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Inhibition of the Sodium Pump in Squid Axons by the Cardioactive Drug AR-L 57

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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 inhibition of ouabain-sensitive Na efflux from axons of *Loligo forbesi* loaded with ^{22}Na by microinjection. The half-maximally effective concentration is 58 μM. AR-L 57 also inhibits ouabain-sensitive ^{86}Rb influx. Raising the external K does not diminish the degree of Na-pump inhibition by AR-L 57. AR-L 57 also inhibits the ouabain-sensitive Na–Na exchange seen in axons partially poisoned with dinitrophenol. In both Na and choline seawaters, the inhibition of ouabain-sensitive Na efflux displays similar rates of onset and similar concentration–response relationships. Injection of AR-L 57 into an axon produces only a transient inhibition of Na efflux, suggesting that AR-L 57 can cross the axolemma. The onset and extent of Na-efflux inhibition by externally applied AR-L 57 are unaltered at an external pH of 6. The results show that AR-L 57 is a Na-pump inhibitor like the cardioactive glycosides, but the lack of dependence on external Na ions suggests a difference in the molecular mode of interaction with the Na pump.

INTRODUCTION

It has recently been shown that the imidazopyridine derivative AR-L 57[1] has a positive inotropic action on the heart.[2] The action of this nonsteroidal drug (Fig. 1) resembles that of the cardiac glycosides because it inhibits the active Na–K exchange of erythrocytes and the Na,K-ATPase.[2] The present study characterizes the effect of AR-L 57 on the Na pump using the squid giant axon as a model which allows the direct measurement of unidirectional transmembrane fluxes in a single cell. AR-L 57 inhibits the active Na–K exchange of squid axons, but the inhibition differs in detail from that induced by the cardiac glycosides. This suggests a difference in the molecular mode of interaction with the Na pump. Furthermore, AR-L 57 was found to lack the selectivity of action displayed by the cardiac glycosides: The effects on certain ouabain-insensitive ion movements are the subject of a later publication.[3] Some of the results have been communicated to the British Physiological Society (1).

METHODS

Material. Giant axons from *Loligo forbesi* were isolated and cleaned by the usual methods. The axons, 500 to 910 μm in diameter, were obtained mainly from refrigerated mantles, although live squid were used in a few experiments. The experiments were performed at room temperatures of 18–23°C.

Na efflux. After cannulation, the axons were loaded with radioactive isotope by intracellular microinjection (2, 3). An aqueous solution of $^{22}NaCl$ (200,000–500,000 cpm) was introduced axially over a length of normally 10 mm and at least 2 cm from either end of the axon. A good diphasic action potential recorded with external electrodes from each end of the axon both before and after injection was taken as adequate evidence of excitability. Experiments were rejected if, at any stage, the injected region showed block or decrement (for one exception to these criteria, see the legend to Fig. 9). The axon was continuously superfused with ASW by mounting it in a glass tube (internal diameter, 3 mm) which was connected to a peristaltic pump (flow rate, 1.5 ml/min). The superfusate was collected at intervals of 2 min.

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[1] Abbreviations used: AR-L 57, 6-(2,4-dimethoxyphenyl)-imidazo-(4,5-β)-pyridine hydrochloride; ASW, artificial seawater (see Table 1); DNP, 2,4-dinitrophenol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Pipes, piperazine-*N,N'*-bis(2-ethanesulfonic acid).

[2] Kobinger, W., A. Czongrady and C. Lillie, manuscript submitted for publication.

[3] Honerjäger, P., M. Reiter and P. F. Baker, manuscript in preparation.

