Transmembrane Chloride Flux in Tissue-cultured Chick Heart Cells

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ABSTRACT To evaluate the transmembrane movement of chloride in a preparation of cardiac muscle lacking the extracellular diffusion limitations of natural specimens, intracellular chloride concentration ($[Cl]_i$) and transmembrane ^{36}Cl efflux have been determined in growth-oriented embryonic chick heart cells in tissue culture. Using the method of isotopic equilibrium, $[Cl]_i$ was 25.1 ± 7.3 mmol·(liter cell water)$^{-1}$, comparable to the value of 24.9 ± 5.4 mmol·(liter cell water)$^{-1}$ determined by coulometric titration. Two cellular ^{36}Cl compartments were found; one exchanged with a rate constant of 0.67 ± 0.12 min^{-1} and was associated with the cardiac muscle cells; the other, attributed to the fibroblasts, exchanged with a rate constant of 0.18 ± 0.05 min^{-1}. At 37°C, transmembrane Cl flux of cardiac muscle under steady-state conditions was 30 pmol·cm^{-2}·s^{-1}. In K-free, normal, or high-K_o solutions, the responses of the membrane potential to changes in external Cl concentration suggested that chloride conductance was low. These results indicate that Cl transport across the myocardial cell membrane is more rapid than K transport and is largely electrically silent.

INTRODUCTION

Evaluation of anion transport in cardiac muscle physiology has largely been concerned with the contribution of chloride to electrical activity (for review, see Carmeliet and Vereecke, 1980). The membrane current attributed to chloride in naturally occurring cardiac muscle is small (Fozzard and Lee, 1976; Kenyon and Gibbons, 1977), as is the transmembrane chloride (^{36}Cl) flux (e.g., Fong and Hinke, 1981). However, in cell types other than cardiac muscle (e.g., red blood cells, epithelia, nerve), the transport of chloride is large and also appears to contribute significantly to the regulation of intracellular pH and to changes in cell volume (for review, see Brodsky, 1980).

The mechanisms of anion transport are best understood by the combined application of microelectrode, ion content, and isotopic tracer techniques to the same preparation. In cardiac muscle, such studies have been difficult to

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accomplish because the morphology of the preparations limits the ability to separate the exchange characteristics of the various cell types from the contribution of the extracellular matrix. Indeed, complex kinetics of chloride tracer exchange in cardiac muscle have been reported by Sekul and Holland (1959) and Carmeliet and Verdonck (1977). To circumvent the extracellular diffusion delays and tracer reflux that slow the rates of tracer exchange in these preparations (MacDonald et al., 1974; Attwell et al., 1979), embryonic chick heart cells of muscle and nonmuscle origin were grown in tissue culture around nylon monofilaments (Horres et al., 1977). Previously, the simple geometry and short extracellular diffusion distances (<50 μm) of the "polystrand preparation" allowed a more complete analysis of tracer exchange from the two cellular compartments as well as a better estimate of true transmembrane fluxes than was possible in naturally occurring preparations (^{42}K, Horres and Lieberman, 1977; ^{24}Na, Wheeler et al., 1982).

This study is a multidisciplinary evaluation of the transmembrane movement of chloride in the polystrand. A comparison of the large chloride flux with the small effects of chloride removal on membrane potential indicates that chloride transport across the cardiac cell membrane is largely electroneutral.

METHODS

Tissue Culture

The techniques for producing the polystrand have been described in detail (Wheeler et al., 1982) and are reviewed briefly here. Whole hearts were dissected from 10–11-d-old chick embryos, mechanically minced, and disaggregated with 0.025% trypsin (Gibco Laboratories, Grand Island, NY). The proportion of muscle cells was increased by using a differential cell attachment technique (Blondel et al., 1971; Horres et al., 1977), after which the cells were suspended in Medium 199 with a base of Earle's solution (Gibco Laboratories), 5% fetal bovine serum (Sterile Systems Inc., Logan, UT), and 2% chick embryo extract. The substrate for cell growth consisted of 20-μm-diam nylon monofilament (Nylon-66; Monsanto Research Corp., Monsanto Co., Dayton, OH) wound 35 turns around a U-shaped silver wire. An aliquot of the suspension was placed in growth chambers with the substrate and maintained at 37° C in a 96% air/4% CO_2 atmosphere for 3 d. Polystrand preparations were then transferred onto a silicone rubber insert in culture dishes containing fresh culture media to prevent the formation of cellular bridges between strands on adjacent nylon monofilaments. In this manner, 70 spontaneously beating sheaths of muscle were formed around the nylon core, each up to 5 mm in length and with the outside diameter typically <100 μm. Tissue dry weight of the total preparation was in the range of 0.15–0.35 mg.

Noncontractile polystrands, consisting primarily of nonmuscle (fibroblast-like) cells, were produced in the growth chambers by seeding a suspension of cells obtained from those that had preferentially adhered to the culture dish surface during the muscle enrichment procedure 1 wk earlier.

Experimental Solutions

Control perfusate for all experiments was a modified Earle's solution with the following composition (mM): 118 NaCl, 5.4 KCl, 26 $NaHCO_3$, 0.81 NaH_2PO_4, 0.81

$MgSO_4$, 2.7 $CaCl_2$, and 5.6 dextrose. All solutions contained 1.4 gm·liter^{-1} bovine serum albumin (Fraction V; Sigma Chemical Co., St. Louis, MO). The solution was maintained at pH 7.4 by gassing with 96% air/4% CO_2. High-K and K-free solutions were made by equimolar substitution of KCl and NaCl. Low-Cl solutions were obtained by equimolar substitution of Na-methanesulfonate and K-methanesulfonate (made with methanesulfonic acid; Eastman Kodak Co., Rochester, NY) for NaCl and KCl. Ca^{2+} activity in methanesulfonate solutions shows little change (Kenyon and Gibbons, 1977). Cl-free solutions were obtained by substituting 2.7 mM Ca-methanesulfonate for $CaCl_2$.

