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Na^+ movement in a single turnover of the Na pump

(Na,K-ATPase/membrane transport/rapid kinetics/membrane vesicles)

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ABSTRACT **Ouabain-sensitive ^{22}Na efflux from right-side-out membrane vesicles prepared from dog kidney has been examined with a time resolution of 30 msec. The vesicles are preloaded with ^{22}Na and caged ATP [P^3-1-(2-nitro)phenylethyl adenosine triphosphate], so that transport by the Na pump can be initiated by light. After a brief illumination, which releases less ATP than the number of catalytic sites, a burst of ^{22}Na extrusion is observed corresponding to a single turnover of the Na pump. By the use of a rapid filtration apparatus, with which a continuous record of the rate of efflux is obtained, it has been possible to resolve the efflux burst in the time range of 20–1500 msec. The rate of efflux rises rapidly, but not instantaneously, to a peak and then decays, with a time constant of $\approx 6\ sec^{-1}$ at 15°C. The time course of Na efflux is unaffected by extracellular K^+, as predicted by models of the Na pump in which Na is released early in the cycle. Unphotolyzed caged ATP is found to bind to the catalytic site of Na,K-ATPase, in competition with ATP that is produced in the flash, and the possibility has not been excluded that dissociation of unphotolyzed caged ATP and binding of ATP are involved in the Na efflux time course. It seems most likely that binding of ATP and translocation of ^{22}Na are involved in the increase in the ^{22}Na efflux rate in the single turnover and that the release of transported ^{22}Na from extracellular pump sites limits the slow decay.**

Over the last 20 yr, a detailed description of the steady-state characteristics of ion transport by the Na pump has been developed in a number of laboratories, complemented by studies of ATP hydrolysis by Na,K-ATPase (for review, see ref. 1). Although in the past decade transient-state analysis of the conformational changes and phosphorylation events in Na,K-ATPase has provided a view of the sequence of events in the enzymatic cycle (cf. refs. 2–4), parallel measurements of rapid ion movements have not been carried out; thus, the assignment of the ion translocation steps within the various kinetic schemes remains uncertain. At the most basic level, the kinetic data are insufficient to conclude whether Na^+ and K^+ ions are transported simultaneously, or if first Na^+ and then K^+ ions are transported in a "ping-pong" manner; although the studies of partial reactions of the pump have been most easily interpreted in terms of the latter mechanism, the concerted model of ion movement has found recent support (5, 6). At a more detailed level, it is not known at exactly which steps in the phosphorylation–dephosphorylation cycle the ions are moved across the membrane. These questions could be answered if ion translocation could be resolved within a single pump turnover.

Rapid kinetic analysis of ion movements requires (*i*) a biological preparation in which Na pumps are found at a high density and situated in membranes surrounding a closed compartment and (*ii*) an apparatus for resolving ion fluxes on the time scale of 5–1000 msec. I have previously reported a characterization of tight right-side-out membrane vesicles prepared from dog kidney outer medulla (7). The membranes are rich in Na,K-ATPase, so that a significant fraction of intravesicular Na^+ can be pumped from the vesicles in a single turnover: the number of catalytic sites per vesicle volume is >100 μM. It has been possible to incorporate caged ATP [P^3-1-(2-nitro)phenylethyl adenosine triphosphate] (8) within the vesicles so that Na efflux can be fueled by ATP released upon exposure to long wavelength ultraviolet light.

Techniques for the measurement of rapid ion movements in membrane vesicles have been described recently. For the measurement of isotopic fluxes, a quench reagent is used to stop ion transport rapidly by chelating the transported species (9) or by blocking an ion channel (10); this approach is not immediately applicable to the study of Na^+ and K^+ transport by the Na pump. In addition, the quench-flow method has the disadvantage of consuming a substantial quantity of membrane vesicles.

This paper presents measurements of Na movement in a single turnover of the Na pump, resolved on the time scale of tens of milliseconds. Using kidney vesicles loaded with ^{22}Na and caged ATP, a pump cycle is initiated by generation of ATP as a result of a bright flash of light. The rate of ^{22}Na efflux from the vesicles is then monitored as a function of time, using a rapid filtration apparatus. The time course of ^{22}Na efflux at 15°C is found to be consistent with models of the Na pump in which extracellular Na release is an early step. A preliminary report of some of these results has been presented (11), and a more detailed description of the apparatus is given elsewhere (12).

