Na/K Pump Current in Aggregates of Cultured Chick Cardiac Myocytes

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ABSTRACT Spontaneously beating aggregates of cultured embryonic chick cardiac myocytes, maintained at 37°C, were voltage clamped using a single microelectrode switching clamp to measure the current generated by the Na/K pump (I_p). In resting, steady-state preparations an ouabain-sensitive current of 0.46 ± 0.03 $\mu A/cm^2$ ($n = 22$) was identified. This current was not affected by 1 mM Ba, which was used to reduce inward rectifier current (I_{K1}) and linearize the current-voltage relationship. When K-free solution was used to block I_p, subsequent addition of K_o reactivated the Na/K pump, generating an outward reactivation current that was also ouabain sensitive. The reactivation current magnitude was a saturating function of K_o with a Hill coefficient of 1.7 and $K_{0.5}$ of 1.9 mM in the presence of 144 mM Na_o. The reactivation current was increased in magnitude when Na_i was increased by lengthening the period of time that the preparation was exposed to K-free solution prior to reactivation. When Na_i was raised by 3 μM monensin, steady-state I_p was increased more than threefold above the resting value to 1.74 ± 0.09 $\mu A/cm^2$ ($n = 11$). From these measurements and other published data we calculate that in a resting myocyte: (*a*) the steady-state I_p should hyperpolarize the membrane by 6.5 mV, (*b*) the turnover rate of the Na/K pump is 29 s^{-1}, and (*c*) the Na influx is 14.3 $pmol/cm^2 \cdot s$. We conclude that in cultured embryonic chick cardiac myocytes, the Na/K pump generates a measurable current which, under certain conditions, can be isolated from other membrane currents and has properties similar to those reported for adult cardiac cells.

INTRODUCTION

The sodium/potassium pump (Na/K pump) is an active transport mechanism that is generally believed to be electrogenic because for each molecule of ATP that is hydrolyzed 3 Na ions are transported out of the cell in exchange for 2 K ions moving into the cell. The rate of pumping is stimulated by Na_i and K_o such that perturbations of these ions away from their steady-state levels increases or decreases pump activity accordingly.

The Na/K pump generates a measurable current, which can modulate cardiac activity (for review, see Eisner et al., 1984). In voltage-clamped Purkinje fibers,

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Na/K pump blockade leads to an increase in intracellular Na activity (a^i_{Na}) and an inward deflection of the holding current (I_h), while reactivation results in a slowly decaying outward current (Gadsby and Cranefield, 1979; Eisner et al., 1981*a*; Cohen et al., 1987) and a parallel decay of a^i_{Na} (Eisner et al., 1981*a*). Eisner et al. (1981*a*) have shown that a^i_{Na} and the outward current decay with the same time course and that the magnitude of the current is linearly related to a^i_{Na} over the range studied. The outward current transient is completely blocked by cardiac glycosides (Gadsby and Cranefield, 1979) which further supports the idea that the current is due to Na/K pump activity. Similar changes in membrane current have been reported in guinea pig ventricular muscle (Daut and Rudel, 1982; Daut, 1983).

In cultured chick cardiac myocytes, electrogenicity of the Na/K pump was suggested by Horres et al. (1979). They found that the membrane hyperpolarized beyond the K reversal potential (E_K) when K_o was restored. These results were subsequently confirmed by Lobaugh et al. (1987) by making simultaneous measurements of a^i_K and membrane potential in response to similar manipulations. Measurements of ouabain-sensitive K influx (Lobaugh and Lieberman, 1987) and Na efflux (Wheeler et al., 1982) also suggest that the Na/K coupling ratio in this preparation is 3:2, which is consistent with an electrogenic transport mechanism. Until now there has been no direct measurement in chick cardiac myocytes of a membrane current related to the Na/K pump.

Since the Na/K pump in cultured cardiac myocytes is electrogenic, the resultant Na/K pump current (I_p) should provide a direct measure of Na/K pump turnover assuming that the coupling ratio is constant and that the pump is operating primarily in its forward mode. By measuring I_p we should then be able to determine the physiological properties and contributions of the Na/K pump to electrical activity in chick cardiac muscle. Use of cultured chick cardiac myocytes offers several advantages over intact or freshly isolated adult cardiac preparations (for review, see Horres et al., 1987). An important advantage for these studies is that the cultured chick cardiac myocyte preparation has relatively unrestricted extracellular spaces (Wheeler et al., 1982), which minimizes accumulation and depletion of K in extracellular spaces during Na/K pump stimulation or inhibition. The aims of this study are to demonstrate that (*a*) a current due to Na/K pump activity can be measured in spherical aggregates of chick cardiac myocytes and (*b*) this current reasonably quantitates Na/K pump activity in this preparation. Preliminary reports of some of these results have appeared (Stimers et al., 1986, 1989).