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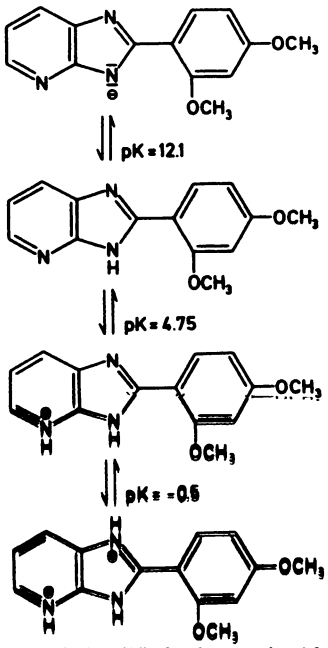

FIG. 1. *Structure and simplified scheme of acid-base equilibria of AR-L 57 (A. Reuter, Thomae Research Laboratories, private communication)*

In the experiment shown in Fig. 10 the axon was mounted in a chamber, and the external solution was replaced every 3 min by means of a syringe. At the end of the experiment the fiber was removed and the total quantity of tracer in the fiber measured. Effluxes are normally expressed in units of the fraction of the total activity lost per minute. Injection of ^{22}NaCl raised the axoplasmic Na concentration by only a few μmol/liter. ^{22}Na was determined with a Packard Auto-Gamma spectrometer.

Rb influx. Partially cleaned axons were equilibrated in the inactive control or test solution for 15 min. Subsequently, ^{86}RbCl was added in small amounts as a tracer for K ions that were present at 10 mM in these solutions. Exposure to isotope-containing solutions lasted 30 min. Thereafter the axons were soaked in several changes of inactive solution over some 30 min. During this time the diameter of the axon was measured at three points along its length, and a 1-cm length of axon at one end was cleaned. After blotting the axon on filter paper, axoplasm was extruded through the cleaned end onto a tared piece of Parafilm. Care was taken to ensure that axoplasm from within 1.5 cm of each end was not included with the axoplasm taken for analysis. The axoplasm was weighed, dispersed in 0.5 M $CaCl_2$, and counted in a Packard Auto-Gamma spectrometer. The surface area of the axon was calculated from the diameter of the axon and the weight of the extruded axoplasm. In general, two treatments were compared using paired axons from the same squid.

Solutions and drugs. The main external solutions used are listed in Table 1. Ouabain was obtained from British Drug Houses, AR-L 57 from Dr. Karl Thomae GmbH, lithium chloride from Fisher Scientific Co., and radiochemicals from the Radiochemical Centre, Amersham.

RESULTS

Inhibition of active Na–K exchange. The cardioactive drug AR-L 57 produced a rapid and reversible inhibition of Na efflux from axons loaded with ^{22}Na by microinjection. This is illustrated in Fig. 2, which is a plot of the fraction of ^{22}Na lost by an axon per minute vs time. In order to test whether AR-L 57 reduced the rate of ^{22}Na loss by inhibiting the sodium pump, the axon shown in Fig. 2 was again challenged with AR-L 57 at a time when this component was already inhibited either by withdrawing external K or by adding a fully blocking concentration of ouabain. Under both conditions AR-L 57 did not reduce Na efflux further, indicating that the drug inhibits the active transport of Na.

As shown in Fig. 2 inhibition reached a steady state after 10 min of exposure to ASW containing 100 μmol/liter of AR-L 57, and after removal of the drug the rate constant for ^{22}Na loss recovered to its predrug value in about 20 min. The kinetics of the effect were similar in three other axons treated with the same concentration. The experiments are summarized in Fig. 3, which is a plot of the ouabain-sensitive fraction of Na efflux relative to the predrug value during and after exposure to AR-L 57. The experiments include one axon in which free intracellular Ca had been buffered close to the physiological level by the injection of a mixture of $CaCl_2$ and EGTA (4). This procedure prevents the effect of AR-L 57 on free intracellular Ca and Ca efflux (1) but, as shown in Fig. 3, does not interfere with the ability of the drug to inhibit ouabain-sensitive Na efflux.

Since the active extrusion of Na ions is normally coupled to the uptake of K ions, AR-L 57 should also inhibit ouabain-sensitive K influx. To test this point we measured the influx of ^{86}Rb added to 10-K(Na) ASW in tracer amounts. It is known that Rb and K are transported similarly by the Na–K exchange pump (5). The results shown in Fig. 4 demonstrate that 200 μM AR-L 57 reduced ^{86}Rb influx by 62%. The small residual ^{86}Rb influx of ouabain-treated axons, on the other hand, was not significantly altered by AR-L 57. The extent of inhibition of

TABLE 1

Composition of the main external solutions

All concentrations are millimolar. All solutions were pH 7.8.