^{36}Cl Efflux

Isotope was obtained as a 1.5-N solution of [^{36}Cl]HCl (6 mCi/gm) from New England Nuclear (Boston, MA) and was neutralized with aqueous NaOH. The resulting stock solution was used to make an isotopic loading solution with a chemical composition equivalent to the control solution and a specific activity of ~8 μCi/ml.

After 4 d in culture, polystrands were selected for uniformity of growth and placed in the loading solution under controlled humidity (~95%) at 37°C in an incubator with 96% air/4% CO_2 for a time sufficient to achieve isotopic equilibrium (typically 25 min). Removal of the loading solution was achieved by placing the polystrand in a tube of fluorocarbon liquid (FC-80; 3M Co., St. Paul, MN). This procedure minimized isotopic losses from rapidly exchanging compartments during transfer to the flux chamber (Wheeler et al., 1982). The flux apparatus allowed the cells to be continuously superfused at 20 ml·min^{-1} with nonradioactive solution and permitted rapid switching between two perfusates with a chamber half-time of 2 s. An in-line planar flow pH electrode (model 2000; Owens-Illinois Corp., Toledo, OH) continuously monitored solution pH. Once superfusion began, the chamber was aligned with a television microscopy system (Olympus X, Tokyo, Japan, and Shibaden HV-16SU, Hitachi Electronics, Melrose Park, IL) to observe the contractile activity of the preparations throughout the efflux period. Preparations were field stimulated at 150 beats·min^{-1} by platinum-plate electrodes mounted in the walls of the flux chamber. The superfusate was collected at 24-s intervals for the duration of an efflux experiment after which the preparation was quickly removed from the flux chamber and placed in 4 ml of control solution for 1 h to equilibrate the residual intracellular isotope (typically <2% of total tissue counts). After determination of the residual activity, some preparations were returned to the ^{36}Cl loading solution to repeat the efflux experiment. Next, the polystrand was air-dried, removed as a continuous loop from the silver wire, placed overnight in a vacuum oven at 110°C (National Appliance Co., Portland, OR), and weighed on an electrobalance (Cahn G-2; Ventron Instruments, Paramount, CA). The mean weight (± SD) of monofilament without tissue (0.322 ± 0.010 mg; n = 5) was subtracted from the total weight of the preparation to obtain tissue dry weight. Superfusate and residual samples were assayed in 10 ml of Hydrofluor (National Diagnostics, Inc., Somerville, NJ) using liquid scintillation techniques (7500 Liquid Scintillation System; Beckman Instruments, Inc., Fullerton, CA) with an overall counting efficiency of 91%. Data were corrected for background and summed using a PDP 11/44 computer (Digital Equipment Corp., Marlboro, MA) to generate efflux curves as a function of time (Keynes, 1954). The criteria for curve-fitting the data involved a program that used a nonlinear technique without derivatives (Brent, 1973) and the sum of weighted squares of residuals (Grinvald and Steinberg, 1974).

The contribution of Cl exchange from cell-free supports was determined by ^{36}Cl efflux experiments performed as with polystrand preparations. The kinetics of ^{36}Cl

exchange were characteristic of a two-component process (Fig. 1). The initial component represented washout of the superficial adherent film, whereas the second component was associated with the slow exchange of ^{36}Cl with the substrate. When normalized to the specific activity of the loading solution, the size of the second component indicates that the substrate at equilibrium contained ~7 × 10^{-10} mol of Cl (<5% of the cellular Cl content). The mean value of the substrate component (n = 3) was subtracted from each experiment before applying the curve-fitting procedure.

Cellular Chloride Content

Chloride content was determined by two independent methods. The first one involved preparations loaded to isotopic equilibrium with ^{36}Cl. After superfusion in 4°C control

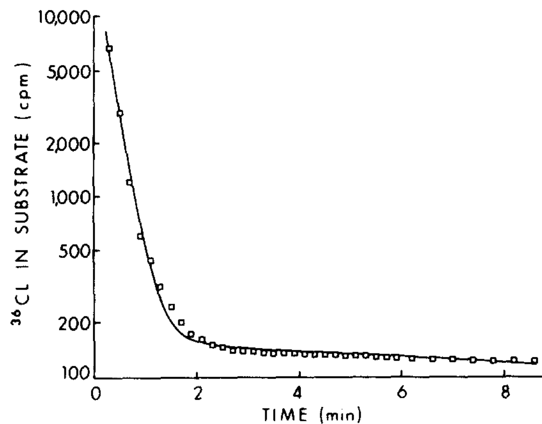

FIGURE 1. ^{36}Cl efflux of a nylon substrate in control solution at 37°C. The fitted curve represents a two-compartment model described by the following equation: cpm = $20{,}224e^{-4.00t} + 165e^{-0.03t}$. The size of the second component indicates that the substrate at equilibrium contained ~7 × 10^{-10} mol of Cl.