METHODS

Tissue Culture

11-d-old chick embryo hearts were disaggregated by a series of incubations in 0.05% trypsin (GIBCO, Grand Island, NY) as previously described (Jacob et al., 1987). The resultant cell suspension was incubated in a 10-cm culture dish (Falcon 3003; Becton Dickinson and Co., Oxnard, CA) for 1 h at a density of 5–8 × 10^6 cells/5 ml of culture medium (60% medium 199, 5% fetal bovine serum, and 2% chick embryo extract with a base of Earle's solution) to allow the preferential attachment of fibroblasts (Blondel et al., 1971). The resulting myocyte-enriched supernatant was seeded into 60-mm culture dishes (Falcon 3002; Becton Dickinson and Co.) at 1 × 10^6 cells/3 ml of culture medium. After 3–4 d in culture, spontaneously

contracting (1–2 Hz) cardiac cell aggregates formed and attached in 50–75-μm-diameter holes that were made in a thin coat of 1% agar on the bottom of the culture dishes (Ebihara et al., 1980). Aggregates were used on days 3–10 in culture.

Solutions

Control perfusate was a modified Hanks solution (H/TBSS) with the following composition in millimolar: 144 Na, 5.4 K, 0.8 Mg, 2.7 Ca, 156 Cl, 5.6 HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 4.2 Trizma Base, and 5.6 dextrose (pH 7.4 ± 0.1). Altered K solutions were made by equimolar substitution with Na. Ba and ouabain were added to solutions as indicated in the results. Monensin was dissolved in ethanol to make a 4 or 6 mM stock solution that was diluted into the final solution at a concentration of 3 μM (ethanol concentration $\leq 0.1\%$). Ethanol added to the control solution had no effect on I_h in control experiments.

A rapid perfusion system was designed to allow solutions bathing the preparation to be changed within 1–2 s. A micromanipulator was used to mount a solenoid valve close to the culture dish. A short glass capillary tube (1.0 mm i.d.), attached to the common port of the valve, served as the inlet to the culture dish. The opening of this tube was positioned within a few millimeters of the preparation so the entering solution would rapidly engulf the aggregate. Two lines, from solution reservoirs, were attached to the two inlets of the solenoid valve by T-connectors so that one line flowed through the valve into the culture dish at a rate of ~1 ml/min, while the other line drained to waste. The continuous flow of both lines allowed for proper temperature regulation even when the solution was changed. All experiments were performed at 37°C.

Voltage-Clamp Technique

A switching single microelectrode voltage-clamp amplifier (model 8100; Dagan Corp., Minneapolis, MN) was used to perform the electrophysiological experiments. Microelectrodes with tip resistances of <10 MΩ were used to impale the aggregates. The low resistance was necessary to obtain both an adequate frequency response of the clamp and to provide sufficient current to the membrane. A switching frequency of 1 kHz was used in all experiments. To further improve the performance of the voltage clamp, 1 mM Ba was added to all solutions, except for the experiment shown in Fig. 1 *A*, which illustrates the fundamental observation. The effects of Ba were to reduce the current through the inward rectifier (I_{K1}) (Standen and Stanfield, 1978; DiFrancesco, 1981; Cohen et al., 1983; DiFrancesco et al., 1984; Shah et al., 1987) and to stabilize the clamp by linearizing and flattening the current-voltage relationship (unpublished observations). All experiments were done with a holding potential of −70 mV, which is near the resting potential of these preparations. An additional benefit of holding at −70 mV is that since experiments were done with the membrane potential near the K equilibrium potential this would tend to minimize K channel currents.

Both membrane potential and current were filtered at 300 Hz and continuously recorded on video tape using a Digital Acquisition and Storage System (Unitrade Inc., Philadelphia, PA). Portions of the data were later transferred to an IBM PC/AT microcomputer using AXOLAB (Axon Instruments, Inc., Burlingame, CA) for analysis. Currents are shown using the standard convention of inward current being negative.

To compare data between preparations we normalized for membrane area by measuring both the input resistance and time constant of a voltage relaxation in response to a 1–5 nA current step and by calculating the capacitance of each preparation. Capacitance was converted to membrane area by assuming a specific capacitance of 1.3 μF/cm^2 (Mathias et al., 1981). Statistical comparisons of data were made with a paired *t* test using Statgraphics (STSC Inc., Rockville, MD).

RESULTS

Response to K-free Solution or Ouabain

When an aggregate of cultured cardiac myocytes was voltage clamped at −70 mV and exposed to solutions that inhibit the Na/K pump (K-free or ouabain-containing solutions) I_h became more negative (Fig. 1 *A*). After a rapid solution change from H/TBSS to K-free H/TBSS the resting I_h (−1.8 nA) rapidly became more inward by −3.9 nA and then quickly returned to the control level when K_o was restored (Fig. 1 *A*, *upper trace*). This finding is consistent with a steady-state outward Na/K pump current that is blocked by removal of K_o and reactivated by the addition of