Reference	Na	K	Li	Choline	Mg	Cl	HCO_3	Ca
10-K(Na)	402.5	10	—	—	100	630	2.5	10
10-K(choline)	2.5	10	—	400	100	630	2.5	10
0-K(Na)	402.5	0	—	—	100	620	2.5	10
10-K(Na), 0-Ca	402.5	10	—	—	100	610	2.5	—
10-K(choline), 0-Ca	2.5	10	—	400	100	610	2.5	—
0-K(Li), 0-Ca	2.5	0	400	—	100	600	2.5	—

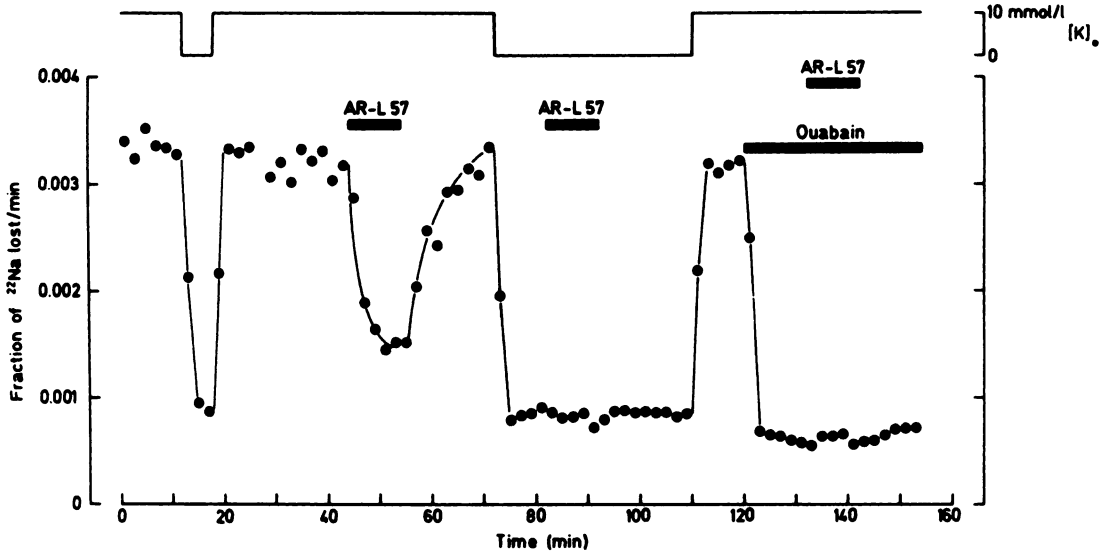

FIG. 2. *Inhibition of Na efflux by AR-L 57*

The axon was superfused with 10-K(Na) ASW or 0-K(Na) ASW as indicated and the superfusate collected at 2-min intervals. AR-L 57 was applied at 100 μM and ouabain at 10 μM during the periods indicated. Axon diameter, 800 μm. Temperature, 21°C.

ouabain-sensitive Rb influx (68%) is similar to that of ouabain-sensitive Na efflux (see Fig. 5). These results support the conclusion that the drug inhibits active Na–K exchange.

Figure 5 summarizes data from seven axons on the relation beteeen the degree of inhibition of ouabain-sensitive Na efflux and the concentration of AR-L 57. Due to the limited solubility of AR-L 57 at physiological pH, the highest possible test concentration was 300 μM. Inhibition of ouabain-sensitive Na efflux was still incomplete at this concentration. The maximum inhibition observed was 84% in one experiment using 200 μmol/liter of the drug. The continuous curve in Fig. 5 was drawn to fit the experimental points on the assumption of a reversible one-to-one binding reaction of AR-L 57 molecules to all Na-pump sites with an apparent affinity of K_i = 58 μM.