solution to clear isotope from the extracellular space (see below for experimental verification), counts remaining within the preparation were normalized for the specific activity of the loading solution and tissue dry weight. The second technique determined chloride content by coulometric titration (model F-25; W-P Instruments, Inc., New Haven, CT) of tissue extracts (Cotlove, 1963). After clearing extracellular space of Cl by vigorously rinsing preparations for 30–45 s in ice-cold isotonic $NaNO_3$ lightly buffered with 0.3 mM HEPES to pH 7.4 (Aull et al., 1977), the preparations were removed from their silver supports, dried, and weighed, and two to four polystrands were pooled to obtain enough material for each determination (total dry weight equaled 0.3–1.2 mg). Overnight oxidation in 0.2 M Na perborate minimized possible interference at the silver electrode during titration from reducing agents such as sulfhydryl or sulfide groups released during the alkaline extraction procedure (Polimeni and Page, 1980). Appropriate calibration solutions and correction for Cl contamination from glassware, reagents, and substrate were made by the titration of parallel "blank" specimens without tissue. Cl concentration was calculated using the cell water determinations of Horres et al. (1977).

Clearance of Extracellular Space

Preparations were loaded to isotopic equilibrium with the extracellular marker [^{14}C]carboxyldextran (New England Nuclear) dissolved in control solution (5.2 μCi$\cdot$ml^{-1}). After the customary passage through fluorocarbon, one group of preparations was immediately removed for processing, while another group was further rinsed for 30 s in ice-cold Cl-free solution (isotonic $NaNO_3$). Preparations were then air-dried, weighed, extracted for 1 h in 0.5 ml in 1-N HNO_3, and assayed in 10 ml of Hydrofluor for ^{14}C activity.

Electrical Recording

Transmembrane potentials were recorded from preparations placed in a chamber designed to simulate the perfusion conditions of the ^{36}Cl efflux experiments (Horres et al., 1979). Transmembrane potentials were recorded by standard techniques using glass microelectrodes (20–30 MΩ) filled with 3 M KCl and referenced to a flowing KCl calomel electrode (13-639-56; Fisher Scientific Co., Pittsburgh, PA) positioned downstream of the preparation. Larger-diameter microelectrodes filled with 2% agar in normal saline were used to stimulate the preparations at 150 beats$\cdot$min^{-1}. Signals were channeled to an oscilloscope (5111; Textronix, Inc., Beaverton, OR) and records were obtained either photographically with an oscilloscope camera (model CU-5; Polaroid Corp., Cambridge, MA) or a chart recorder (7702B; Hewlett-Packard Co., Palo Alto, CA). Data were tabulated from stable transmembrane potentials that returned to within 2 mV of the zero baseline after removal of the microelectrode.

RESULTS

^{36}Cl Efflux in Contractile Preparations

The kinetics of ^{36}Cl efflux of contractile polystrands in control solution at 37°C is a process in which at least three exponential components can be identified (Fig. 2). In the example shown, the initial component is described by a rate constant of 4.9 min^{-1} that could be separated from the second and third components, which have rate constants of 0.67 min^{-1} and 0.13 min^{-1}, respectively. Under these conditions, a quantitative description of the separate compartments was not always possible because the larger initial component of the efflux curve often obscured the second component.

To separate temporally the initial compartment from the others, preparations were superfused by cold 4°C control solution for 1.1–2.8 min and then by the same solution at 37°C (Fig. 3). At 4°C, the efflux curve comprises an initial, rapid component similar to that seen at 37°C, followed by a very slow exchange (a plateau), whereas on rewarming to 37°C, the efflux curve is characterized by two components. The fast and slow components obtained upon rewarming to 37°C were similar in rate constant and size (normalized for tissue dry weight) to the second and third components obtained when the entire experiment was performed at 37°C (Fig. 2).

If it is assumed that the initial component represents the superficially adherent film (extraneous) and extracellular isotope (see below and Carmeliet, 1962; Wheeler et al., 1982), then the washout in cold solution accomplishes two objectives. First, extracellular washout can be separated in time from

transmembrane efflux. Second, if a solution other than control is used during the efflux, the time in the cold would be sufficient to allow exchange of extracellular space with the new experimental solution.

To verify the clearance of extracellular and extraneous space by an independent method, $[^{14}C]$carboxyldextran was used as a tracer of the extracellular compartment (Paton, 1975). $[^{14}C]$Dextran space (microliters per milligram dry weight) after 30 s of cold, Cl-free rinse was 0.25 ± 0.02 $\mu l \cdot mg$ dry wt^{-1} compared with 5.0 ± 0.2 $\mu l \cdot mg$ dry wt^{-1} before the rinse (mean ± SD; $n = 3$). Assuming first-order kinetics, a rate constant of 5.9 min^{-1} ($t_{1/2}$ = 7 s) for clearance of extracellular and extraneous dextran space can be calculated

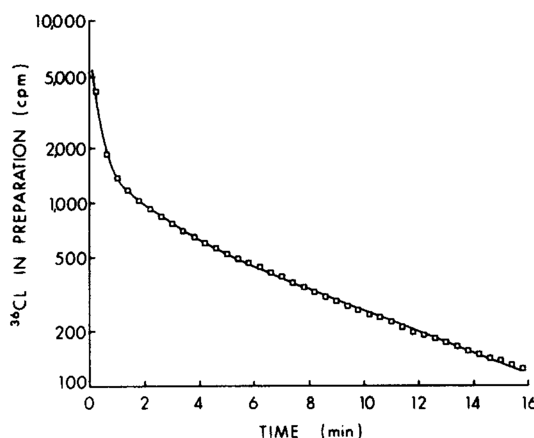

FIGURE 2. ^{36}Cl efflux of a contractile polystrand in control solution at 37°C. The fitted curve represents a three-compartment model described by the following equation: cpm = $6{,}15le^{-4.09t} + 974e^{-0.67t} + 934e^{-0.13t}$.

from these data. This compares well with the measured rate constant of 4.9 min^{-1} for the initial, relatively temperature-insensitive ^{36}Cl component.