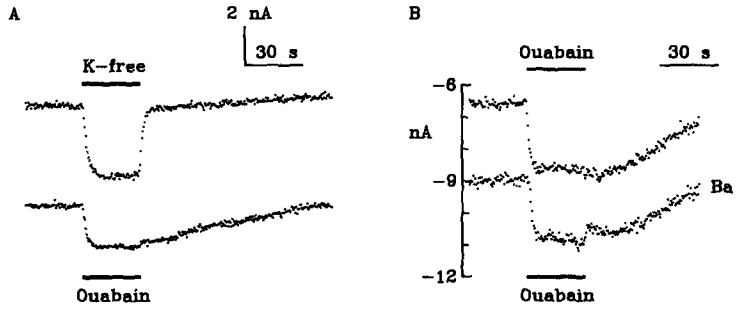

FIGURE 1. Changes in I_h after Na/K pump inhibition. (*A*) The upper trace shows the change in I_h induced by K-free perfusion. The time course of the current changes reflects the speed of the solution change. The lower trace shows the response of the same aggregate to addition and removal of 1 mM ouabain. Notice that the change in holding current induced by ouabain is smaller than the change induced by K-free perfusion and that the recovery of the current after removal of ouabain is much slower than the recovery after the addition of 5.4 mM K_o. This relatively slow recovery reflects the kinetics of ouabain unbinding. The upper trace was recorded 11 min after the lower trace. (*B*) The two current traces in this panel are plotted on the same absolute current scale. The upper trace shows the response of an aggregate to 1 mM ouabain similar to the lower trace in *A*. In the presence of 1 mM Ba (*lower trace*) I_h is more negative. Subsequent exposure to ouabain elicits a change in I_h that is similar to the change seen in the absence of Ba (*upper trace*). The lower trace was recorded 15 min after the upper trace and 7 min after the addition of 1 mM Ba. The bars at the top and bottom of each panel represent solution changes for the upper and lower traces, respectively. All currents were recorded at a holding potential of −70 mV.

K_o. However, removal of K_o can also affect other K-sensitive currents. For example, K-free solution shifts the voltage dependence of the conductance of I_{K1} so that it would be reduced in magnitude at −70 mV, producing an inward shift of I_h (Shah et al., 1987). That removal of K_o inhibits I_p and affects other K-sensitive currents is confirmed by the lower trace in Fig. 1 *A* which shows a much smaller inward shift of I_h (−2.3 nA) when the same preparation was exposed to 1 mM ouabain in H/TBSS. Notice that when ouabain was removed from the bath I_h returned to the baseline level in 3–5 min. This recovery of I_h reflects the dissociation of ouabain from the Na/K pump and has a half-time of ~1 min. The magnitude of the resting ouabain-sensitive current at −70 mV was 0.46 ± 0.05 $\mu A/cm^2$ ($n = 22$). From the average

membrane resistance of 14.3 ± 1.5 kΩ/cm^2 (n = 18) we calculate that the ouabain-sensitive current should hyperpolarize the membrane by 6.5 mV.

Before proceeding with the identification of the ouabain-sensitive current as I_p, it is necessary to consider potential artifacts that may be associated with this type of experiment. When the Na/K pump is inhibited by either K-free or ouabain-containing solution, K can accumulate in the extracellular spaces of diffusion-limited preparations. In K-free solution, accumulation of K may partially reactivate the Na/K pump. In either K-free or ouabain-containing solutions, K accumulation may also change the magnitudes of other K-sensitive currents (e.g., I_{K1} or I_f [hyperpolarizing-activated current, DiFrancesco, 1985]). In adult cardiac preparations (e.g., Purkinje fiber), Na/K pump inhibition leads to a rise in K in the intercellular spaces (Deitmer and Ellis, 1978; Eisner and Lederer, 1980; Gadsby, 1980; Eisner et al., 1981*a*; January and Fozzard, 1984). To preclude this from happening in this study, I_{K1}, a major pathway for K efflux, was blocked by adding 1 mM Ba to all solutions (Standen and Stanfield, 1978; DiFrancesco 1981; Cohen et al., 1983; DiFrancesco et al., 1984; Shah et al., 1987). Fig. 1 *B* shows that the magnitude of the ouabain-sensitive current is not affected by Ba, e.g., compare the response of the preparation when exposed to 1 mM ouabain in the absence (upper trace, −2.0 nA) or presence (lower trace, −1.9 nA) of 1 mM Ba. In four such experiments, the ouabain-sensitive current did not differ significantly (P = 0.01) in the absence (0.44 ± 0.06 μA/cm^2) or the presence (0.47 ± 0.05 μA/cm^2) of 1 mM Ba. The shift in I_h (I_h = −6.6 nA without and −9.0 nA with 1 mM Ba_o) was due to the Ba effect on the background conductance of I_{K1}.

Artifacts resulting from accumulation and depletion of K in the extracellular spaces are expected to be negligibly small in the cultured chick cardiac myocyte preparation. Wheeler et al. (1982) found that diffusion between the intercellular spaces and the bulk solution is more than 10 times faster than in other multicellular (adult) cardiac preparations. Furthermore, as shown in Fig. 2, after 5 min of total pump inhibition by K-free solution, the addition of 1 mM ouabain did not cause any change in holding current (Fig. 2 *B*). Identical results were obtained in four preparations. If K accumulation due to passive leak of intracellular K in the extracellular spaces was a significant problem we would have detected it in these experiments since the data in Fig. 3, *A* and *B* (see below) show that as little as 0.3 mM K_o produces an easily measurable activation of Na/K pump current. Since in K-free solution, ouabain had no effect on membrane currents, K accumulation within extracellular spaces must be much less than 0.3 mM. This observation also shows that ouabain does not affect K_o-insensitive membrane currents, i.e., Na and Ca currents, and that removal of K_o blocks all the ouabain-sensitive current.