Activation of the Na pump by external potassium. An inhibitor of the Na pump might act by decreasing the affinity of the external activation site for K ions. Figure 6 shows the ouabain-sensitive fraction of Na efflux as a function of the external K concentration in both the absence and presence of AR-L 57. It is evident that the half-maximally activating concentration of K was reduced rather than increased by AR-L 57 (from 8.5 to 2.5

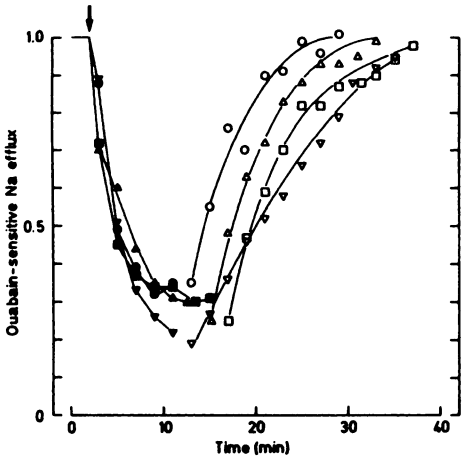

FIG. 3. *Reversible inhibition of ouabain-sensitive Na efflux by AR-L 57*

The ordinate shows the ouabain-sensitive part of the rate constant of ^{22}Na loss relative to the predrug value. This part was obtained by subtracting the residual Na efflux after equilibration with 10 μM ouabain from the total Na efflux. Ouabain was applied at the end of each experiment. AR-L 57 was applied at 100 μM in 10-K(Na) ASW. Filled and open symbols of identical shape denote the times of drug exposure and washout, respectively, of the same axon. The data include the axons shown in Figs. 2, 7, and 8. The axon characterized by the symbols ▲ and △ was initially injected over a length of 2 cm with a mixture containing 200 mM EGTA and 80 mM $CaCl_2$ (pH 7.2 with KOH) to a final axoplasmic concentration of 11 mM EGTA; this column overlapped the subsequently introduced patch of ^{22}Na by about 5 mm at each end. Axon diameters, 690–910 μm. Temperature, 18–21°C.

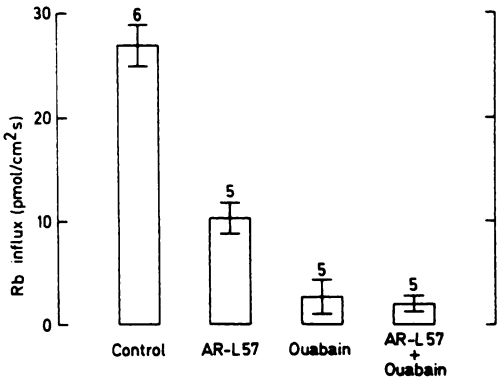

FIG. 4. *Inhibition of ouabain-sensitive ^{86}Rb influx by AR-L 57*

^{86}Rb was added to 10-K(Na) ASW in tracer amounts (see Methods). The results are presented as pmol/cm²/s Rb influx on the assumption that the Na–K exchange pump does not discriminate between K and Rb (5). Five of the control axons and all axons treated only with AR-L 57 represent pairs of axons obtained from the same animal; the same holds for the two groups exposed to ouabain and to both ouabain and AR-L 57, respectively. AR-L 57 was applied at 200 μM, ouabain at 10 μM. Mean values ± SD and the number of axons in each group are shown. Temperature, 20°C.

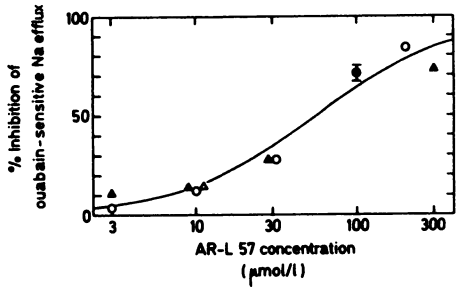

FIG. 5. *Concentration–response relationship for the inhibition of the ouabain-sensitive Na efflux by AR-L 57*

Ouabain-sensitive Na efflux was determined as described in the legend to Fig. 3. Pooled results from seven axons which were exposed to a single concentration of AR-L 57 (100 μM) only or to cumulatively increasing concentrations. At 100 μM the mean value (±SD) of all axons is shown. Three axons, each characterized by a different symbol, were also exposed to the other indicated concentrations. The curve has been drawn on the assumption of Michaelis–Menten kinetics with V_{max} = 100% inhibition and K_i = 58 μM. Axon diameters, 690–910 μm. Temperature, 18–22°C.

mM) and that the drug reduced the maximum rate of Na pumping achievable by increasing the external K. Similar effects were observed in another axon; a rather large ouabain-sensitive Na efflux into 0-K(Na) ASW prevented the estimation of half-maximally activating K concentrations in this second preparation.