METHODS

Membrane Vesicles. The isolation and characterization of membrane vesicles, which are right-side-out and rich in Na pump, and the loading of membrane vesicles by osmotic shock following equilibration with glycerol have been described (7). The loading medium contained 5 mM caged ATP/32 mM glutathione/1 mM NaCl/^{22}Na (300 Ci/mol, unless otherwise noted; 1 Ci = 37 GBq) (New England Nuclear)/0.1 mM KCl/5 mM $MgCl_2$/32 mM Tris/Hepes, pH 7.2. The procedure is apparently effective in equilibrating the vesicular contents with the extravesicular loading medium with regard to small molecules, because the volume of distribution of trapped ^{22}Na (≈ 2 μl per mg of protein) is roughly the same as the vesicular volume determined in [^{14}C]sucrose/^3H_2O distribution studies. On the other hand, the equilibration of the vesicular space with regard to large molecules is far from complete. This is reflected in the fact that, in the flux experiments reported here, only a fraction of the vesicular ^{22}Na was pumped out of the vesicles when all of the caged ATP was photolyzed, even though the caged ATP had been included in the loading medium in 10-fold excess over ^{22}Na. When the standard loading procedure was used, it was

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Abbreviation: caged ATP, P^3-1-(2-nitro)phenylethyl adenosine triphosphate, a protected analog of ATP that releases ATP on exposure to long wavelength ultraviolet light.

found that 9%–12% of the vesicular ^{22}Na could be pumped out. The fractional release of ^{22}Na on photolysis of caged ATP (≈10%) was independent of the relative concentrations of ^{22}Na (1–10 mM) and caged ATP (5–10 mM) in the loading medium. This is consistent with the hypothesis that ≈10% of the vesicles were loaded with caged ATP rather than that all of the vesicles were loaded with a small amount of caged ATP. [Since 20%–30% of the membrane protein in the preparation is Na,K-ATPase (7), it is not a reasonable alternative that only 10% of the vesicles have Na pumps.] However, even within the population of vesicles that are loaded with caged ATP there may be heterogeneity, and the caged ATP concentration may be considerably below the concentration at which it was loaded; evidence presented below indicates the intravesicular caged ATP concentration is closer to 1–2 mM than to 5 mM. Recently, we have been able to increase the caged ATP "loading efficiency" (as evidenced by fractional ^{22}Na release) to 20%–35% by sonication for 1 min during the osmotic shock.

Rapid Efflux Measurement. A new rapid filtration method was used to determine the time course of ^{22}Na efflux after release of ATP in a brief flash of light. The method uses a pressure filtration device, described in detail elsewhere (12), in which the membrane vesicles are immobilized on the surface of a cellulose ester filter (Gelman GN-6); efflux medium is forced through the filter at high velocity and out of the filter support funnel in a steady stream. This fluid is collected in a rapidly moving ring of 56 plastic cuvettes mounted on the platter of a phonograph turntable. The amount of ^{22}Na collected in each cuvette is a direct measure of the rate of ^{22}Na efflux over the collection time period of one cuvette, which at 33.3 rpm rotational velocity is 32.5 msec.

Light from a high-intensity continuous arc lamp strikes the sample only when admitted through a hole (one cuvette position long) in a skirt surrounding the turntable, yielding a 32-msec flash (at 33.3 rpm). With the optical arrangement used here, one such flash was sufficient to photolyze 2.6% of the caged ATP, releasing ATP with 80% yield (8). Thus with ≤5 mM caged ATP incorporated in the vesicles, ≤100 μM ATP is produced per 32-msec flash. Since only a small fraction of caged ATP is photolyzed in each flash, a number of successive flashes can be delivered in successive revolutions, each one releasing additional ATP; the resulting ^{22}Na efflux bursts are thus "signal-averaged" as the fluid is collected in the same set of 56 cuvettes. Unless otherwise noted in the figure legends, 40 μg of membrane protein was used for each run, the turntable was operated at 33.3 rpm, flashes were 32.5 msec long, and efflux was monitored for 15 sec (8 revolutions; 8 flashes). The time resolution of the method is determined solely by the characteristics of fluid flow between the sample and the collection cuvettes, because it is a simple matter to spin the turntable fast enough to provide collection periods as short as 2 msec. In this apparatus, the active filter diameter was 7 mm, the fluid flow rate was 1.3 ml/sec at 37°C (1.0 ml/sec at 5°C), and the filter funnel had a volume of 20 μl. There was a fixed delay of ≈25 msec for fluid flow between the filter and the collection cuvette, and there was dispersion due to fluid mixing, which spread an instantaneous pulse over a 25- to 30-msec period (Fig. 3 and unpublished data).