K_o restoration after a period of time in K-free solution increases Na/K pump activity (Eisner et al., 1981*a*). In diffusion-limited preparations this maneuver could reduce K_o in intercellular spaces and result in artifacts due to a reduction of Na/K pump current and changes in other K-sensitive currents (Eisner and Lederer, 1980). The increased flux rate due to diffusion between intercellular spaces and the bulk solution in the cultured cardiac myocyte preparation and the presence of Ba to block I_{K1} would tend to minimize possible depletion artifacts. Indeed, we found that the magnitude of the current stimulated by addition of K_o after a prolonged period

of Na/K pump inhibition in K-free solution was maximal in 5.4 mM K_o (experiments described below), which suggests that even if there were some depletion of K_o in 5.4 mM K, it would not affect the results.

Reactivation Current Is Ouabain Sensitive

If the inward shift of I_h induced by K-free or ouabain-containing solution is due to the block of the steady-state outward I_p, then the outward current activated by restoring K_o or removal of ouabain must be caused by reactivating the Na/K pump (reactivation current). Furthermore, the reactivation current must be sensitive to ouabain and depend on K_o and Na_i. Initially, we will operationally define the magnitude of the reactivation current as the change in holding current measured when K_o is restored after exposure to K-free, Ba-containing solution. In the following sections we will establish this measurement of reactivation current as a reasonable

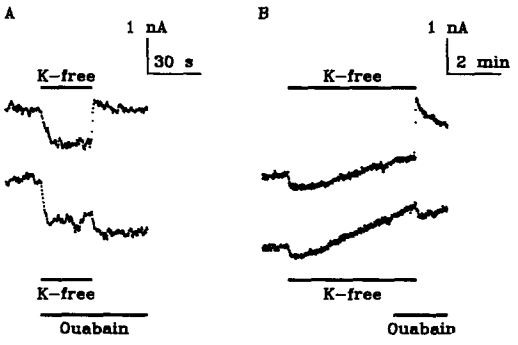

FIGURE 2. Reactivation current is ouabain sensitive. (*A*) In the upper trace the steady-state I_p was blocked by a 30-s perfusion of K-free solution. Restoring K_o to 5.4 mM reactivates I_p. In the lower trace (same aggregate, 4 min after the upper trace) the reactivation current is blocked by ouabain applied 30 s before restoring K_o. The small downward shift in I_h seen when K_o is restored in the presence of ouabain reflects a K-sensitive, ouabain-insensitive current that is recorded in the presence of 1 mM Ba. (*B*) Protocol was similar to that of *A* but with a 5-min exposure to K-free solution. The reactivation current is much larger due to the rise in Na_i (*upper trace*); however, it is still blocked by 1 mM ouabain (*lower trace*). The lower trace was recorded 12 min after the upper trace. The bars at the top and bottom of each panel represent solution changes for the upper and lower traces, respectively. All currents were recorded in the presence of 1 mM Ba and at a holding potential of −70 mV.

estimate of the current generated by reactivation of the Na/K pump. Fig. 2 *A* (*upper trace*) shows that after a brief exposure (30 s) to K-free solution (a minimal change in Na_i is expected), readdition of K_o restored I_h to the prior resting level. However, the lower trace in Fig. 2 *A* shows that 1 mM ouabain completely blocked this reactivation current. Similar results were obtained in three experiments. In Fig. 2 *B*, the upper trace shows the response of another aggregate exposed to K-free solution for 5 min (Na_i should increase significantly, see Lobaugh et al., 1987). We would expect that when normal K_o is restored, the elevated Na_i should stimulate the Na/K pump above its resting level and generate a larger outward reactivation current. This expectation was confirmed by the upper trace when restoration of K_o stimulated a large transient outward current which decayed back to the control level of I_h within 5 min (data not shown). Similar to the results in Fig. 2 *A*, prior application of 1 mM ouabain completely blocked the reactivation current (*lower trace*). When ouabain was

removed, I_h recovered to its resting level within 10 min (data not shown). Similar results were obtained in four experiments. These data show that the reactivation current after both short and long exposures to K-free solution is ouabain sensitive.

The presence of a K-sensitive, ouabain-insensitive current introduces an error in estimating the Na/K pump reactivation current by this method. The lower trace of Fig. 2 *B* shows that in the presence of 1 mM Ba and 1 mM ouabain, restoring K_o elicited a small K_o-sensitive, ouabain-insensitive current. This current may be residual I_{K1} not blocked by Ba, I_f, or an undefined passive membrane conductance. In Na-loaded cells held at −70 mV, the observed K_o-sensitive, ouabain-insensitive current is -0.09 ± 0.02 $\mu A/\mu F$ ($n = 6$), which in comparison with the upper trace in Fig. 2 *B* is a small fraction (and of opposite polarity) of the ouabain-sensitive current (2.52 ± 0.19 $\mu A/\mu F$, $n = 11$). Preliminary whole-cell patch-clamp experiments on small (two to three cell) aggregates show that the magnitude of K-sensitive, ouabain-insensitive current is linearly dependent on K_o between 0 and 10.8 mM, and increases linearly with hyperpolarization between −50 and −100 mV. Thus, measuring reactivation current as the difference in I_h between K-free and K-containing solutions will slightly underestimate the magnitude of the Na/K pump reactivation current, with the underestimate being greater for larger K_o (see Discussion).