The flux remaining after clearing the initial compartment and rewarming to 37°C was not characterized by a single exponential, but could be consistently fitted by the sum of two exponentials. Rate constants derived individually from efflux curves of 32 contractile polystrands averaged to 0.67 ± 0.12 min^{-1} and 0.18 ± 0.05 min^{-1} (mean ± SD) for the fast and slow compartments, respectively. In four preparations that were reloaded with isotope and perfused a second time, essentially the same rate constants were obtained, which is indicative of the integrity of these preparations throughout the experimental manipulations.

^{36}Cl Efflux in Nonmuscle Polystrands

Nonmuscle preparations in 4°C control solution also showed a temperature-insensitive efflux that approached a plateau. After rewarming to 37°C, the ^{36}Cl efflux could now be approximated by a single exponential curve (Fig. 4).

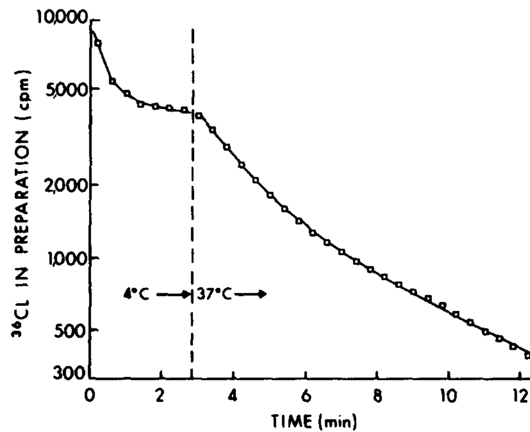

FIGURE 3. ^{36}Cl efflux of a contractile polystrand in control solution. Superfusion of preparation at 4°C for first 2.8 min and at 37°C thereafter. Solid lines represent fitted curves given by cpm = $5{,}754e^{-3.60t} + 4{,}862e^{-0.07t}$ for the first part; for times >2.8 min, cpm = $2{,}610e^{-0.68t} + 1{,}760e^{-0.16t}$. The initial efflux into cold solution represents extracellular tracer, whereas efflux into warm solution is associated with cellular compartments, the faster of which is cardiac muscle (see text).

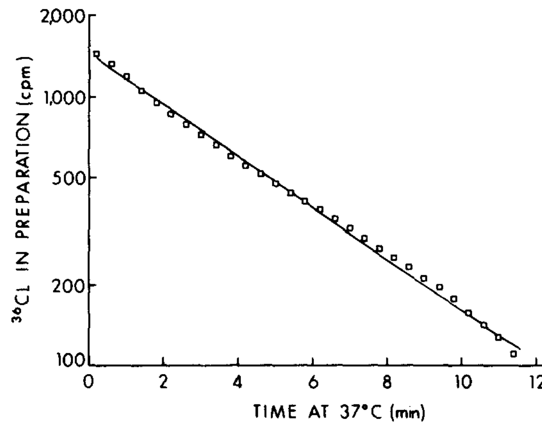

FIGURE 4. ^{36}Cl efflux of a noncontractile polystrand in control solution. The initial superfusion performed at 4°C for 1.4 min is omitted; time zero is defined as the time of switch to 37°C solution. Solid line represents fitted curve given by cpm = $1{,}457e^{-0.22t}$.

The mean rate constant for 11 nonmuscle polystrands in control solution was 0.23 ± 0.03 min^{-1} (mean ± SD).

Identification of Efflux Curve Components

For the reasons given below, the temperature-insensitive component of ^{36}Cl efflux can be attributed to the isotope leaving the extraneous and extracellular compartment. First, this component is present in contractile and noncontrac-

tile preparations, as well as the nylon substrate. Second, the size of this component is consistent with analytical data characterizing the polystrand preparation (Horres et al., 1977). For example, from the ^{36}Cl content of the initial component, an estimate can be made of the extraneous and extracellular Cl content. The initial component in Fig. 2 corresponds to 5×10^{-7} mol Cl·(mg dry wt)$^{-1}$ or ~80% of total tissue Cl content when normalized for the specific activity of the loading solution and preparation dry weight. After removal of polystrand preparations from fluorocarbon, the amount of extracellular and extraneous solution was calculated to be equivalent to 5.6 μl·(mg dry wt)$^{-1}$ (Lieberman et al., 1981) based on the results obtained with the extracellular marker [^{125}I]iothalamic acid (Horres et al., 1977). Determinations with [^{14}C]dextran in the present study estimate this compartment to be 5.0 μl·(mg dry wt)$^{-1}$. These values, combined with the extracellular Cl concentration of 128 mM (see Methods), lead to an estimate of the extraneous and extracellular Cl content of 6–7 $\times 10^{-7}$ mol Cl·(mg dry wt)$^{-1}$, which compares favorably with the measured extraneous and extracellular ^{36}Cl space of 5×10^{-7} mol Cl·(mg dry wt)$^{-1}$. Third, a reasonable value for the effective diffusion coefficient for Cl movement through the extracellular space is obtained when the rate constant associated with the initial component at 4°C is substituted into the Hill equation (1928) for a cylindrical muscle bundle. The calculated Cl diffusion coefficient of 0.5×10^{-6} cm^2·s^{-1} at 4°C should be considered a lower limit since the effluent sample interval of 24 s prohibits the accurate resolution of a very rapid compartment. Analysis of a similar compartment, continuously monitored for ^{24}Na activity (Wheeler et al., 1982), revealed a rate constant at least two to three times that detected by effluent sampling. Fourth, washout of the extracellular marker [^{14}C]dextran resulted in an estimated rate constant similar to that of the initial Cl component.