In a typical efflux experiment, 10–20 efflux time courses were obtained. Each "run" was carried out as follows: a 5- to 10-μl sample of membrane vesicles loaded with ^{22}Na and caged ATP was diluted into 200 μl of efflux medium at 0°C, filtered on a cellulose ester filter on a conventional vacuum filtration apparatus (with a "chimney" of 7 mm inside diameter), and washed with another 200 μl of cold medium. While still wet, the filter and sample were transferred to the rapid pressure filtration apparatus and clamped in place. After flow of efflux medium was begun (90 psi pressure; 1 psi = 6.895×10^3 Pa) fluid was diverted to a waste vessel for 5–10 sec; then, at the beginning of a cycle (8 cuvette positions prior to the flash position) the diversion funnel was removed and the manual shutter in front of the arc lamp was opened. After 15 sec of sample collection (8 flashes at 33.3 rpm), the diversion funnel was replaced, the shutter was closed, flow was turned off, and the filter cuvette contents were transferred to scintillation vials. Unless otherwise noted, the efflux medium contained 0.1 mM KCl/125 mM sucrose/0.1 μM valinomycin (similar results were obtained with 0 and 1 μM valinomycin)/33 mM Tris/Hepes, pH 7.5.

[^{3}H]ATP Binding to Na,K-ATPase. Crude Na,K-ATPase was prepared as detergent-washed microsomes. The pellet from the differential centrifugation at 48,000 × *g* (7) was treated with 0.7 mg of $NaDodSO_4$ per ml in 1% bovine serum albumin/25 mM imidazole, pH 7.2, for 10 min at 20°C, then pelleted, washed, and resuspended in imidazole buffer. [^{3}H]ATP binding was determined by a filtration assay in a procedure similar to a method previously used by Yamaguchi and Tonomurra (13) for ion binding. Na,K-ATPase (1.5 mg/ml) was mixed with 0.4 μM [^{3}H]ATP, various concentrations of caged ATP, 6 mM Na/19 mM EDTA/25 mM imidazole, pH 7.2, in a vol of 25 μl. The sample was immediately transferred without dilution to a Nuclepore filter (0.08-μm pore) on top of a Millipore filter (HAWP) and spread over the surface during the 30–45 sec required for filtration to dryness under vacuum. The Nuclepore filter was immediately transferred to a scintillation vial for counting without rinsing, with the Millipore filter serving to blot it completely dry.

RESULTS AND DISCUSSION

^{22}Na Efflux After a Pulse of ATP. The time course of ^{22}Na release from membrane vesicles at 15°C is shown in Fig. 1*A*. After a 30-msec flash of light to release <90 μM ATP from caged ATP, a burst of ^{22}Na efflux is seen as Na is pumped from the vesicles. The ion movement is due to operation of the Na pump, because no burst is observed if vesicles are preincubated with 2 mM ouabain (Fig. 1*B*). In other experiments (not shown), it was found that the presence of caged ATP was required for the increase in ^{22}Na efflux, as was the flash, and that there was no detectable change in efflux rate for $^{42}K^+$ or $^{35}SO_4^{2-}$. Since the concentration of Na pumps per vesicle volume is 100–300 μM (7), in the same range as