Inhibition of ouabain-sensitive Na–Na exchange. In axons partially poisoned with alkaline DNP, the normal Na–K exchange mode of operation of the Na pump is changed to a mode in which the Na pump exchanges extracellular Na for intracellular Na in a one-for-one manner (5). The experiment illustrated in Fig. 7 was performed to examine whether the Na pump would retain its sensitivity to AR-L 57 despite the change in mode of operation. As shown here, an initial exposure to AR-L 57 resulted in the typical reduction of Na efflux. During the subsequent simultaneous withdrawal of external K and addition of 0.4 mM DNP at pH 8, the Na efflux first declined and later increased to about half the value observed under control conditions. The flux remained stable at this level afterward when the concentration of DNP was halved. Exposure to AR-L 57 resulted again in a marked and reversible inhibition of Na efflux. The subsequent replacement of external Na by Li and withdrawal of Ca, which otherwise would have activated Ca_o-dependent Na efflux (3), removed most of the Na efflux, confirming that the major part of this flux is activated by external Na. Finally, ouabain eliminated this Na_o-dependent Na efflux, indicating as expected that the Na–Na exchange is produced by the Na pump (5). The ineffectiveness of AR-L 57 in the presence of ouabain shown near the end of the experiment demonstrates that the drug has a selective inhibitory action on the ouabain-sensitive Na–Na exchange under these conditions.

Effect at low external sodium concentrations. It is well recognized that the presence of external Na ions plays a critical role in the inhibitory effect of cardiac glycosides on the Na pump. In squid axons the rate of action of ouabain is reduced to less than one-tenth if external Na is replaced by choline, and the rate of ouabain binding is also reduced (6). The experiment shown

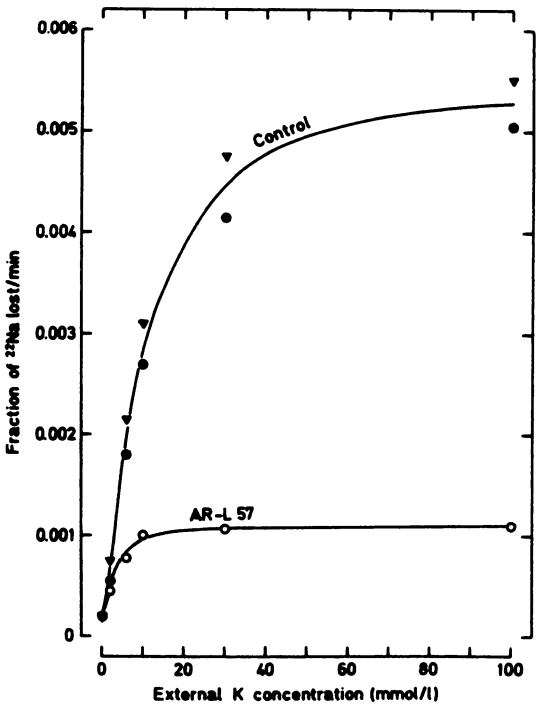

FIG. 6. *The dependence of ouabain-sensitive Na efflux on external K concentration in the absence and presence of AR-L 57*

The ordinate represents the ouabain-sensitive part of the rate constant for loss of ^{22}Na. The axon was exposed to cumulatively increasing concentrations of K in the absence of drugs (●), in the presence of 100 μM AR-L 57 (○), after washout of AR-L 57 (▼), and during exposure to both AR-L 57 and 10 μM ouabain in this sequence. The residual fluxes measured under the latter conditions were subtracted from all other corresponding fluxes to obtain the values shown after correction for a slight time-dependent increase evident from the repeated checks of the flux into O-K(Na) ASW. It has been observed previously that the residual ouabain-insensitive Na efflux tends to increase with time (5). The residual efflux amounted to about 14% of the total Na efflux into 10-K(Na) ASW in the absence of drugs. Each K concentration was applied for 6 min, during which time the Na efflux stabilized under all conditions. The K concentration was increased as shown at a constant Na concentration of 402.5 mM without osmotic compensation. Axon diameter, 750 μm. Temperature, 22.5°C.