In the continued presence of K-free solution, I_h gradually became more outward, usually surpassing its original resting level (Fig. 2 *B*). This current cannot be attributed to the reactivation of the Na/K pump by K_o accumulation because the addition of 1 mM ouabain had no effect on the current. In several experiments (data not shown), after 5 min in K-free solution, removal of Ca_o returned I_h to approximately the level of the maximum inward current attained just after the switch to K-free solution. Possible sources of this current are Na/Ca exchange operating in its reverse mode or a Ca-activated K, Cl, or nonspecific conductance.

K_o Dependence of Reactivation Current

In the next two sections we evaluate the K_o and Na_i sensitivity of the reactivation current to determine the extent to which this current provides a reasonable estimate of the ouabain-sensitive Na/K pump reactivation current. If the reactivation current is related to Na/K pump activity, then its magnitude should depend on the concentration of K_o and the apparent affinity of K_o to activate the Na/K pump. The effect of K_o on the reactivation current and the apparent affinity of K_o was measured in voltage-clamped preparations that were Na-loaded by exposure to K-free solution. Fig. 3 *A* shows that, under these conditions, I_h reached a new steady level. Then the Na/K pump was briefly activated by 30-s exposures to various concentrations of K_o (0.3–10.8 mM). The preparation was returned to K-free H/TBSS between exposures to K-containing solutions and I_h was allowed to recover to its previous level before the next K_o exposure. The difference between the current measured immediately before and after the switch to a K-containing solution is plotted vs. the concentration of K in the reactivating solution (Fig. 3 *B*). The K_o dependence of the reactivation current is best fit by the Hill equation (Hill, 1910) (Fig. 3 *B*, *inset*) with a Hill coefficient (n) of 1.7 and a $K_{0.5}$ of 1.9 mM. Considering the nature of the K-sensitive, ouabain-insensitive current described above, our method of measuring the

reactivation current could overestimate the Hill coefficient while the $K_{0.5}$ could be underestimated (see Discussion).

Na_i Dependence of Reactivation Current

The Na/K pump was stimulated by increasing Na_i using either of two protocols to raise Na_i: preparations were exposed to either K-free solution for varying durations

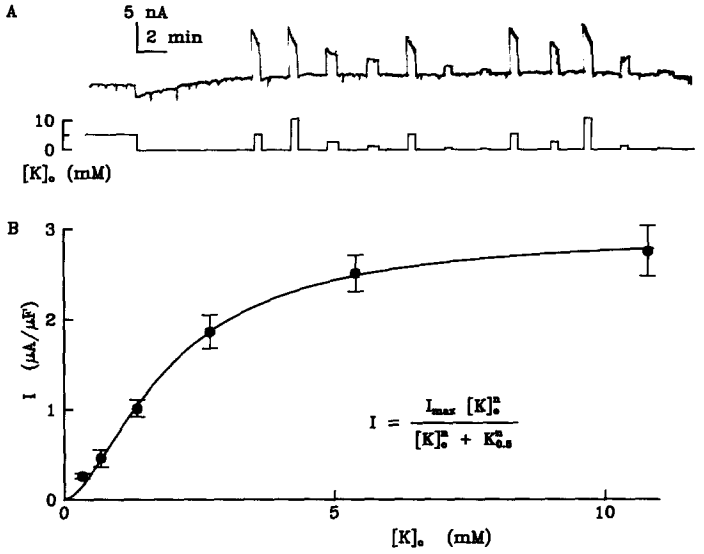

FIGURE 3. Reactivation current is K_o sensitive. (*A*) The upper trace is a continuous current record from an aggregate voltage clamped at −70 mV. Below this is a scale indicating the changes in K_o. From control conditions (5.4 mM K_o) the preparation was exposed to K-free solution for 8 min and then alternately exposed to a K-containing and K-free solution. The concentrations of the K-containing solutions in millimolar were from left to right: 5.4, 10.8, 2.7, 1.35, 5.4, 0.67, 0.34, 5.4, 2.7, 10.8, 1.35, and 0.34. This record shows that the magnitude of the peak of the reactivation current depends on the concentration of K_o. With time of exposure to K_o, the reactivation can be seen to start decaying as expected if Na_i is decreasing after Na/K pump reactivation. The decay of the reactivation current is faster with increasing K_o as expected for a substrate-limited reaction. (*B*) Data from four experiments similar to that shown in *A* were pooled and the magnitude of the reactivation current is plotted (mean ± SEM) vs. K_o. The solid curve represents the best fit of the Hill equation (*inset*) to the data. The values of the best-fit parameters were I_{max} = 2.9 μA/μF, n = 1.7, and $K_{0.5}$ = 1.9 mM.

or monensin for 5–10 min. At present, we are unable to measure a^i_{Na} in a voltage-clamped aggregate of cardiac myocytes; however, these protocols must increase Na_i above resting levels. Activity (Lobaugh et al., 1987) and ion content (Jacob et al., 1987) measurements in non–voltage-clamped preparations of cultured cardiac myocytes have shown that Na_i increased two to threefold during a 10–20 min exposure to 0.5 mM or K-free solution. In voltage-clamp experiments, after perfusion with K-free solution, I_h reached a new steady level in 5–10 min (Fig. 4 *A*, *bottom trace*).

