarly nonselective, since the drug does block sodium channels if their ability to inactivate is prevented by veratridine.

Veratridine and other toxins that maintain an open form of the sodium channel are frequently used to measure chemically sodium fluxes through sodium channels in cell membranes. The differential effect of AR-L 57 on the ion current through sodium channels with a normal gating mechanism and on the ^{22}Na flux through sodium channels opened by veratridine shows that interference with the gating mechanism may alter the sensitivity of sodium channels to drugs.

AR-L 57 increased axoplasmic calcium binding both during direct application to extruded axoplasm and during external application to intact axons. This implies that AR-L 57 molecules readily penetrate the axolemma, which is understandable because most of the molecules are present in the uncharged form at physiological pH.[5]

[5] The pK_a values of AR-L 57 are 12.1, 4.75, and −0.5 (A. Reuter, Thomae Research Laboratories, private communication).

We do not know which axoplasmic constituent is responsible for the decrease in free intracellular calcium. AR-L 57 itself does not seem to bind calcium in significant amounts.[6] In cardiac muscle cells, an increased calcium uptake by a cellular store that releases calcium during contraction could explain a positive inotropic effect. Indeed, with the possible exception of the effect on the sodium pump (2), none of the additional effects on the axolemma, if presumed to be present at the sarcolemma of mammalian myocardial cells, provides an obvious explanation for the cardiotonic action of AR-L 57.

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REFERENCES

1. Diederen, W., and R. Kadatz. Comparative cardiovascular effects of three benzimidazole derivatives, AR-L 57 BS, AR-L 100 BS, and AR-L 115 BS. *Arzneim. Forsch. Drug Res.*, in press.
2. Honerjäger, P., M. Reiter, and P. F. Baker. Inhibition of the sodium pump in squid axons by the cardioactive drug AR-L 57. *Mol. Pharmacol.* **17**:350–355 (1980).
3. Baker, P. F., M. P. Blaustein, A. L. Hodgkin, and R. A. Steinhardt. The influence of calcium on sodium efflux in squid axons. *J. Physiol. (Lond.)* **200**: 431–458 (1969).
4. Akera, T., and T. M. Brody. The role of Na^+,K^+-ATPase in the inotropic action of digitalis. *Pharmacol. Rev.* **29**:187–220 (1978).
5. Baker, P. F., P. Honerjäger, and M. Reiter. Reversible inhibition of the sodium pump in squid axons by the cardioactive drug AR-L 57. *J. Physiol. (Lond.)* **287**:22–23P (1979).
6. Honerjäger, P., M. Reiter, and P. F. Baker. Effects of the cardioactive drug AR-L 57 [6-(2,4-dimethoxyphenyl)-imidazo-(4,5-β)-pyridine] on ouabain-insensitive ion fluxes in squid axons. *Naunyn-Schmiedebergs Arch. Pharmacol.* **311**:R38 (1980).
7. Baker, P. F., A. L. Hodgkin, and E. B. Ridgway. Depolarization and calcium entry in squid giant axons. *J. Physiol. (Lond.)* **218**:709–755 (1971).
8. Baker, P. F., H. Meves, and E. B. Ridgway. Calcium entry in response to maintained depolarization of squid axons. *J. Physiol. (Lond.)* **231**:527–548 (1973).
9. Baker, P. F., D. E. Knight, and R. D. D. Pattni. Porous cellulose acetate tubing provides a suitable support for isolated protoplasm during studies under controlled conditions. *J. Physiol. (Lond.)* **266**:6P (1977).
10. Kimura, J. E., and H. Meves. The effect of temperature on the asymmetrical charge movement in squid giant axons. *J. Physiol. (Lond)* **289**:479–500 (1979).
11. Luxoro, M., and E. Yañez. Permeability of the giant axon of *Dosidicus gigas* to calcium ions. *J. Gen. Physiol.* **51**:115–122 (1968).
12. Blaustein, M. P., and A. L. Hodgkin. The effect of cyanide on the efflux of calcium from squid axons. *J. Physiol. (Lond.)* **200**:497–527 (1969).
13. Baker, P. F., and P. A. McNaughton. Kinetics and energetics of calcium efflux from intact squid giant axons. *J. Physiol. (Lond.)* **259**:103–144 (1976).
14. Baker, P. F., and H. G. Glitsch. Does metabolic energy participate directly in the Na^+-dependent extrusion of Ca^{2+} ions from squid giant axons? *J. Physiol. (Lond.)* **233**:44–46 P (1973).
15. Baker, P. F., and P. A McNaughton. The influence of extracellular calcium binding on the calcium efflux from squid axons. *J. Physiol. (Lond.)* **276**:127–150 (1978).
16. Baker, P. F., and P. A. McNaughton. Selective inhibition of the Ca-dependent Na efflux from intact squid axons by a fall in intracellular pH. *J. Physiol. (Lond.)* **269**:78–79 P (1977).
17. Bezanilla, F., and C. M. Armstrong. Negative conductance caused by entry of sodium and cesium ions into the potassium channels of squid axons. *J. Gen. Physiol.* **60**:588–608 (1972).
18. Baker, P. F., and J. S. Willis. Inhibition of the sodium pump in squid giant axons by cardiac glycosides: dependence on extracellular ions and metabolism. *J. Physiol. (Lond.)* **224**:463–475 (1972).
19. Khodorov, B. Some aspects of the pharmacology of sodium channels in nerve membrane. Process of inactivation. *Biochem. Pharmacol.* **28**:1451–1459 (1979).

[6] The calcium activity of an aqueous solution of 100 μmoles/liter of $CaCl_2$ did not decrease when AR-L 57 was added to a final concentration of 1 mmole/liter. A calcium-sensitive ion exchanger embedded in a polyvinyl chloride membrane as described by Ruzicka *et al.* (26) was used to measure calcium activity.

20. Ulbricht, W. The effect of veratridine on excitable membranes of nerve and muscle. *Ergeb. Physiol. Biol. Chem.* **61**:18–71 (1969).
21. Baker, P. F. Sodium-calcium exchange across the nerve cell membrane, in *Calcium and Cellular Function* (A. W. Cuthbert, ed.) Macmillan, London, 96–107 (1970).
22. DiPolo, R. Calcium influx in internally dialyzed squid giant axons. J. Gen. Physiol. **73**:91–113 (1979).
23. Meves, H., and Y. Pichon. The effect of internal and external 4-aminopyridine on the potassium currents in intracellularly perfused squid giant axons. *J. Physiol. (Lond.)* **268**:511–532 (1977).
24. Armstrong, C. M. Interaction of tetraethylammonium ion derivatives with the potassium channels of giant axons. *J. Gen. Physiol.* **58**:413–437 (1971).
25. Rojas, E., and B. Rudy. Destruction of the sodium conductance inactivation by a specific protease in perfused nerve fibres from *Loligo*. *J. Physiol. (Lond.)* **262**:501–531 (1976).
26. Ruzicka, J., E. H. Hansen, and J. C. Tjell. Selectrode—the universal ion-selective electrode. Part VI. The calcium(II)selectrode employing a new ion exchanger in a nonporous membrane and a solid-state reference system. *Anal. Chim. Acta* **67**:155–178 (1973).

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