in Fig. 8 demonstrates that the rate of action of AR-L 57 on Na efflux is not notably changed when the axon is bathed in 10-K(choline) ASW rather than 10-K(Na) ASW. Furthermore, the relation between the steady-state inhibition of ouabain-sensitive Na efflux and the AR-L 57 concentration is practically unaltered in choline seawater. This is illustrated in Fig. 9. The filled symbols in this figure were obtained from two axons exposed to 10-K(choline), 0-Ca ASW. External Ca was reduced to avoid the possible activation of Ca_o-dependent Na efflux in the absence of external Na. We have found[3] that this Na-efflux component is also inhibited by AR-L 57. It is therefore essential to suppress ouabain-insensitive, Ca_o-dependent Na efflux for an evaluation of the effect of AR-L 57 on the Na pump. Withdrawal of external Ca per se does not interfere with the inhibition of the Na pump by AR-L 57, as shown by the open circles in Fig. 9, which were obtained from another axon in 10-K(Na), 0-Ca ASW.

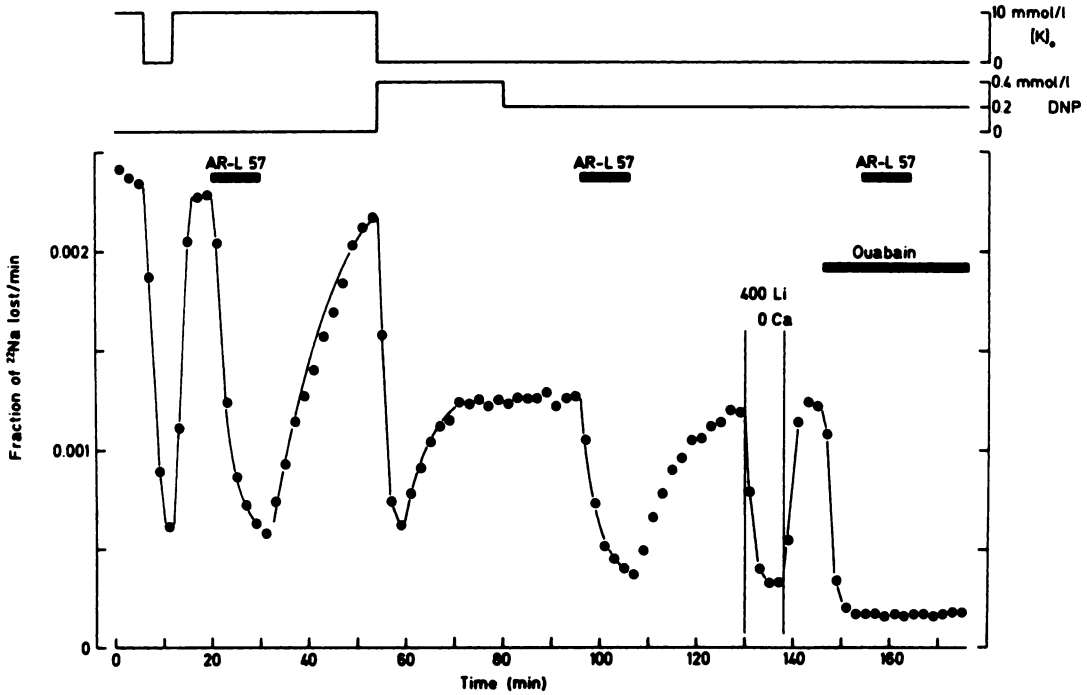

FIG. 7. *Inhibition of ouabain-sensitive Na–Na exchange by AR-L 57*

The axon was superfused with 10-K(Na) ASW or 0-K(Na) ASW or 0-K(Li), 0-Ca ASW as indicated. AR-L 57 was applied at 100 μM, ouabain at 10 μM. The DNP-containing ASW was buffered to pH 8 with Tris. Axon diameter, 910 μm. Temperature, 18°C.

Intracellular application. Ouabain fails to inhibit the Na pump when it is injected into squid giant axons (7). Intracellular application of AR-L 57, on the other hand, caused a marked but transient reduction of the K-activated Na efflux, as shown by the experiment in Fig. 10.