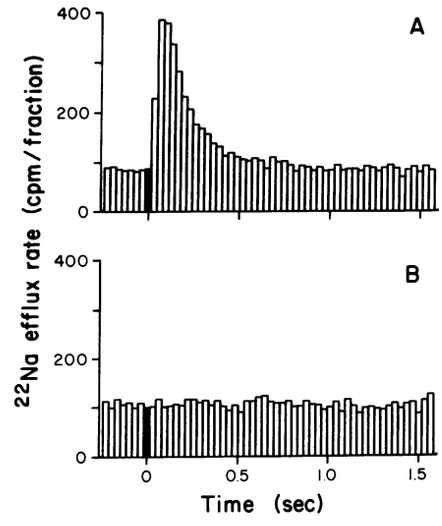

FIG. 1. Na efflux from right-side-out membrane vesicles after a bright flash of light at 15°C. The time period during which the sample was illuminated is shown by solid bars. Six cycles were averaged. (*A*) Control. (*B*) The sample was incubated in 2 mM ouabain throughout the vesicle loading procedure.

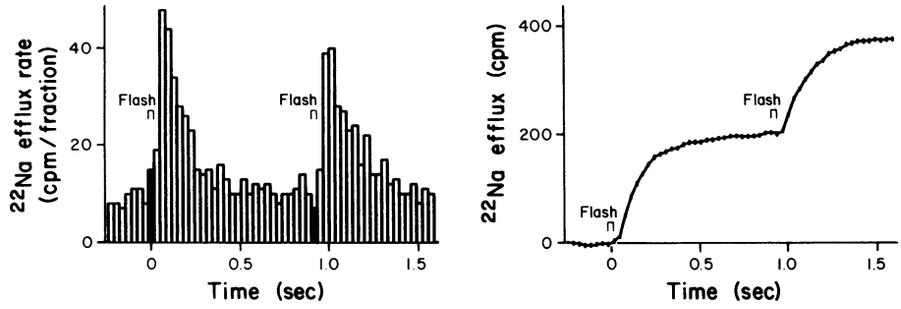

FIG. 2. Na efflux after two light flashes at 15°C. Fluid was collected for one cycle of the turntable during which two flashes were delivered, as shown by solid bars. (*Left*) ^{22}Na efflux rate reflected as radioactivity in each cuvette. (*Right*) Total ^{22}Na efflux as a function of time, calculated as the integral of the ^{22}Na efflux rate (*Left*), subtracting the background efflux prior to the flash.

the concentration of released ATP, it is reasonable to propose that the ^{22}Na efflux burst represents the operation of a single turnover of the Na pump. This hypothesis is supported by other experiments discussed below.

For the "signal averaging" approach to provide useful data, it is important that each of the efflux cycles in a run have the same time course. This was tested in an experiment in which fluid was collected for only one revolution of the turntable; two holes in the turntable skirt admitted two flashes in the single cycle. As shown in Fig. 2, within the scatter in the data there appears to be no significant difference between the first two ^{22}Na efflux bursts or between these bursts and the six averaged bursts shown in Figure 1*A*. In Fig. 2 (*Right*) is shown the integral of the ^{22}Na efflux rate with time, or the total efflux resulting from the flash; the preflash baseline has been subtracted. The data also show that the efflux rate returns to the baseline after a flash: note that a maintained increase in rate would be obscured in Fig. 1*A* by the cyclic nature of the signal averaging process.

The temperature dependence of the single turnover burst of ^{22}Na efflux is illustrated in Fig. 3. Since at 37°C it is expected that a single turnover would be complete in ≈7 msec (14), Fig. 3*A*, showing ^{22}Na efflux at this temperature, is presumed to illustrate the time response of the rapid filtration apparatus. As seen here, the time delay from "flash to cuvette" is about one cuvette period, or about 30 msec, while the dispersion is also limited to one cuvette period. Thus, the results at lower temperatures, shown in Fig. 3 *B–D*, are clearly not limited by the apparatus. As the temperature is lowered from 15°C to 10°C and to 5°C, the decay of the ^{22}Na efflux burst is slowed markedly: at these temperatures, the first-order rate constant is 6.0, 3.7, and 2.3 sec^{-1} (from semilogarithmic plots similar to those in Fig. 4, not shown). Note that at 5°C the actual rate constant may be lower than observed: in the cyclic signal averaging process, efflux maintained longer than 1.8 sec simply results in an increased background.