These results suggest that the system has equilibrated and that Na_i has therefore reached a new steady level that is probably two to threefold higher than control.

Since Na_i increases when preparations are in K-free solution, I_p should also be increased when reactivated by addition of K_o. To relate the stimulation of the reactivation current to the rise in Na_i, aggregates were exposed to K-free solution for varying periods (Fig. 4 *A*). When the preparation was returned to 5.4 mM K_o solution the reactivation current increased in magnitude with increased duration in K-free solution. The magnitude of the reactivation current was measured as the difference between the current just before and at the peak just after the switch from K-free to 5.4 mM K_o solution. The data are plotted in Fig. 4 *B* as a function of the duration in K-free solution. Since this increase in magnitude of the reactivation cur-

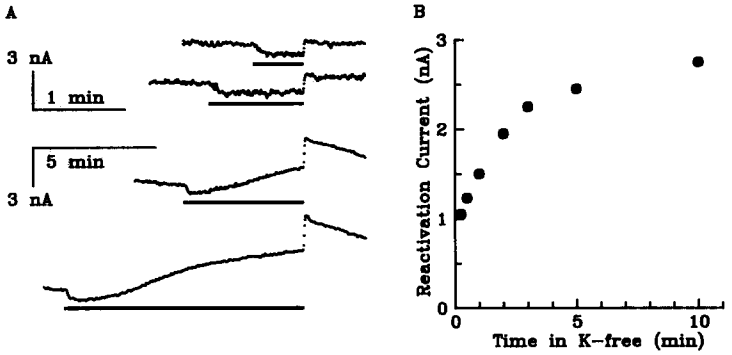

FIGURE 4. Reactivation current is Na_i sensitive. (*A*) The four records shown illustrate the effect of increasing the duration of K-free exposure on the magnitude of the reactivation current. All records are segments from a continuous recording from an aggregate voltage clamped at −70 mV and bathed in H/TBSS + 1 mM Ba. During the time indicated by the solid bar below each trace the solution was switched to K free + 1 mM Ba. On restoring K_o it can be seen that the magnitude of the reactivation current increases with increased duration of K-free solution. (*B*) The magnitude of the reactivation current vs. time in K-free solution for the same aggregate as in *A* is shown here. Similar results were obtained from more than 10 other experiments, but none were done with as complete a time course.

rent parallels the expected increase in Na_i, this observation is consistent with the reactivation current being due to Na_i stimulation of the Na/K pump.

The second method used to raise Na_i was to apply 3 μM monensin to the preparation (Hume and Uehara, 1986; Stimers et al., 1990) and compare the ouabain-sensitive current with and without monensin (Fig. 5). Without monensin, the ouabain-sensitive current was −1.4 nA. After washing out ouabain, application of monensin caused a marked outward shift in I_h (compare I_h in the two traces). In the presence of 3 μM monensin, application of 1 mM ouabain caused a much larger change in I_h, −6.0 nA. Notice that with and without monensin the absolute current level in the presence of ouabain was nearly identical. As described for the resting value of I_p previously, we calculate that I_p stimulated by 3 μM monensin was 1.74 ± 0.09 $\mu A/cm^2$ (n = 11), more than a threefold increase over the resting I_p. In summary, the data shows that stimulation of a ouabain-sensitive current by elevating Na_i is consistent with identifying this current as the steady-state Na/K pump current.

DISCUSSION

Identification of Na/K Pump Current

We have presented evidence that the ouabain-sensitive current measured in an aggregate of cultured chick heart cells voltage clamped at −70 mV has properties consistent with the Na/K pump current under both steady-state resting conditions and when the preparation is Na loaded. To identify a membrane current as being due to Na/K pump activity, the known physiological properties of the Na/K pump must be reflected by this current in predictable ways. First, Na/K pump activity should be a saturable function of K_o (as expected for an enzyme substrate reaction). We found I_p to be half maximally stimulated by 1.9 mM K_o, in agreement with previous measurements of 2–2.7 mM K_o in this laboratory (Lieberman et al., 1982; Lobaugh et al., 1987) as well as with values of 0.8–10 mM K_o reported in the literature for other cardiac preparations (Deitmer and Ellis, 1978; Gadsby, 1980; Glitsch et al., 1981; Eisner et al., 1984; Cohen et al., 1987). In all cases, K_o or Rb_o activation of the Na/K pump appeared hyperbolic despite the fact that a sigmoidal

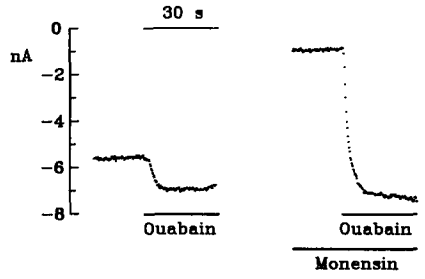

FIGURE 5. Ouabain-sensitive current is stimulated by monensin. The two records are segments of a continuous recording from an aggregate voltage clamped at −80 mV and bathed in H/TBSS + 1 mM Ba. Both records are plotted on the same absolute current scale. The left trace shows the response to the addition of 1 mM ouabain (*bar*). After the removal of ouabain, I_h recovered to the baseline level of −5.6 nA (not shown). Addition of 3 μM monensin, 5 min before the trace shown on the right, caused a large outward shift in I_h. Addition of 1 mM ouabain in the continued presence of monensin caused I_h to return to nearly the same level as ouabain did without monensin.

