Control of Ion Distribution in Isolated Smooth Muscle Cells

I. Potassium

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ABSTRACT We describe a technique for examining unidirectional ion movements in suspensions of enzymatically disaggregated smooth muscle cells derived from stomach muscle of the toad. This technique has been used to analyze the movement of $^{42}$K across these cells. This analysis was greatly simplified by the finding that the cells were in a steady state with respect to $K^+$ distribution after isolation. The potassium contents of the isolated cells were identical to those of intact smooth muscle (131 mM/liter intracellular fluid) and stable for over 4 h; moreover, the unidirectional influx and efflux rates were equal. An additional simplification was provided by the finding that virtually all the $K^+$ exchanges in a manner predicted for a simple two-compartment system consisting of an extracellular and an intracellular space. Transmembrane $K^+$ flux in these cells averaged 1.2 pmol·cm$^{-2}$·s$^{-1}$ at room temperature. A large portion (~80%) of $^{42}$K influx appeared to be mediated by a saturable transport system with an apparent $K_m$ of 0.6 mM and an apparent $V_{max}$ of 1.3 pmol·cm$^{-2}$·s$^{-1}$. The calculated resting membrane permeability to $K^+$ in these isolated smooth muscle cells, assuming a membrane potential of $-50$ mV, was $2.9 \times 10^{-8}$ cm/s. The calculated $g_{K}$ was $2.7 \mu$mho/cm$^2$ constituting only a small fraction of the total membrane conductance as measured electrophysiologically. The latter finding suggests that the resting membrane potential in the isolated cells must be determined by ions in addition to $K^+$. We propose that these methods for studying ion movements in smooth muscle should aid in unraveling the mechanisms responsible for controlling the distribution of ions both at rest, as in the present study, as well as in response to neurotransmitters.

INTRODUCTION

In the past considerable effort has been devoted to the study of transmembrane ion movements and ion distribution in smooth muscle tissue preparations. The purpose of these studies was to define the cellular processes responsible for the distribution of ions across the smooth muscle membrane at rest and the changes that take place in these processes in response to excitatory and inhibitory agents. The interpretation of these studies was complicated, how-
ever, by certain inherent properties of tissue preparations. The heterogeneity of cell types, the complex geometry of the large extracellular space, and the presence of an extracellular fibrous matrix which avidly binds ions all served to complicate the kinetic analyses of ion movements in smooth muscle. As a result only the kinetics for K⁺ exchange in smooth muscle are relatively uncomplicated and even for this ion some uncertainties have remained. For example, some studies of K⁺ efflux from smooth muscle suggested the presence of two slowly exchanging, presumably intracellular compartments (Goodford and Hermansen, 1961), and it has been difficult to determine whether or not the second compartment is real or simply an artifact of tissue deterioration (Brading, 1973). Similarly, it has been difficult to determine the dependence of K⁺ influx on the extracellular K⁺ concentration since it is virtually impossible to set the extracellular K⁺ to a precise level in tissues. The advent of techniques for enzymatically disaggregating smooth muscle from the toad stomach to produce suspensions of functionally intact, single smooth muscle cells (Bagby et al., 1971; Fay and Delise, 1973) has opened the possibility of investigating transmembrane ion movements in smooth muscle uncomplicated by the access limitations and the extracellular binding characteristically seen in intact tissue. The present paper describes a technique for studying ion movements in suspensions of isolated smooth muscle cells and the application of this technique to investigate the control of K⁺ distribution in smooth muscle.

MATERIALS AND METHODS

The stomach muscle of the toad Bufo marinus was dissected free of connective tissue and mucosa, sliced into thin sheets, and digested enzymatically as previously described (Fay and Delise, 1973). For some experiments the disaggregated smooth muscle cells were collected by low speed centrifugation (40 min at ~11.5 g) and resuspended in enzyme-free amphibian physiological saline (APS: 108.9 mM NaCl, 3 mM KCl, 0.56 mM Na₂HPO₄, 0.14 mM Na₂HPO₄, 19.98 mM NaHCO₃, 1.8 mM CaCl₂, 0.975 mM MgSO₄, 11.11 mM glucose, equilibrated with 95% O₂: 5% CO₂, pH 7.4). In other experiments the cells were not removed from their digestion medium (0.5 mg type I Sigma collagenase/ml APS, Sigma Chemical Co., St. Louis, Mo.) prior to use. This transfer into enzyme-free medium did not alter the K⁺ contents or the unidirectional influx rate for ⁴²K in isolated smooth muscle cells. In cells that had not been transferred, ⁴²K influx averaged 1.01 ± 0.10 pmol·cm⁻²·s⁻¹, and the potassium contents averaged 133.7 ± 6.2 mM/liter intracellular fluid (mean ± SEM, 16 experiments). These parameters were virtually identical in cells that had been collected by low speed centrifugation and resuspended in enzyme-free medium; the ⁴²K influx rate averaged 1.04 ± 0.09 pmol·cm⁻²·s⁻¹ and the potassium contents averaged 129.4 ± 5.4 mM/liter intracellular fluid (mean ± SEM, 16 experiments). Results using both experimental protocols were thus pooled below.