It is also apparent from the results shown in Figs. 2 and 3 that the rate of ^{22}Na efflux does not reach a peak instantaneously after the flash but is delayed longer than can be explained by the characteristics of the apparatus (seen at 37°C). Although these data do not afford much precision, from the intercept of the semilogarithmic plots of the total ^{22}Na efflux, the time delay is 18, 37, and 63 msec at 15°C, 10°C, and 5°C, respectively (in addition to the delay in the apparatus seen at 37°C). The significance of the biphasic nature of the time course will be discussed below.

It is important to compare the rate constant for decay of Na efflux in the single turnover to the expected steady-state rate of pump turnover. When the Na,K-ATPase activity of vesicles rendered permeable by detergent treatment (15) was measured under conditions (1 mM Na^+/0.1 mM K^+/2 mM Mg^{2+}/2 mM caged ATP/10 mM glutathione/50 μM [γ-^{32}P]ATP, 15°C) similar to those of the rapid efflux experiments and compared to the activity under optimal conditions (100 mM Na/25 mM K/4 mM Mg·ATP, 37°C) the ratio was found to be 0.0035. Since the maximal turnover rate of the Na pump is ≈150 sec^{-1} at 37°C (14), this indicates that the steady-state turnover rate under the conditions of Fig. 1 is ≈0.5 sec^{-1}, which is lower than the observed rate constant for decay of Na efflux (6 sec^{-1}) by a factor of ≈10. Thus, the time course of Na efflux in a single turnover is consistent with extracellular Na release from the Na pump as an early step in the pump cycle. In the same set of experiments, Na,K-ATPase activity measured under optimal conditions at 15°C was found to be 0.036 of the activity at 37°C, indicating that the maximal steady-state turnover rate at 15°C is ≈5 sec^{-1}. Under these conditions, not yet attainable in the rapid

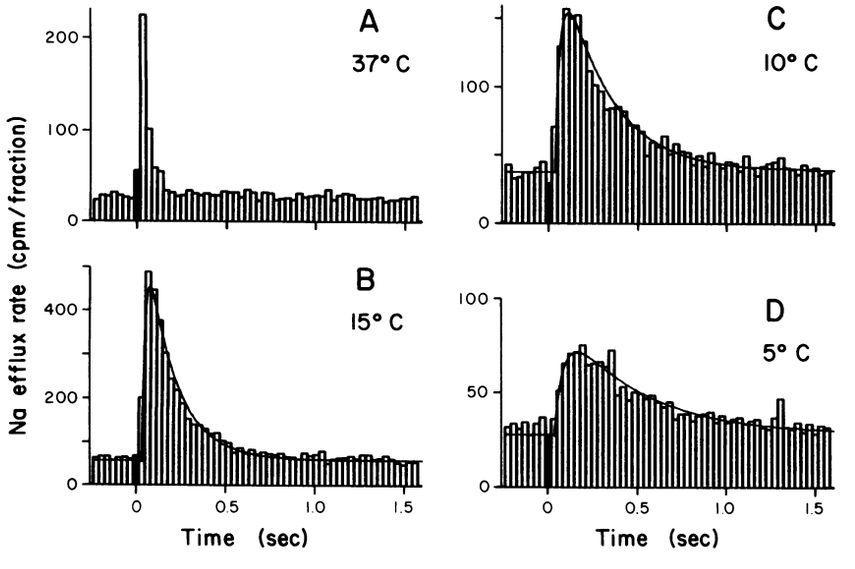

FIG. 3. Na efflux burst at various temperatures. Procedures were as in *Methods* and in previous figures. (*B–D*) Time courses are from a single experiment; 8 cycles were summed in each run. (*A*) The 37°C run is from a different experiment in which ^{24}Na (9 Ci/mol) was used and 16 cycles were summed. The continuous lines are analytical solutions to the two-step reaction sequence $X \xrightarrow{k_1} Y \xrightarrow{k_2}$ and 14 sec^{-1} for k_1 and 6.0, 3.7, and 2.3 sec^{-1} for k_2 at 15°C, 10°C, and 5°C, respectively, and with arbitrary baselines; note that the same solutions are obtained if the values of k_1 and k_2 are interchanged.