Contractile polystrands contain two cell types, one having the morphological characteristics of cardiac muscle and the other having the characteristics of nonmuscle or fibroblast-like cells (Horres et al., 1977). Once extracellular and extraneous ^{36}Cl was cleared from the preparation, contractile polystrands comprised two distinct Cl components. On the other hand, noncontractile polystrands, which contained only fibroblast-like cells, are described by a single Cl component. Furthermore, the rate constant for ^{36}Cl from the noncontractile polystrands is comparable to the slow-component ^{36}Cl rate constant of contractile polystrands. Thus, it is concluded that the slow component of ^{36}Cl efflux from contractile polystrands represents fibroblast-like cells and the fast compartment represents cardiac muscle cells. The slight difference between the rate constant of the fibroblast component of contractile preparations and the rate constant of fibroblasts in noncontractile preparations may reflect differences in the age of the cells and/or culture techniques.

Cellular Cl Content and Concentration

Intracellular Cl content can be estimated by extrapolating the isotopic activity of the plateau level back to the time when the preparation was first exposed

to nonradioactive solution (time zero in Fig. 3). The mean steady-state intracellular Cl concentration determined by isotopic equilibrium was 25.1 ± 7.3 mmol·(liter cell water)$^{-1}$ (mean ± SD) for 26 contractile preparations and 24.4 ± 6.2 mmol·(liter cell water)$^{-1}$ for 7 noncontractile preparations (Table I). Values for intracellular chloride were independent of the duration of isotopic equilibration for loading times >20 min. Coulometric titration of tissue extracts resulted in an intracellular Cl concentration of 24.9 ± 5.4 mmol·(liter cell water)$^{-1}$ for 14 contractile preparations and 27.3 ± 5.7 mmol·(liter cell water)$^{-1}$ for 8 noncontractile preparations. Comparison between all values for intracellular chloride concentration showed no significant differences based on one-way analysis of variance (Wallenstein et al., 1980).

TABLE I

Cl CONTENT AND Cl CONCENTRATION IN TISSUE-CULTURED HEART CELLS

	Isotopic equilibrium		Coulometric titration	
	Contractile	Noncon-tractile	Contractile	Noncon-tractile
Cell Cl content, nmol·(mg dry weight)$^{-1}$	107.6±30.2 (26)	87.6±20.8 (7)	106.7±22.0 (14)	98.2±18.3 (8)
Cell water content, µl·(mg dry weight)$^{-1}$	4.29±0.33 (12)	3.59±0.34 (8)	4.29±0.33 (12)	3.59±0.34 (8)
$[Cl]_i$, mmol·(1 cell water)$^{-1}$	25.1±7.3	24.4±6.2	24.9±5.4	27.3±5.7

(*n*) = number of determinations.

Values are means ± SD. Cell water content is recalculated from the data of Horres et al. (1977). Comparison between all values for $[Cl]_i$ showed no significant differences based on one-way analysis of variance (Wallenstein et al., 1980).

Cl Flux

Transmembrane Cl flux can be calculated by combining the above data with the volume-to-surface area ratio (Horres et al., 1977) using the following equation (Keynes and Lewis, 1951):

$$J_{Cl} = k_{Cl}[Cl]_i V/A, \tag{1}$$

where J_{Cl} = transmembrane chloride flux (pmol·cm^{-2}·s^{-1}); k_{Cl} = cellular chloride rate constant (min^{-1}); $[Cl]_i$ = intracellular chloride concentration (mmol·liter cell water^{-1}); and V/A = volume-to-surface area ratio (cm).

Table II summarizes the Cl flux data for the contractile and noncontractile polystrands. A transmembrane Cl flux of 3.5 pmol·cm^{-2}·s^{-1} was calculated from a V/A of 0.48×10^{-4} cm, an $[Cl]_i$ of 24.4 mmol·(liter cell water)$^{-1}$, and a rate constant of 0.18 min^{-1} for the nonmuscle compartment of contractile polystrands. From a V/A of 1.06×10^{-4} cm, an $[Cl]_i$ of 25.1 mmol·(liter cell water)$^{-1}$, and a rate constant of 0.67 min^{-1}, a transmembrane Cl flux of 30

pmol·cm^{-2}·s^{-1} was calculated for the muscle compartment of contractile polystrands.[1]

Dependence of Membrane Potential and Rate Constant on Cl_o

One method for determining whether the Cl flux can contribute significantly to membrane current is to render the preparation quiescent and measure the change in membrane potential upon switching to a low-Cl solution. When quiescence was achieved by raising $[K]_o$ to 30 mM, switching to 15 mM $[Cl]_o$ caused a small transient hyperpolarization of only 3.9 ± 0.3 mV (mean ± SD; n = 3), after which the membrane potential returned to a quasi-steady state within 1–2 mV of its original potential (Fig. 5). Re-addition of Cl_o gave

TABLE II

STEADY-STATE TRANSMEMBRANE Cl FLUX IN TISSUE-CULTURED HEART CELLS

Preparation	Contractile		Noncontractile
Cell Type	Muscle	Fibroblast	Fibroblast
	(n = 32)	(n = 32)	(n = 11)
^{36}Cl efflux rate constant (min^{-1})	0.67±0.12	0.18±0.05	0.23±0.03
V/A ratio* (cm)	1.06×10^{-4}	0.48×10^{-4}	0.48×10^{-4}
Cl flux (pmol·cm^{-2}·s^{-1})	30	3.5	4.4

Means ± SD. (n) = number of determinations.