Ion influx experiments were carried out at 20-22°C as follows: ⁴²K (1 mCi/ml New England Nuclear, Boston, Mass.) was added to a suspension of isolated smooth muscle cells and at various times after tracer addition, triplicate 10-ml aliquots of cells (~10⁶ cells/ml) were removed and separated from the bathing medium using a modification of the silicone oil technique of Manno and Schachter (1970). This technique involves centrifugation of the cells through 2 ml of silicone oil (Dow Corning Corp., Midland, Mich., sp gr 1.04) into 0.5 ml of lysing solution (0.5 N HCl in sucrose, 9 mg/ml). The
buffer and oil phases above the cell pellets were then removed by aspiration, and the centrifuge tubes (15 ml conical, Nalgene, Nalge Co., Rochester, N.Y.) were cut above the sucrose phase. The latter procedure was adopted in order to minimize carryover of labeled buffer phase to the cell pellets. The bottom of the tube containing the cell pellet and sucrose was then transferred to a plastic scintillation vial containing 5 ml of CsCl (0.1 mg/ml in 0.5 N HCl) and the cells were disrupted by sonication. A portion of this cell digest was then analyzed for $^{42}$K contents using a liquid scintillation counter. Another portion of the cell digest was analyzed for total K$^+$ contents by flame emission spectroscopy. In all ion flux studies, $[^{14}C]urea$ and $[^{3}H]inulin$ were introduced along with the $^{42}$K as markers for the total water and the extracellular fluid volume (ECF) associated with each cell pellet. The intracellular fluid volume (ICF) was calculated as the difference between the total H$_2$O (the volume in which $[^{14}C]urea$ was distributed) and the extracellular water (the volume in which $[^{3}H]inulin$ distributed). The $^{42}$K contents of the intracellular fluid was then calculated by subtracting ECF $^{42}$K (that distributed in the $[^{3}H]inulin$ volume) from the total $^{42}$K content of the pellet. Changes in the ICF $^{42}$K were monitored as a function of time. Influx data were corrected for radioactive decay during counting and for backflux of isotope (see Appendix for details).

For efflux experiments, the dispersed smooth muscle cells were collected by centrifugation at low speed and resuspended in approximately 0.1 their original volume in APS containing $^{42}$K. Cells were allowed to incorporate isotope for several hours at room temperature and then the specific activity of the bathing medium was reduced by diluting the cells 15–20-fold with APS containing nonradioactive K$^+$, $[^{14}C]urea$, and $[^{3}H]inulin$. The final cell density was approximately $10^5$ cells/ml. At various times after dilution, triplicate 10-ml aliquots were centrifuged through oil as described above, and the specific activity of $^{42}$K in the intracellular fluid was assessed as a function of time. Details regarding the analysis of flux data are provided in the appendix.