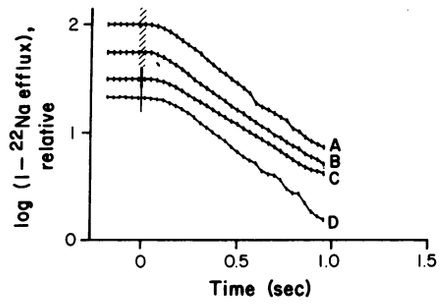

FIG. 4. Na efflux at different levels of released ATP at 10°C. The amount of light delivered in each flash was varied by decreasing the shutter opening or by increasing the effective light intensity (by decreasing the amount of optical filtering at 310 nm). The total amount of light delivered in each was kept reasonably the same from one run to the next by reciprocally varying the number of cycles for which fluid was collected. To ensure that a slow process was not missed, a flash was given only every 3.6 sec, fluid being collected in every other cycle (33.3 rpm). The ^{22}Na efflux rate was integrated with time, subtracting the rate prior to the flash (as in Fig. 3 *C* and *D*), and the data are plotted as log (total release − release$_t$), separated on the log axis for clarity. Curves: A, flash intensity 1.5, duration 32 msec, 3 cycles averaged; B, intensity 1.0, duration 32 msec, 6 cycles (as in Fig. 1); C, intensity 1.0, duration 11 msec, 15 cycles; D, intensity 1.0, duration 4.5 msec, 30 cycles.

efflux experiments, either Na efflux events must be rate limiting or, more likely, the rate constant of Na release must be substantially higher than observed in the experiment of Fig. 1.

If Na release from extracellular sites is an early step in the pump cycle, the simplest models predict that release in the first turnover of the pump would be unaffected by extracellular Na^+ or K^+. In fact, that is what was found: in three separate experiments (not shown) in which duplicate runs were carried out at each K^+ concentration, there was no significant difference in the time course of ^{22}Na efflux burst from that shown in Fig. 1, whether the efflux medium contained 0, 0.1, or 10 mM K^+, or 0 or 100 mM Na^+ (0.1 mM K^+). This result is not easily reconciled with simple concerted models of pump action in which Na^+ efflux and K^+ influx are mediated in the same step of the pump cycle.

Single vs. Multiple Turnovers. Since, under the conditions used in the present experiments, the duration of the ^{22}Na efflux burst is ≈1/10th that of the estimated steady-state turnover time of the Na,K-ATPase, it does not seem likely that multiple turnovers of the pump could occur during the observed efflux burst. However, the possibility of multiple turnovers is an important point and deserves further consideration. Thus, it is useful to compare the number of Na ions extruded after a flash to the number of available active pumps. The amount of ^{22}Na released after each flash in the experiment of Fig. 1*A* was ≈13 pmol per mg of protein. Even if it is assumed that only 10% of the vesicles contain caged ATP and thus only 10% of the pumps contribute to the burst (see *Methods*), the relevant ratio of ions pumped to membrane protein is only 130 pmol per mg of protein involved per flash. Since the catalytic site concentration is 500–1000 pmol/mg in these membranes [from maximal [^3H]ouabain binding after $NaDodSO_4$ treatment to permeabilize vesicles (7, 12); there is no reason to believe that a significant fraction of pumps that are "counted" by this procedure would be inactive in the intact vesicles], this corresponds to 0.13–0.26 ions transported per catalytic site per flash, well below the ratio of 3 ions per site maximum expected if one flash supplied enough ATP to fuel all of the pumps.

A second estimate of the fraction of pumps involved after each flash can be obtained by comparing the fraction of intravesicular Na (1 mM) that is released to an estimate of the intravesicular pump concentration (100–300 μM; see ref. 7). We have regularly compared the amount of ^{22}Na extruded in a run of six to eight flashes (Fig. 1) to the amount of ^{22}Na released in a 2-sec exposure that photolyzed all of the caged ATP (for an example, see ref. 7) and found that only 0.5–0.7 of the "extrudable ^{22}Na" is released in eight flashes. This indicates that 65–85 μmol of intravesicular Na per liter is extruded in each cycle, or 0.2–0.9 ions per pump per flash, in reasonable agreement with the above estimate. Thus, although we have only a rough estimate of vesicular contents, using either of the two approaches it is seen that after a flash, a single turnover by <30% (4%–30%) of the available pumps is required to account for the efflux burst.