* V/A ratio: values are from Horres et al. (1977).

Cl flux is calculated from Eq. 1.

rise to identical changes in the depolarizing direction. Similarly, when polystrands were depolarized to −40 mV by the removal of K_o (see also Horres et al., 1979; Lieberman et al., 1982), the subsequent complete removal of Cl_o transiently hyperpolarized the membrane by 1.7 ± 0.3 mV (n = 7); this effect was also reversible upon re-addition of Cl_o. Moreover, as shown in Fig. 6, 2 min after the removal of chloride (5.4 mM $[K]_o$) the duration of the action potential increased by only 16% when measured at the 50% level of repolarization. Accompanying changes in the initial phase of repolarization and the maximum diastolic potential were barely detectable and quickly reversed upon return of normal $[Cl]_o$. Thus, transmembrane potentials recorded from cultured chick heart cells are relatively unresponsive to sudden changes of $[Cl]_o$ in K-free, normal, or high-$[K]_o$ solutions.

The effect of Cl removal on ^{36}Cl efflux rate constants was also determined after loading to isotopic equilibrium in control solution. The mean rate constants for three contractile polystrands in 5.4 mM $[K]_o$, Cl_o-free (methanesulfonate) media were 0.67 ± 0.02 min^{-1} and 0.15 ± 0.02 min^{-1} for the muscle

[1] No significant differences exist between $[Cl]_i$ in contractile preparations (composed of muscle cells and fibroblasts) and noncontractile preparations (chiefly composed of fibroblasts); therefore, use of the mean $[Cl]_i$ in contractile preparations as an estimate for $[Cl]_i$ in the muscle compartment should not introduce any large errors into the flux calculation.

and nonmuscle compartments, respectively. These rate constants, essentially unchanged from control values (Table II), directly indicate the continued rapid transmembrane efflux of Cl under a condition shown to produce little change in membrane current (Fig. 6).

DISCUSSION

Multicellular preparations of cardiac muscle have provided a formidable challenge to investigators studying the transport mechanisms associated with

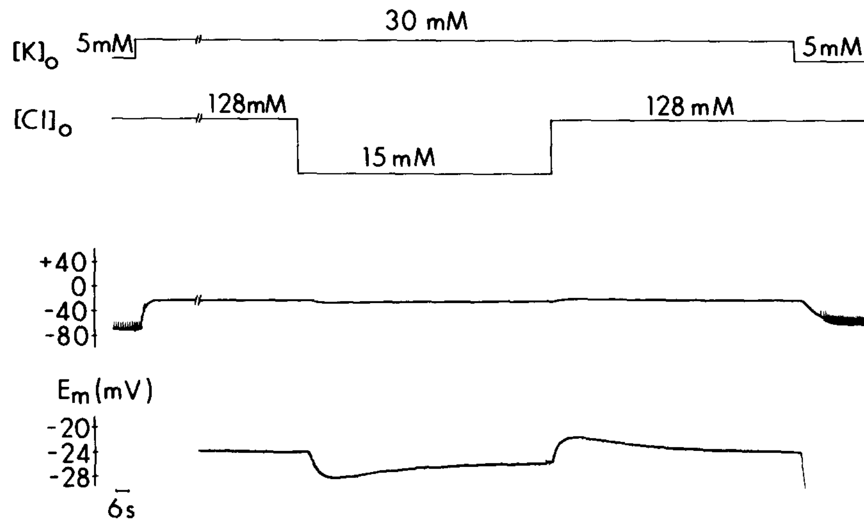

FIGURE 5. A continuous impalement of an unstimulated polystrand illustrating the effects of sudden changes in $[K]_o$ and $[Cl]_o$ on the membrane potential. The top two panels show solution $[K]_o$ and $[Cl]_o$, whereas the bottom two panels show membrane potential at low and high gain. The break in the record represents 5 min.

the ionic regulation of cardiac activity. For ions such as chloride, which are largely distributed extracellularly, the restricted spaces and tortuous diffusion paths can complicate the interpretation of tracer kinetics obtained from naturally occurring preparations of cardiac muscle (for discussion, see Horres and Lieberman, 1977; Attwell et al., 1979). Although the problems of extracellular diffusion delays in multicellular preparations have been approached by applying mathematically formulated corrections to the kinetic data (Keynes, 1954; Solomon, 1960), the values needed to compensate for the errors inherent in the complexities of cardiac preparations have been considerable (Horres and Lieberman, 1977) and bring into question the physiological significance of corrected tracer data. An alternative approach to resolve the tracer kinetics was to simplify the extracellular morphology and reduce diffusion distances by growth-orienting heart cells in tissue culture (Horres et al., 1977). The results of combining high superfusion rates with such a preparation show that chloride exchange across the cardiac cell membrane is

rapid (k = 0.67 min^{-1}; $t_{1/2}$ = 1.0 min) compared with previously published values for cardiac muscle, and, as discussed below, has been found to be largely electroneutral in nature.