RESULTS

Centrifugation through Silicone Oil

The goal of the present studies was to develop a system with which to examine unidirectional ion movements in suspensions of single smooth muscle cells that had been freed of their complex interconnections by enzymatic disaggregation. A cell separation technique that would effectively halt transmembrane ion exchange at the time the cells were separated from the bathing medium was required. Simple centrifugation or filtration of the cells could not be utilized since these procedures contract the cells and/or fail to effectively halt exchange with the bathing medium. Therefore, we adapted the silicone oil technique of Manno and Schachter (1970) for use with the isolated cells. This technique affords a number of advantages for the study of ion contents and/or ion movements in a suspension of cells. For one, the procedure rapidly removes the cells from the surrounding medium thus halting further transmembrane exchange. We calculate that a smooth muscle cell at the top of the centrifuge tube would take less than 2 s to reach the oil phase when the centrifuge is at full speed; half speed was achieved within 5 s after starting the centrifuge motor. A second advantage is that the oil phase appears to reduce the amount of extracellular fluid associated with the cell pellet by effectively wiping the
cells free of adherent fluid. We calculate, by using the space markers described below, that this procedure reduces the ECF to a 1.4-μm "sleeve" such that the ratio of intracellular to extracellular fluid in the cell pellet is approximately 1:1. In addition, the oil phase seems to exclude small particulate debris and thus reduces the amount of noncellular contamination in the cell pellets. This effect is illustrated in Fig. 1b in which the Coulter Counter (Coulter Electronics Inc., Hialeah, Fla.) was used to compare a population of cells that had not been spun through oil (top right) to a population that has been spun through oil (bottom right) (Singer and Fay, 1977). The abscissa indicates the amplitude of the voltage pulse (related to the cell size) produced as a nonconducting particle traverses the orifice of the Coulter Counter. The ordinate indicates the relative proportion of cells (or particles) of a given size in the population. The very small amplitude pulses to the left of the arrow in each panel reflect small noncellular, particulate debris, and as is evident in this figure, centrifugation of cells through oil apparently reduced the amount of particulate debris associated with the cell pellet. In the experiment depicted in Fig. 1, the particulate debris constituted 34% of the total particles in the cell suspension that had been spun through oil whereas this component constituted 56% of the total particles in the original cell suspension. Although this component still represents a large percentage of the total number of particles, the volume per particle is quite small (~0.3% of the volume of an intact smooth muscle cell, cf. Fig. 1a); thus it is estimated that the particulate debris contributes < 0.2% of the total volume associated with the cell pellet. The silicone oil procedure thus provided a useful means for rapidly separating the cells from their bathing medium and halting transmembrane exchange. With this procedure we could assess the amounts of labeled ion associated with the cell pellets at various times after tracer addition. However, we could not distinguish between intracellular label and label associated with the trapped extracellular fluid. Thus, we included radioactive markers for the total water and the extracellular water associated with each cell pellet.

\[^{14}\text{C}]\text{Urea and }[^{3}\text{H}]\text{Inulin as Space Markers}\n
The validity of various space markers was first examined in whole tissue preparations that had not been subjected to enzymatic digestion. \[^{14}\text{C}]\text{Urea was chosen to monitor the total cell water associated with the tissue. This marker appeared to equilibrate rapidly with the tissue water such that uptake was completed within 15 min. The total tissue water associated with four slices of stomach muscularis (dimensions: 10 × 10 × 0.5 mm) averaged 33.7 μl when determined from the distribution of }[^{14}\text{C}]\text{urea, and this was virtually identical to that determined from the difference between wet and dry weights (33.6 μl). Thus }[^{14}\text{C}]\text{urea appeared to provide a reliable estimate of the total tissue water. }[^{3}\text{H}]\text{Inulin was then examined as a possible marker for the extracellular space within the tissue. This marker equilibrated more slowly than }[^{14}\text{C}]\text{urea, but within 1 h the uptake of }[^{3}\text{H}]\text{inulin had reached a plateau. To confirm that this marker had access to all of the extracellular space within the tissue, the extracellular fluid volume estimated with }[^{3}\text{H}]\text{inulin was compared with that obtained using the smaller, but presumably nonpermeating compound,}
[14C]sucrose. The extracellular fluid volume (ECF) calculated from the distribution of [3H]inulin was 22.46 ± 4.04 µl (n = 8) whereas that calculated from the distribution of [14C]sucrose was 23.67 ± 3.42 µl (n = 8) for the same group of tissue slices. Thus it would seem that [3H]inulin can provide a reliable estimate of the extracellular compartment in smooth muscle.