Finally, the multiple turnover hypothesis was further tested in the following experiments. If multiple turnovers occurred, it would be expected that the length of the burst of Na release would depend on the amount of ATP released and, hence, on the intensity and duration of the flash. Thus, experiments were carried out in which the length and intensity of the flash were varied, and the time course of ^{22}Na efflux was examined. The data from one of four experiments with similar results are shown in Fig. 4, plotted in logarithmic format for purposes of comparison. It is seen that over a 10-fold range in amount of ATP released per flash, there is no systematic change in the time course of ^{22}Na efflux; this is the result expected if ≤1 turnover were involved. [If the efflux rate were proportional to ATP concentration, similar time courses would be expected for different ATP concentrations, even for multiple turnovers. However, we have obtained the same result shown in Fig. 4 when there was no K^+ in the extravesicular medium, and under these conditions the Na^+ pump is saturated at micromolar levels of ATP (ref. 1; confirmed in unpublished studies of Na,K-ATPase activity for 2 mM caged ATP/1 mM Na/10–100 μM ATP).] In addition, the results at the lowest ATP releases (Fig. 4, curve D) extend the quantitative considerations to the point where only ≤5% of available pumps (≤0.5% of total pumps) must turn over once to account for the burst (from the magnitude of the ^{22}Na efflux; not shown).

Caged ATP Interactions with the Na Pump. Two possible modes of interaction of caged ATP with the Na pump must be considered in relation to the observed time course. The first of these, inhibition of the Na pump by products of the photoreaction, is easily ruled out. Kaplan *et al.* (8) have shown that in the presence of an excess of glutathione, photolysis of 1 mM caged ATP does not lead to loss of either Na,K-ATPase activity or Na pump activity; in the present case, a single flash involves less than one-tenth this much photoreaction. Furthermore, even if a photoproduct were to inhibit the Na pump stoichiometrically, by the above calculation only 10%–20% of the pumps would be inhibited in one flash.

The possibility that unphotolyzed caged ATP binds to the catalytic site has not been previously investigated. This was examined in an experiment testing the ability of caged ATP to compete with the high affinity binding of [^3H]ATP to Na,K-ATPase. It was found that caged ATP binds to Na,K-ATPase in the absence of Mg^{2+} with an affinity of ≈43 μM (data not shown; the affinity for [^3H]ATP was ≈0.5 μM). The significance of this with regard to ^{22}Na efflux experiments is that at the start of each cycle the catalytic sites of the Na pumps are already occupied by unphotolyzed caged ATP. As a result of a 30-msec flash, only ≈2% of the bound caged ATP molecules are converted to ATP, so that contribution to ^{22}Na efflux from ATP produced at catalytic sites is relatively small. Instead, most of the ATP is produced in solution, but before it can bind to Na pumps, unphotolyzed caged ATP must dissociate. Since the binding of caged ATP is much weaker than that of ATP, it is likely that the rate of caged ATP dissociation is at least as fast as that of ATP un-

der similar conditions (i.e., 20–30 sec^{-1}; 20–25°C; refs. 2 and 3); if so, the apparent rate constant of E·ATP formation is estimated to be at least 50 sec^{-1} in the following calculation. Note that because the concentration of available ATP is small compared to the number of sites, the apparent rate constant (the inverse of the observed pseudo-first-order time constant) for E·ATP formation can exceed the rate constant for caged ATP dissociation. The analytical solution is not a single exponential; the apparent time constant can be estimated from the initial rate of E·ATP formation ($v_{E\cdot ATP}$). Free sites are assumed to become available as caged ATP dissociates at a rate of 20 sec^{-1}. Caged ATP and ATP compete for the sites in proportion to the product of their concentration ratio ([released ATP]/[caged ATP] = 0.02) and the ratio of their association rate constants; if the dissociation rate constants are assumed to be similar, the latter is given by the inverse ratio of K_a values (43 μM/0.5 μM = 86). Thus, the rate of E·ATP formation is $\approx 20\ sec^{-1} \times v_{E\cdot ATP}/(v_{E\cdot ATP} + v_{E\text{-caged ATP}}) = 20\ sec^{-1} \times 1.7/(1 + 1.7) = 12\ sec^{-1}$. Assuming that the concentration of sites is 250 μM, and the concentration of released ATP is 50 μM, the apparent rate constant of E·ATP formation will be $12 \times 250/50 = 60\ sec^{-1}$. Alternatively, a slightly higher apparent rate constant is obtained by assuming that caged ATP binding is in rapid equilibrium and that the expected rate of E·ATP formation (4) is decreased by the ratio of free catalytic sites to total sites.