Compartmental Analysis

The polystrand preparation, although only 100 μm in overall diamter, has an extracellular compartment in series with rapidly exchanging cellular compartments that could potentially introduce errors into the analysis of the efflux data. Using the series compartmental model of MacDonald et al. (1974) for

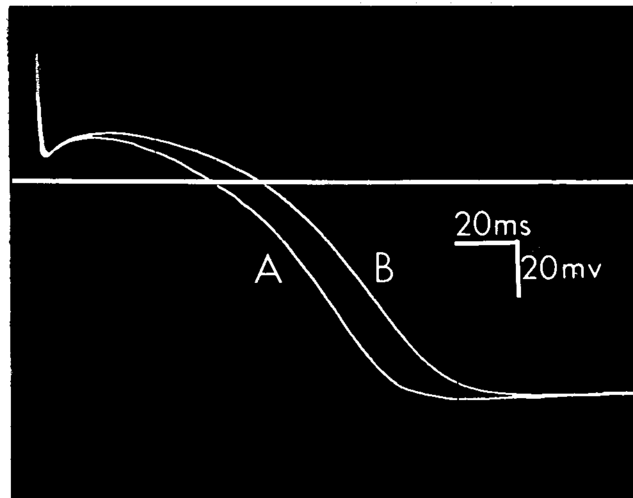

FIGURE 6. Superimposed action potentials from a polystrand stimulated at 150 min^{-1} in control solution (A) and after 2 min in Cl-free (methanesulfonate) solution (B). The horizontal line shows the zero reference potential. Stimulus artifact obscures the first 5 ms of each action potential.

a 100-μm-diam cylindrical bundle of muscle, the error in detecting the true rate constants for ^{36}Cl in the polystrand was computed to be <2%. This suggests that the rate-limiting step for Cl diffusion is at the cell membrane, not in the extracellular matrix, which validates the assumption of parallel cellular compartments for analyzing the tracer efflux data (for further discussion, see Horres and Lieberman, 1977; Wheeler et al., 1982).

Because muscle and nonmuscle compartments contain similar $[Cl]_i$'s (Table I), the relative size of each compartment in a contractile polystrand could be estimated from the magnitude of the corresponding ^{36}Cl component. For example, from the equation describing the cellular efflux in Fig. 3, the size of the ^{36}Cl component corresponding to cardiac muscle was 60% of the cellular isotope. Previous histological analysis of contractile polystrands indicated that the muscle compartment typically comprises 50–70% of the tissue (Horres, 1975), which correlates well with the size of the ^{36}Cl muscle component. Similar conclusions regarding compartmental analysis have been reported for

^{42}K and ^{24}Na studies with these preparations (Horres et al., 1979; Wheeler et al., 1982).

^{36}Cl Efflux Rate Constant

The diffusion limitations in the large and complex extracellular space of naturally occurring preparations of cardiac muscle, when in series with cellular compartments, would obscure the detection of fast cellular exchange processes observed in the polystrand. Most ^{36}Cl rate constants assigned to the cardiac cell compartment of intact preparations are one order of magnitude smaller than the value reported in the present study (Sekul and Holland, 1959; Carmeliet and Verdonck, 1977; Macchia and Golnick, 1981; Fong and Hinke, 1981). However, ^{36}Cl rate constants in qualitative agreement with the present result were achieved when the diffusional limitations were reduced either by using a fast perfusion protocol applied to the rat ventricle (0.29 min^{-1}, Polimeni and Page, 1980) or a monolayer preparation of noncontractile Girardi heart cells (0.34 min^{-1}, Lamb and McCall, 1972).

Intracellular Cl Concentration

Determination of $[Cl]_i$ did not require the use of extracellular markers because this compartment was cleared by cold perfusion before collecting the ^{36}Cl effluent from the preparations. Nevertheless, the measurements of Cl concentration, based both on compartmental analysis and on electrotitration of tissue extracts, agree with many reported values for vertebrate cardiac muscle (Lamb, 1961; Page, 1962; Carmeliet and Janse, 1964; Page and Page, 1968; Caille et al., 1981). If an activity coefficient of 0.7 is assumed (Robinson and Stokes, 1970), the present value is also consistent with many recent determinations of $[Cl]_i$ activity by ion-selective microelectrodes (Ladle and Walker, 1975; Spitzer et al., 1978; Baumgarten and Fozzard, 1978; Vaughan-Jones, 1979*a*; Caille et al., 1981; Fong and Hinke, 1981). Although the $[Cl]_i$ reported for preparations of amphibian and rodent hearts is significantly lower than that for the polystrand (Macchia et al., 1978; Polimeni and Page, 1980), the differences may be attributed either to species variation or to the use of various extracellular markers (Vaughan-Jones, 1979*a*).

The chloride equilibrium potential (E_{Cl}) in the polystrand is −42 mV, far from a maximal diastolic potential (MDP) of −75 mV, but virtually identical to the time-averaged potential (TAP) of −41 mV (Horres et al., 1979) for this spontaneously beating preparation. This observation should not, however, be construed as proof that Cl is at equilibrium with the TAP of cardiac muscle cells. For example, a similar correlation between E_{Cl} and TAP has been reported for frog cardiac muscle beating at 120 min^{-1} (Ladle and Walker, 1975). However, when the frog myocardium was rendered quiescent for as long as 18 h at MDP, E_{Cl} was maintained positive to MDP, which suggests that Cl was actively accumulated and that the correlation between E_{Cl} and TAP was coincidental.