[14C]Urea and [3H]inulin were then utilized to estimate the total water and extracellular water associated with isolated smooth muscle cells that had been centrifuged through oil. These markers appeared to equilibrate more rapidly with the isolated cells than with intact tissue. [14C]Urea equilibrated with the isolated cells within 1 min. [3H]Inulin, which presumably labels only the sleeve of extracellular fluid adhering to the cells as they traverse the layer of silicone oil, reached complete equilibration with the cells in less than 1 min. Our standard procedure was thus to add [14C]urea to cell suspensions 10 min prior to collection through silicone oil; [3H]inulin was added 1 min before centrifugation. The volume of intracellular fluid (ICF) in each cell pellet was assumed to be equal to the [14C]urea volume minus the [3H]inulin volume. In order to check the validity of this assumption we compared this
estimate of the ICF with the cell volume estimated by morphometric analysis as follows. The length and width of approximately 240 cells in a single suspension was determined from photomicrographs, and the average volume per cell (5.76 μl) was calculated assuming the cells to be prolate spheroids. The number of cells per pellet was then determined using a hemocytometer and the volume predicted for this number of cells was calculated. The volume predicted in this manner was 3.79 ± 0.87 μl (four cell-aliquots), a value statistically indistinguishable from the ICF volume measured in four other cell-aliquots using radioactive space markers (3.05 ± 0.35 μl). The agreement between these methods is probably even better than this owing to the fact that the morphometric analysis yields an estimate of total cell volume (solids + water) whereas the radioactive space markers estimate the intracellular water compartment alone. We conclude that the inclusion of [14C]urea and [3H]inulin in the cell suspension permitted an accurate assessment of the intracellular and extracellular fluid volumes associated with the cell pellets.

\[ [K^+]_{IN \text{ in Tissue vs. Cells}} \]

The agreement between the estimate of intracellular water obtained with [14C]urea and [3H]inulin with that obtained from morphometric analysis indicated that the cells were capable of excluding inulin. Such studies could not, however, determine whether the cells were also capable of maintaining normal transmembrane ion gradients after isolation. To obtain this information, we examined the K⁺ contents of pellets of isolated smooth muscle cells. The intracellular K⁺ concentration in these pellets averaged 131.5 ± 4.01 mM/liter intracellular water (n = 32 cell suspensions); in tissue this averaged 130.8 ± 3.85 mM/liter intracellular water (n = 61 tissue slices). Thus, it would seem that the cell isolation and separation procedures employed did not compromise the cells' ability to maintain normal intracellular K⁺ levels. Furthermore, the cells' K⁺ contents remained stable for at least 4 h after isolation (Table I) indicating that the cells are in a steady state with respect to K⁺ contents throughout this period. Since all flux experiments were carried out within 4 h after cell isolation, we assumed that steady-state kinetic analyses could be employed in analyzing the flux data.

Compartmental Analysis

For ⁴²K exchange in a simple two-compartment system involving exchange between an intracellular and an extracellular compartment, the specific activity of the cellular K⁺ would be expected to increase exponentially with time. Thus, a semilog plot of (one - fractional equilibrium) vs. time should be linear. Fig. 2 shows ⁴²K uptake in a suspension of isolated smooth muscle cells plotted in this manner. The lower curve depicts the total (extracellular plus intracellular) ⁴²K associated with the cell pellets at various times after tracer addition. As can be seen in this graph, there is an initial rapid phase of K⁺ influx during which ⁴²K accumulation is nonlinear, followed by a slower phase which is constant with time. This initial brief phase of ⁴²K accumulation seems to be attributable in large part to equilibration of the isotope in the trapped extracellular fluid associated with the cell pellets. If one assumes that
this trapped extracellular $^{42}\text{K}$ will be distributed in a fluid compartment which is accessible to $[^3\text{H}]\text{inulin}$, and if one corrects the total $^{42}\text{K}$ at each time point for the $^{42}\text{K}$ distributed in this $[^3\text{H}]\text{inulin}$ "space," one obtains the upper curve in Fig. 2. Inherent in this correction is the assumption that within the time required to obtain the first sample following $^{42}\text{K}$ addition (1 min) this ion equilibrates fully in the inulin space. This assumption seems well justified because the uptake into this space of inulin, a much larger molecule than $^{42}\text{K}$, is complete in less than 1 min. Note that this estimate of extracellular $^{42}\text{K}$ accounts for the majority of rapidly exchanging $^{42}\text{K}$. We did, however, consistently find a small, rapidly exchanging pool of $\text{K}^+$ ($\sim 2\%$ of the total $\text{K}^+$ in the cell pellet) that could not be accounted for as $^{42}\text{K}$ trapped in the inulin space. This would suggest that there is some rapidly exchanging compartment that is accessible to $^{42}\text{K}$, but not to inulin. The location and properties