relation is expected from two external K-binding sites (Baker et al., 1969; Robinson and Flashner, 1979). Data obtained from cultured chick cardiac myocytes grown either as aggregates (this report) or confluent mass cultures (Lobaugh et al., 1987) demonstrate a sigmoidal relationship between I_p and K_o with a Hill coefficient of ~2. Baker et al. (1969) discussed several artifacts in some of their data that could account for a decrease in the Hill coefficient. Similar artifacts may have also been present in the work on other cardiac preparations cited above.

A potential problem with our measurement of reactivation current as the change in I_h measured when K_o is changed from K-free to K-containing in the presence of 1 mM Ba is that of a small K-sensitive, ouabain-insensitive current that has an opposite polarity to that of the Na/K pump reactivation current. We know this current is -0.09 ± 0.02 μA/μF ($n = 6$) when switching between K-free and 5.4 mM K_o and that it is linearly dependent on K_o between 0 and 10.8 mM. From this we can calculate a correction for the data shown in Fig. 3 *A* for each concentration of K_o used. Fitting the Hill equation to the corrected data we obtain a Hill coefficient of 1.6 and

$K_{0.5}$ of 2.1 mM. Since these numbers are very near those calculated from the raw data we conclude that the K-sensitive, ouabain-insensitive current is negligible in these experiments. If the K-sensitive, ouabain-insensitive current were significantly larger than that measured in our experiments, the Hill coefficient would tend toward 1, giving a hyperbolic shape to the K_o vs. reactivation current curve. In the absence of Ba, to block I_{K1}, the K_o-sensitive currents were much larger (Fig. 1 *A*). In this case the reactivation current would have been a poorer estimate of the Na/K pump reactivation current.

The activity of an electrogenic Na/K pump should also depend on the concentration of Na_i as has been shown in other cardiac preparations (Eisner et al., 1981*a*; Glitsch et al., 1982; Gadsby and Nakao, 1986; Sejersted et al., 1988). Using two methods to increase Na_i, duration in K-free and monensin exposure, we could demonstrate that I_p increases as a result of maneuvers expected to increase Na_i. While we are unable to quantify the Na_i dependence of I_p at this time, the manipulations of Na_i produced changes in I_p that are consistent with an effect on the Na/K pump. The one discrepancy between our results and the results in other cardiac preparations is that we did not find a single-exponential decay of the reactivation current after perfusion with K-free solution (Gadsby and Cranefield, 1979; Eisner and Lederer, 1980; Eisner et al., 1981*b*). Since a time-dependent outward current, which appears to be a Ca-dependent mechanism (Na/Ca exchange or a Ca-activated conductance), was clearly activated by K-free perfusion (Figs. 2 *B*, 3 *A*, and 4 *A*), this current must contribute a time-varying component as the reactivation current decayed back to the control level. Others have used the time course of decay of the reactivation current as a measure of Na/K pump activity (Eisner et al., 1981*b*; Glitsch et al., 1982); however, we were unable to make this measurement in our preparation because the Ca-dependent current decays concurrently with the reactivation current. Whether the Ca-dependent current is larger in chick cardiac myocytes than in other cardiac preparations requires further study.

Ouabain sensitivity of I_p was demonstrated in several ways: (*a*) ouabain blocked the reactivation current generated when K was restored to a K-free medium; (*b*) ouabain blocked the same current that was blocked by the removal of K_o in the presence of 1 mM Ba; (*c*) ouabain did not change I_h when applied to preparations already in K-free solution; and (*d*) ouabain had a dose-dependent effect on I_p (Stimers et al., 1990). Thus, these data support the identification of the measured current as Na/K pump current.

Steady-State Properties of the Na/K Pump

We were able to measure the steady-state properties of the Na/K pump because the rapid perfusion system allowed changes of solution within 1–2 s. When a preparation was exposed to 1 mM ouabain, the resultant change in I_h reflects the block of a steady-state outward I_p. This change in I_h reached a steady-state within 20 s. If we assume that no change in Na_i occurred in this time (limitations of this assumption will be discussed below), then the change in I_h is a measure of the resting I_p, which was 0.46 ± 0.05 $\mu A/cm^2$ ($n = 8$). This value for resting I_p agrees well with 0.31 $\mu A/cm^2$ calculated from rate of change in a^i_{Na} (Lobaugh et al., 1987). In other cardiac preparations, estimates of resting I_p range from 0.15 to 0.8 $\mu A/cm^2$ (Eisner et al.,

1981*a*; Daut and Rudel, 1982; Glitsch and Krahn, 1986; Cohen et al., 1987). Multiplying the resting I_p by the membrane resistance measured at −70 mV, we calculate that under control conditions I_p should hyperpolarize the membrane by 6.5 mV, close to calculated values of 4.2 mV for isolated canine Purkinje myocytes (Cohen et al., 1987) and 5 mV for sheep Purkinje fiber (Glitsch and Krahn, 1986). The contribution of the Na/K pump to the resting membrane potential should be small, but at depolarized potentials, i.e., during the plateau of the action potential, when the membrane resistance is higher than at rest, the Na/K pump should have a larger hyperpolarizing effect on the membrane potential (for reviews, see DeWeer, 1986; Cranefield and Aronson, 1988). Thus, stimulation of I_p should foreshorten the cardiac action potential (Gadsby and Cranefield, 1979). In support of this conclusion, action potentials in Na/K pump-induced preparations were found to be shorter in comparison with those measured in control preparations because of the enhanced activity of the Na/K pump (Lobaugh et al., 1987).