The close agreement between the isotopic equilibrium method and the coulometric titration method in determining $[Cl]_i$ (25.1 and 24.9 mmol·liter

cell water^{-1}, respectively) illustrates the freely exchangeable nature of Cl. In this regard, the equivalence of the isotopic and titrated values for $[Cl]_i$, and the correlation between extracellular ^{36}Cl space, [^{14}C]dextran space, and [^{125}I]iothalamate space does not support recent suggestions that a significant amount of bound pericellular Cl exists in cardiac muscle (Fong and Hinke, 1981). It was not possible to detect a compartment that was freely exchangeable with extracellular ^{36}Cl but excluded by extracellular marker.

Cl Flux of Cardiac Cells

The transmembrane Cl flux of 30 pmol·cm^{-2}·s^{-1} is large and one order of magnitude greater than most published values for naturally occurring preparations (Sekul and Holland, 1959; Carmeliet and Verdonck, 1977). Recalculating transmembrane flux in rat ventricle using Eq. 1 and the data of Polimeni and Page (1980) results in a transmembrane Cl flux of 10 pmol·cm^{-2}·s^{-1}, which supports our conclusion that Cl flux in cardiac muscle is rapid.

Membrane Potential Dependence on $[Cl]_o$

Cl permeability of several preparations of adult cardiac muscle (e.g., Carmeliet, 1961; Fozzard and Lee, 1976), as well as the polystrand, appears to be small when determined electrophysiologically. The slight effects of Cl-free solutions on the action potential configuration are virtually identical to those reported in sheep Purkinje fibers by Kenyon and Gibbons (1977). Furthermore, when the polystrand is rendered quiescent in 30 mM $[K]_o$ or K-free solutions, the small deviations in membrane potential observed upon switching to low-Cl solutions are similar in magnitude to those in rabbit ventricular muscle (Fozzard and Lee, 1976). Although the hyperpolarization of 3–4 mV reported in this study was unexpected, similar hyperpolarizations rather than depolarizations have been observed by others using various anion substitutes (Noma and Irisawa, 1975; Vaughan-Jones, 1979*a*). Several explanations have been offered to account for the membrane hyperpolarization observed during anion substitution (Noma and Irisawa, 1975; Vaughan-Jones, 1979*a*; Spitzer and Walker, 1979), including liquid-junction potentials at the reference electrode and the surface of the preparation; permeability of the anion substitute and its effects on cation permeability; and elimination of Cl shunting of electrogenic Na/K transport. Although no explanation is entirely satisfactory, the major observation that the polystrand membrane potential is only slightly influenced by sudden changes in external Cl concentration supports the conclusion that the polystrand, like most cardiac muscle preparations, has a low membrane Cl conductance. The results further suggest that transmembrane Cl movement in cardiac muscle is unlikely to significantly shunt the hyperpolarization of the membrane potential caused by activation of the electrogenic Na/K pump.

Electroneutral Chloride Transport

Since the transmembrane ^{42}K flux in the polystrand is only 16 pmol·cm^{-2}·s^{-1}, most of which is apparently conductive (Horres et al., 1979), the trans-

membrane ^{36}Cl flux of 30 pmol·cm^{-2}·s^{-1}, under similar conditions, is surprisingly large. Assuming that all exchange can be attributed to charge movement across the membrane, absolute values for permeability coefficients can be calculated from efflux data with the use of the following equation (Goldman, 1943; Carmeliet and Verdonck, 1977):

$$P_i = -J_i \frac{RT}{zFE} \cdot \frac{1 - \exp(zFE/RT)}{[c_i] \exp(zFE/RT)}, \qquad (2)$$

where P_i = permeability coefficeint of ion i (cm·s^{-1}); J_i = transmembrane efflux (pmol·cm^{-2}·s^{-1}); E = membrane potential (mV); z = valency; c_i = intracellular concentration (mmol·[liter cell water^{-1}]); and $RT/F \simeq 26$ mV at 37°C.

Combining Eqs. 1 and 2 for both K and Cl efflux yields:

$$\frac{P_{Cl}}{P_K} = \frac{k_{Cl}}{k_K} \exp(EF/RT). \qquad (3)$$

Variations of V/A and intracellular ion concentration will not affect the calculated P_{Cl}/P_K ratio. Combining the ^{36}Cl rate constant with the previously determined ^{42}K rate constant of 0.067 min^{-1} (Horres and Lieberman, 1977) and using the TAP of −41 mV results in a P_{Cl}/P_K ratio of 2.1. This flux-derived value clearly contradicts the observed electrophysiological behavior of the polystrand and the electrophysiologically estimated P_{Cl}/P_K value of 0.11 (Fozzard and Lee, 1976). The present findings imply that the majority of the unidirectional transmembrane Cl flux in cardiac muscle is electrically silent.

Maintaining steady-state intracellular Cl concentration constrains the cardiac cell to match the rapid Cl efflux with an equally rapid Cl influx. This net process of Cl exchange is perhaps not surprising given the widespread distribution of electrically silent chloride transport systems involved in cell volume changes and the regulation of intracellular pH in red blood cells, epithelia, and nerve (for review, see Brodsky, 1980). Reports of bicarbonate-dependent Cl movement sensitive to SITS (4-acetamido-4′-isothiocyano-2,2′-disulfonic acid stilbene) in sheep Purkinje fibers (Vaughan-Jones, 1979*b*) and furosemide-sensitive K influx in cultured chick heart cells (Aiton et al., 1981) are suggestive of anion-dependent counter- and co-transport mechanisms, respectively.

The magnitude of steady-state Cl flux determined in this study indicates that electrically silent, Cl-dependent transport mechanisms may contribute significantly to the homeostatic regulation of myocardial cell function in a manner similar to that observed in other tissues. Efforts are currently underway to characterize the mechanisms and functions of chloride transport in the polystrand preparation.

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