### Table 1

| Time  | [$\text{K}^+$]$_\text{IN}$ mM/liter ICF |
|-------|--------------------------------------|
| min   |                                      |
| 0–30  | 125.1±3.5* (152)                      |
| 30–60 | 136.2±5.8 (68)                        |
| 60–120| 140.2±7.2 (60)                        |
| >480  | 136.7±12.9 (19)                       |

* Time 0 was arbitrarily chosen as the time at which all of the fractions from the tissue digestion procedures were pooled for the flux studies. Aliquots of the pooled cell suspension were centrifuged through oil at various times after this pooling of the isolated cells, and the $\text{K}^+$ contents of the cell pellets were analyzed by flame emission spectroscopy. The data tabulated above represent the mean ± SEM for $n$ cell pellets where $n$ is indicated in parentheses. Data from 25 different cell suspensions are included above.

of this small cellular compartment have not been investigated. It is possible, however, that membrane caveolae, which constitute $\sim 0.3\%$ of the cell volume in intact toad stomach muscle, and/or external binding sites for $\text{K}^+$ could exhibit such properties. With the exception of this small, rapidly exchanging compartment, however, $^{42}\text{K}$ exchange in the isolated smooth muscle cell occurs in a manner that would be predicted for a simple two-compartment system. This is even more evident in Fig. 3 in which influx data from 25 separate experiments are pooled. All time points represent the mean $^{42}\text{K}$ uptake into the intracellular compartment at a given time. A semilogarithmic plot of this data was extremely well fit by a straight line ($R = 0.996$). Furthermore, application of the curve-peeling routine described by Solomon (1960) reveals only one exponential, suggesting that all the intracellular $\text{K}^+$ exchanges as a single compartment.

1 Gabella, G. Personal communication.
**42K Influx and Efflux Rates**

Similarly, 42K efflux from isolated smooth muscle cells behaves as would be expected for exchange in a two-compartment system. As can be seen in Fig. 4, the specific activity of the intracellular K⁺ declined exponentially with time after dilution of the cells with unlabeled medium. The mean rate constant for this decline averaged $5.05 \pm 0.58 \times 10^{-5} \text{s}^{-1}$ (seven experiments). Using this rate constant, our estimate of the intracellular K⁺ concentration in each experiment and an estimate of $V/A$ obtained from morphometric analysis as described above, we calculate an efflux rate ($M_{\text{out}}$) of $1.20 \pm 0.15 \text{pmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$ for K⁺ in the isolated smooth muscle cells. This was virtually identical to the calculated influx rate ($M_{\text{in}}$) in these cells. 42K influx (corrected for radioactive decay during counting and for backflux of isotope) averaged $1.24 \pm 0.11 \text{pmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$ (see Appendix for the formulas employed). The similarity between influx and efflux rates in the isolated smooth muscle cells further supports our conclusion that the cells were in a steady state during the course of these experiments.

**Exchangeability of Cellular K⁺ Contents**

The data for $M_{\text{in}}$ and $M_{\text{out}}$ can be used to test the suggestion that K⁺ exchange involves a simple two-compartment system. Specifically, since $M_{\text{in}}$
\[ M_{\text{OUT}} = (k_{\text{OUT}}) \cdot \frac{[K^+]_{\text{IN}}}{V/A} \]

A paired analysis of the potassium content data measured by flame emission spectroscopy with the content data predicted from efflux data indicated no statistically significant difference between 

\[ \text{K}^+_{\text{IN observed}} = (119.0 \pm 8.2 \text{ mM/liter ICF}) \]

and 

\[ \text{K}^+_{\text{IN predicted}} = (132.3 \pm 18.7 \text{ mM/liter ICF; seven experiments}) \]

This finding confirms the results from compartmental analysis of influx data which suggested a single intracellular compartment for \( \text{K}^+ \). Furthermore, the data suggest that all of the cellular \( \text{K}^+ \) is exchangeable.