If we assume that the Na/K coupling ratio is constant and that the Na/K pump is operating primarily in its forward mode, then from the resting value of I_p, other steady-state parameters can be calculated using constants previously measured in this preparation. A resting turnover rate of the pump can be calculated from the resting pump current as follows:

$$R = N_A I_p/(D_p F)$$

where N_A is Avogadro's number, D_p is the Na/K pump site density that equals 1,000 μm^{-2} (Lobaugh and Lieberman, 1987), and F is 96,500 coulombs. We calculate that the resting turnover rate of the Na/K pump is 29 s^{-1}. This value is comparable to 39 s^{-1} calculated from K influx data in confluent mass cultures of cardiac myocytes (Lobaugh and Lieberman, 1987) and 19 s^{-1} calculated from the initial rate of change a^i_{Na} in polystrands of cultured cardiac myocytes in response to K-free solution (Lobaugh et al., 1987).

Na Influx during Na/K Pump Blockade

Steady-state Na extrusion by the Na/K pump must be balanced by an opposite influx of Na through other mechanisms. Assuming a coupling ratio of 3:2, Na influx is estimated to be 14.3 pmol/cm^2·s. This is similar to the values reported in polystrand preparations of chick cardiac myocytes of 16 pmol/cm^2·s measured as ouabain-sensitive Na efflux (Wheeler et al., 1982) and 9.6 pmol/cm^2·s calculated from initial rates of a^i_{Na} change after Na/K pump blockade by ouabain (Lobaugh et al., 1987). During total Na/K pump blockade this influx becomes unbalanced and causes an increase in Na_i. Assuming a volume to surface area (V/A) ratio of 1.06×10^{-4} cm (Horres et al., 1977), we estimate the rate of change in Na_i as 8.1 mM/min, a value that is comparable to 5.8 mM/min which was measured with Na-selective microelectrodes in cultured chick cardiac myocytes (Lobaugh et al., 1987). While this is higher than the value measured in canine Purkinje fibers (i.e., 1.1 mM/min, Cohen et al., 1987), this can be largely accounted for by the fact that the V/A ratio is 4.5-fold higher in Purkinje fibers than in cultured cardiac myocytes.

Stimulated Na/K Pump Activity

In the presence of 3 μM monensin, I_p is stimulated more than threefold to 1.74 ± 0.09 μA/cm^2 (n = 11), which corresponds to a turnover rate of 109 s^{-1}. Previous measurements of K influx in these cells estimated the maximal turnover rate to be 94 s^{-1} (Lobaugh et al., 1987). These values of maximum turnover rate agree well with 100 s^{-1} found in sheep Purkinje fiber (Sejersted et al., 1988). Although monensin significantly elevates Na_i, it is possible that the Na sites on the Na/K pump may not have been fully saturated in these experiments and so this must be considered a minimum estimate of the maximal turnover rate. While higher concentrations of monensin elevate Na_i to values beyond that induced by 3 μM monensin (Stimers et al., 1990), serious problems with contraction were encountered and impalements were readily lost when, for example, 6 μM monensin was used.

In summary, we have measured a membrane current in voltage-clamped aggregates of cultured chick cardiac myocytes generated by the Na/K pump. The properties of this current are those expected for I_p and are consistent with those reported by others. However, two important differences between our results and those previously reported in other cardiac preparations were found: (*a*) the dependence of I_p on K_o was found to be sigmoidal rather than hyperbolic and (*b*) the decay of the reactivation current did not follow a single-exponential time course. The first difference probably reflects the better diffusion characteristics of the cultured cells, while the second is likely to be a species difference due to an apparently greater Ca-dependent current mechanism. Two areas need further investigation because other membrane currents, which have not been fully identified yet, are activated by elevated Na_i or large potential steps. First, as mentioned above, the time course of the reactivation current decay is complicated by an apparently Ca-dependent current. Second, we have not investigated the nature of the voltage dependence of I_p because of the difficulty in controlling the relatively large currents (compared with the magnitude of the Na/K pump current measured at −70 mV) activated at hyperpolarized (< −100 mV) and depolarized (>0 mV) potentials. This limitation is due at least in part to technical limitations of the switching (single microelectrode voltage clamp to sink and source large currents) and to signal-to-noise ratio artifacts resulting from the necessary subtraction of two large currents to obtain the small difference current due to the Na/K pump. Experiments must be designed to identify and control membrane conductances that are altered by changes in ionic concentrations caused by electrogenic and electroneutral ion transport mechanisms and the ion channels that exist in cardiac cells.

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