Significance of the Biphasic Time Course. The simplest model to fit the biphasic ^{22}Na efflux time course seen in Figs. 3 and 4 is a two-step irreversible process, $X \xrightarrow{k_1} Y \xrightarrow{k_2} Z$, in which the second step involves Na release. In Fig. 3, the continuous lines are analytical fits to this model, where k_1 is 50, 23, and 14 sec^{-1} and k_2 is 6.0, 3.7, and 2.3 sec^{-1} at 15°C, 10°C, and 5°C, respectively. The k_1 rate constant presumably lumps several steps together, including at least dissociation of unphotolyzed caged ATP from the catalytic site, ATP binding, and phosphorylation. Thus we have

$$\underset{\text{caged ATP}}{E\cdot Na} \xrightarrow{k_1} E\cdot Na_o \overset{k_2}{\searrow} \underset{Na}{E}$$

where no attempt has been made to note ligands other than caged ATP and Na. It should be noted that in the analytical solution to the two-step model, the rate constants k_1 and k_2 are symmetrical in their contribution to the ^{22}Na efflux time course, so it is not possible by inspection of a time course to decide whether k_1 or k_2 is more rapid. We have investigated the question of whether the dissociation of caged ATP and binding of ATP is rate limiting in k_1. In the case in which binding of ATP is slowed by competition with free unphotolyzed caged ATP, the apparent rate constant for E·ATP formation should vary inversely with the unphotolyzed caged ATP concentration. [Note that while the absolute rate of formation of E·ATP will increase in proportion to the amount of ATP released in the flash, the "apparent rate constant" (the inverse of the observed pseudo-first-order time constant) should not, because the amount of E·ATP to be formed also increases.] This was tested in an experiment (at 10°C, not shown) in which the concentration of caged ATP in the loading solution was either 1 mM or 10 mM, and the flash duration was reciprocally varied so that the amount of ATP released was approximately the same for each sample. No significant difference was observed between the time courses of ^{22}Na efflux and the time courses shown in Fig. 4 (5 mM caged ATP), suggesting that the formation of E·ATP is not a rate-limiting step in either phase of the ^{22}Na efflux burst.

Summary. The present results represent measurements of ^{22}Na efflux in a single turnover of the Na pump. That only a single turnover is involved is shown by (*i*) comparison of the time course with the estimated steady-state turnover rate, (*ii*) comparison of the amount of Na extruded with the number of catalytic sites, and (*iii*) the finding that the duration of the burst is not dependent on the amount of ATP released in the flash (in the range 10–100 μM). The observed time course of the efflux burst in the temperature range of 5°C–15°C is consistent with models in which Na^+ is released early in the pump cycle; the absence of an effect of extracellular K^+ on the time course of Na efflux is also consistent with models in which Na^+ transport precedes K^+ binding. In these experiments, the presence of excess unphotolyzed caged ATP has presented an ambiguity, because the compound can bind to the high affinity nucleotide binding site of Na,K-ATPase. From the present data, it is not possible to exclude the possibility that caged ATP dissociation is a factor in one of the two phases of the ^{22}Na efflux burst. In future work, the use of high energy flash lamps should enable the photolysis of all of the caged ATP within a single flash, thereby generating ATP within the catalytic site. It is hoped that parallel studies of the time course of K^+ movement and of the binding and release of occluded ions also will be possible so that the mechanism of coupling of Na^+ and K^+ transport can be understood in relation to the enzymatic cycle.

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