**Effect of Ouabain on \( ^{42} \text{K} \) Movements**

Under normal steady-state conditions the transmembrane electrochemical gradient for \( \text{K}^+ \) favors \( \text{K}^+ \) loss from the cytoplasm. The maintenance of cellular \( \text{K}^+ \) levels in most tissues, including smooth muscle (Casteels, 1966; Van Breemen et al., 1973) thus requires the intervention of an ion transport system, presumably the \( \text{Na}^+/\text{K}^+ \) "pump." To assess the contribution of this transport system to steady-state \( ^{42} \text{K} \) influx in these cells, we examined the effects of ouabain, an inhibitor of the \( \text{Na}^+/\text{K}^+ \) transport system, on \( ^{42} \text{K} \) fluxes.
in the isolated cells. As can be seen in Table II, exposure of the cells to $5 \times 10^{-4} \text{M}$ ouabain caused a 49% reduction in $^{42}\text{K}$ influx; thus, at least 49% of influx is presumably mediated by the $\text{Na}^+/\text{K}^+$-dependent transport system in these cells. This fraction is similar to the ouabain-inhibitable portion of $\text{K}^+$ influx observed in frog stomach muscle (Stephenson, 1976). There are, however, several reasons to suspect that this value underestimates the contribution of this transport system to $\text{K}^+$ influx in the toad stomach muscle cells. For one, it is likely that the enzyme was not fully inhibited under these conditions, since tissues derived from the toad are extremely insensitive to ouabain; e.g., the $\text{Na}^+/\text{K}^+$ ATPase from toad heart is 18,000-fold less sensitive to ouabain than the enzyme from dog, cat, human, or mouse heart at 20°C (Repke et al., 1965). Secondly, the apparent increase in efflux rate in the presence of ouabain may signal an increase in $P_{\text{K}^+}$ and as a consequence the passive component of influx may have increased. A precise estimate of $P_{\text{K}^+}$ in the presence of ouabain is difficult to obtain from our data owing to probable decreases in $[\text{K}^+]_\text{IN}$ and resting membrane potential ($V_m$) during the course of ouabain.

**Figure 4.** $^{42}\text{K}$ efflux from smooth muscle cells in a single suspension. At time $t = 0$, the specific activity of the bathing medium was reduced by dilution and the efflux of $^{42}\text{K}$ from the intracellular fluid was monitored as a function of time. Since the specific activity of the intracellular fluid declined asymptotically toward a small but nonzero level, i.e., the specific activity of the extracellular fluid after dilution ($S_{a_{\text{w}}}$), the washout data was plotted as the log ($S_{a_{\text{i}}} - S_{a_{\text{w}}}) / (S_{a_{0}} - S_{a_{\text{w}}})$ vs. time. Note that the intracellular specific activity declined as a single exponential with time as would be expected for exchange in a simple two-compartment system.
exposure. An examination of the ouabain inhibition of $K^+$ influx thus cannot
give a precise estimate of the "pump-mediated" portion of $K^+$ influx in these
isolated smooth muscle cells, and in fact, our data must be viewed as a
minimal estimate of the pump contribution to influx. To examine the contribu-
tion of this transport system further, therefore, we examined the effects of
variations of $[K^+]_{\text{out}}$ on $^{42}\text{K}$ movements.

*Effect of ECF $[K^+]$ on $^{42}\text{K}$ Movements*

The effects of varying the extracellular $[K^+]$ on $^{42}\text{K}$ influx rates is shown in
Fig. 5. The upper trace depicts the total $^{42}\text{K}$ influx into the intracellular
compartments at each $[K^+]_{\text{out}}$. In the lower trace, the "passive" component

\[
\text{TABLE II}
\]

**EFFECT OF OUABAIN ON $^{42}\text{K}$ FLUXES AND K$^+$ CONTENTS OF
ISOLATED SMOOTH MUSCLE CELLS**

| $M_{\text{IN}}$ | $M_{\text{OUT}}$ | $[K^+]_{\text{IN}}$ |
|----------------|-----------------|------------------|
| pmol·cm$^{-2}$·s$^{-1}$ | pmol·cm$^{-2}$·s$^{-1}$ | mM/liter ICF |
| Control | 0.968±0.108 | 1.20±0.16 | 131.5±5.15 |
| (4) | (7) | (4) |
| Ouabain | 0.523±0.086 | 4.11±1.95 | 83.4±8.1 |
| (5×10$^{-4}$ M) | (4) | (3) | (6) |
| $P < 0.025$ | $P < 0.05$ | $P < 0.005$ |