Variation of external pH. Most of the AR-L 57 molecules are present in the uncharged form in the pH range from 6 to 9. We examined whether changes in the external pH over this range, by altering the proportions of the various charged forms (see Fig. 1), would modify the inhibitory effect on the Na pump. The experiment shown in Fig. 11 compares the effect of the same concentration of AR-L 57 in artificial seawaters buffered to pH 6 and 9.

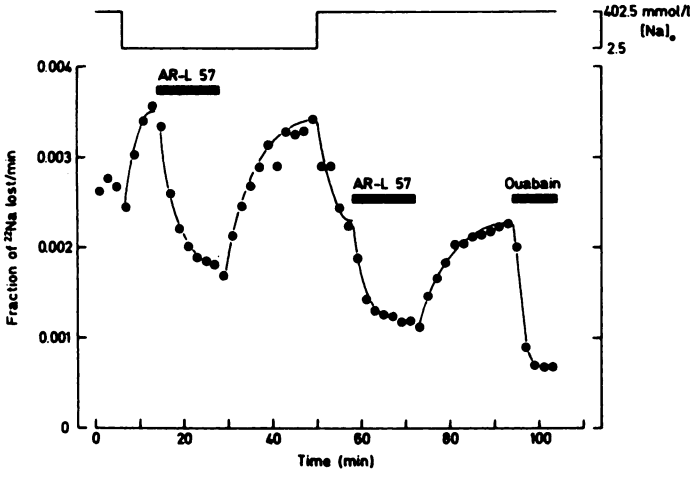

FIG. 8. *Inhibition of Na efflux by AR-L 57 at reduced external Na concentrations*

The axon was superfused with 10-K(choline) ASW or 10-K(Na) ASW as indicated. AR-L 57 was applied at 100 μM, ouabain at 10 μM. Axon diameter, 690 μm. Temperature, 19°C.

At pH 6 the inhibitory effect was very similar in terms of kinetics and extent to that observed in other experiments performed in bicarbonate-buffered ASW at pH 7.8 (see Fig. 3). At pH 9 the situation is complicated by the fact that this pH per se inhibited the sodium pump almost completely. Nevertheless, a reversible inhibition of the residual ouabain-sensitive Na efflux by AR-L 57 is still visible. It may be concluded that changes from pH 6 to 7.8 and probably up to 9 have little influence on the sodium-pump inhibition by AR-L 57.

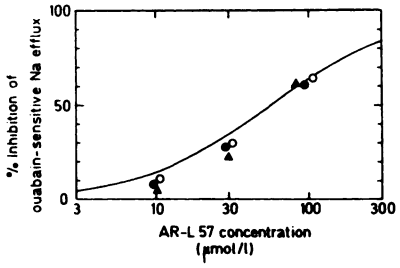

FIG. 9. *Concentration–response relationship for the inhibition of ouabain-sensitive Na efflux by AR-L 57 at reduced external Na concentrations*

Ouabain-sensitive Na efflux was determined as described in the legend to Fig. 3. AR-L 57 was applied at cumulatively increasing concentrations. The filled circles refer to an axon exposed to 10-K(choline), 0-Ca ASW (diameter, 720 μm; temperature, 23°C). The triangles were obtained from an axon superfused with the same medium (diameter, 750 μm; temperature, 20°C). The open circles refer to another axon exposed to 10-K(Na), 0-CA ASW (diameter, 840 μm; temperature, 22.5°C) (although the Na efflux of this preparation showed a normal ouabain sensitivity, it was unexcitable at the end of the experiment, probably due to the prolonged exposure to Ca-free solution). The continuous curve is taken from Fig. 5 to allow comparison with data obtained in 10-K(Na) ASW.