* Cells were preincubated either with or without ouabain at 5×10$^{-4}$ M for
at least 45 min prior to initiation of the flux studies. The influx rates
observed within the first 15 min after tracer addition (before backflux
significantly affected the apparent influx rate for either group) were averaged
above. The rate constant for efflux was obtained from graphical analysis of
efflux data obtained during the first 60 min of efflux and the efflux rate
$M_{\text{OUT}}$ was calculated as described in the Appendix. The K$^+$ content of each
sample was determined by flame emission spectroscopy and corrected for
extracellular K$^+$ and normalized for the estimated volume of ICF associated
with each cell pellet. Data in each column are the mean ± SEM from $n$
experiments where $n$ is designated in parentheses beneath each value.

of influx was subtracted from the total. The "passive" component was
estimated from the Ussing flux ratio, the measured efflux rate ($M_{\text{OUT}}$) at each
$[K^+]_{\text{OUT}}$, and an assumed value for membrane potential of $-50$ mV (see
Appendix). The remaining $^{42}\text{K}$ influx at each $[K^+]_{\text{OUT}}$, (total-passive), was
assumed to be the "nonpassive" transport-mediated influx. For this assump-
tion to be valid, the cells must be in a steady state with respect to K$^+$ contents
throughout the observation period. This criterion appears to hold during the
experimental period studied, i.e., between 5 and 60 min after changing
$[K^+]_{\text{OUT}}$. While the change in $[K^+]_{\text{OUT}}$ in these experiments did cause a small
change in $[K^+]_{\text{IN}}$, the cellular K$^+$ contents rapidly (within 5 min) achieved a
new steady state such that the contents measured between 5 and 30 min after
changing $[K^+]_{\text{OUT}}$ (indicated below as the number preceding the slash) was
approximately equal to that measured between 30 and 60 min after changing \( [\text{K}^+]_{\text{out}} \) (indicated as the number following the slash): at 0.25 mM \( [\text{K}^+]_{\text{out}} \), \( [\text{K}^+]_{\text{in}} = 102.2 \pm 16.9 \) (9) /94.4 \( \pm 14.3 \) (10); at 0.5 mM \( [\text{K}^+]_{\text{out}} \), \( [\text{K}^+]_{\text{in}} = 106.5 \pm 6.1 \) (21) /111.7 \( \pm 11.9 \) (29); at 1.0 mM \( [\text{K}^+]_{\text{out}} \), \( [\text{K}^+]_{\text{in}} = 106.6 \pm 14.9 \) (13) /90.1 \( \pm 9.4 \) (11); at 6.0 mM \( [\text{K}^+]_{\text{out}} \), \( [\text{K}^+]_{\text{in}} = 131.3 \pm 15.4 \) (20) /150.4 \( \pm 17.6 \) (18); and at 9.0 mM \( [\text{K}^+]_{\text{out}} \), \( [\text{K}^+]_{\text{in}} = 114.6 \pm 8.8 \) (23) /121.7 \( \pm 8.9 \) (21). Further support for the contention that the cells are in a steady state with respect to \( \text{K}^+ \) was provided by the finding that a change in \( [\text{K}^+]_{\text{out}} \) caused no significant trends in \( ^{42}\text{K} \) flux rates during this 55-min observation period. Thus the analytical approach described for determining the passive component of \( ^{42}\text{K} \) influx at each \( [\text{K}^+]_{\text{out}} \) appears to be based on

![Figure 5](image-url)