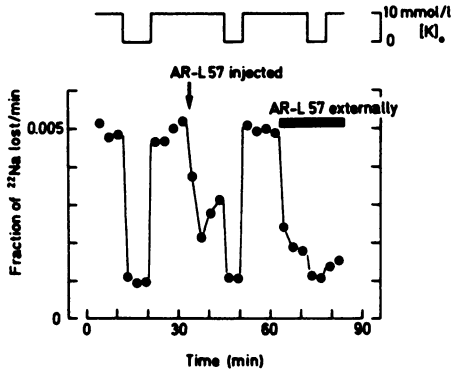

FIG. 10. *Comparison of the effects of intracellular and extracellular application of AR-L 57 on Na efflux*

The axon was placed in 10-K(Na) ASW or 0-K(Na) ASW as shown. At the arrow a 30-mm column of 10 mM AR-L 57 (0.54 μl) was injected into the axon such that it overlapped the patch of ^{22}Na by 10 mm at each end. During the period indicated AR-L 57 was applied externally at 100 μM. Axon diameter, 600 μm. Temperature, 20.5°C.

DISCUSSION

The cardiotonic agent AR-L 57 inhibits the Na pump of squid axons rapidly and reversibly. The concentration–response relationship suggests that this effect is the consequence of a one-to-one binding reaction of AR-L 57 with axolemmal receptors. The degree of Na-pump inhibition by AR-L 57 is not diminished when the external K concentration is increased, indicating that AR-L 57 neither competes with K for the external activation site of the Na pump nor modifies the conformation of this site such as to reduce its affinity for K. This result is similar to that described by Baker and Willis (6) for the interaction between ouabain and external K on the Na pump in squid axons. A further similarity between AR-L 57 and ouabain is that, in addition to Na–K exchange, both inhibit the Na–Na exchange which operates in axons partially poisoned with DNP. On the other hand, our observation that the inhibitory action of AR-L 57 is unchanged when external Na is replaced by choline suggests that the molecular interaction between AR-L 57 and the Na pump differs in detail from that between the cardiac glycosides and the Na pump.

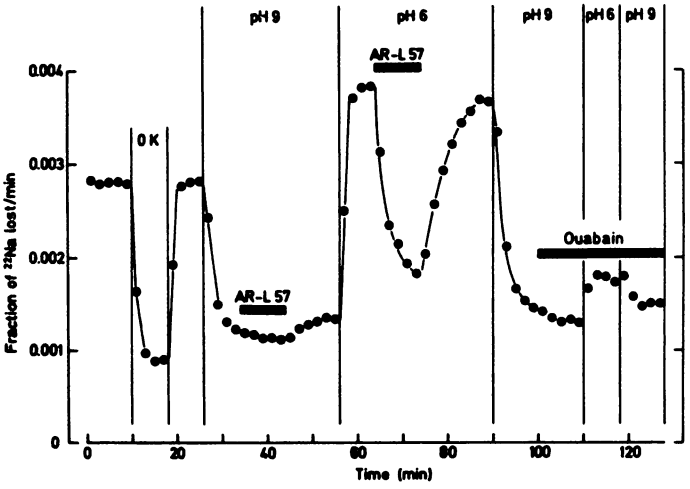

FIG. 11. *Inhibition of Na efflux by AR-L 57 in seawaters of a different pH*

The axon was superfused with 10-K(Na) ASW or 0-K(Na) ASW as indicated. The solutions with pH 6 or 9 were 10-K(Na) ASW to which 5 mM Hepes and 5 mM Pipes had been added. AR-L 57 was applied at 100 μM, ouabain at 10 μM. Axon diameter, 800 μm. Temperature, 23°C.

The transient inhibition of the Na pump that occurs after intracellular injection of AR-L 57 into the axon suggests that the drug can readily escape from the axon through the axolemma. That the drug readily permeates the axolemma is also suggested by the fact that externally applied AR-L 57 decreases both free intracellular Ca and Ca efflux by an action on some constituent of axoplasm, perhaps mitochondria (1). It would be of interest to know whether AR-L 57 blocks the sodium pump by acting at the internal face of the axolemma. Our observations that during external application the inhibitory activity of AR-L 57 apparently resides in the uncharged form is compatible with this possibility but does not prove it.

While AR-L 57 resembles the cardiac glycosides with respect to its cardiotonic action and effect on the transport function of the Na pump, it should be noted that the drug lacks the striking selectivity of action characteristic of the glycosides. In the case of the squid axon, additional effects of AR-L 57 include the blockade of Na, K, and Ca channels, the inhibition of the ouabain-insensitive, Ca_o-dependent Na efflux, and an increase in intracellular Ca binding.[3]

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