**Figure 5.** Effect of extracellular \( \text{K}^+ \) on \( ^{42}\text{K} \) influx in isolated smooth muscle cells. Extracellular \( \text{K}^+ \), \( [\text{K}^+]_{\text{out}} \), was varied between 0.25 and 9 mM and \( ^{42}\text{K} \) influx was measured. For each experiment the \( ^{42}\text{K} \) influx rate at each \( [\text{K}^+]_{\text{out}} \) was taken as the average rate observed between 5 and 60 min after changing \( [\text{K}^+]_{\text{out}} \) from its initial value of 3 mM; the influx rates during this interval were constant. The upper curve depicts the total \( ^{42}\text{K} \) influx (passive plus nonpassive) observed at each \( [\text{K}^+]_{\text{out}} \). The lower curve shows the component of \( ^{42}\text{K} \) influx at each \( [\text{K}^+]_{\text{out}} \) that could not be accounted for by passive diffusion. The component of influx mediated by passive diffusion was calculated using data for \( M_{\text{out}}, [\text{K}^+]_{\text{in}}, \) and membrane potential as described in the Appendix. \( M_{\text{out}} \) at each \( [\text{K}^+]_{\text{out}} \) was calculated using the rate constant for \( ^{42}\text{K} \) efflux observed between 5 and 60 min after dilution of the isotope. These values were as follows: at 0.25 mM \( [\text{K}^+]_{\text{out}} \), \( M_{\text{out}} = 0.4 \pm \) pmol cm\(^{-2}\) s\(^{-1}\) (1); at 0.5 mM \( [\text{K}^+]_{\text{out}} \), \( M_{\text{out}} = 0.52 \) pmol cm\(^{-2}\) s\(^{-1}\) (1); at 1.0 mM \( [\text{K}^+]_{\text{out}} \), \( M_{\text{out}} = 0.4 \pm \) pmol cm\(^{-2}\) s\(^{-1}\) (1); at 6.0 mM \( [\text{K}^+]_{\text{out}} \), \( M_{\text{out}} = 3.28 \times \) pmol cm\(^{-2}\) s\(^{-1}\) (1); at 9.0 mM \( [\text{K}^+]_{\text{out}} \), \( M_{\text{out}} = 2.67 \times \) pmol cm\(^{-2}\) s\(^{-1}\) (2). As can be seen in this figure, the nonpassive component of influx appears to plateau as \( [\text{K}^+]_{\text{out}} \) is raised as would be expected for a saturable, carrier-mediated process. Data from nine separate experiments were pooled for this graph, and the points show the mean ± SEM.
the valid assumption that the cells are in a steady state. The influx rates at each \([K^+]_{OUT}\) after subtraction of this passive component are shown in the lower curve of Fig. 5. Note that the nonpassive component of \(^{42}\)K influx appears to saturate at \([K^+]_{OUT} > 6\) mM. In order to obtain an estimate of the \(K_m\) and \(V_{max}\) for the apparently saturable nonpassive component of \(^{42}\)K influx, these data were analyzed on a Lineweaver-Burke plot (Fig. 6). A \(K_m\) of 0.6 mM \([K^+]_{OUT}\) and a \(V_{max}\) of 1.3 pmol·cm\(^{-2}\)·s\(^{-1}\) were obtained by this analysis.

![Graph](image)

**Figure 6.** The “nonpassive” component of \(^{42}\)K influx at each \([K^+]_{OUT}\) (see Fig. 5 and Appendix) was plotted according to Lineweaver-Burke to obtain an estimate of the apparent \(V_{max}\) (1.3 pmol·cm\(^{-2}\)·s\(^{-1}\)) and \(K_m\) (0.6 mM \([K^+]_{OUT}\)) for K\(^+\) transport in isolated smooth muscle cells. Data from the experiments shown in Fig. 5 were pooled for this graph.

**DISCUSSION**

Results from the present study indicate that isolated smooth muscle cells can provide a useful model system for the study of ion movements and distribution in smooth muscle. This system eliminates the diffusion delays imposed by the complex geometry of intact smooth muscle and minimizes the complications imposed by the binding of ions to sites in the extracellular material of the tissue. As a result, identification of transmembrane ion movements and kinetic analysis of such movements are greatly simplified. Studies of K\(^+\) movements in these isolated smooth muscle cells indicate that virtually all the cellular K\(^+\) exchanges in a manner predicted for a simple two-compartment system: an extracellular and an intracellular compartment. More importantly, these
studies demonstrated that the procedures used during cell preparation and subsequent experimental manipulation do not seriously alter the integrity of the smooth muscle cell membrane or the normal regulatory mechanisms that control K⁺ distribution in these cells. Furthermore, the cells remain stable in this state for several hours after isolation. Thus, studies of ion movements in the isolated smooth muscle cells should provide insight into the mechanisms controlling ion movements and distribution in intact smooth muscle.

Studies of ⁴²K efflux from the isolated smooth muscle cells can provide information regarding the permeability of the smooth muscle membrane to K⁺. If one assumes that, in these cells as in other cells (Sjodin and Henderson, 1964), K⁺ efflux is driven entirely by passive diffusion down an electrochemical gradient, our measurement of K⁺ efflux can be used to estimate P_k⁺ and the role of K⁺ in determining the electrical properties of the membrane at rest. Using our values for \( M_{\text{OUT}} = (1.2 \pm 0.16 \ \text{pmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}) \) and \( [K^+]_m = (131.5 \pm 5.15 \ \text{mM/liter intracellular water}) \), and an estimate of −50 mV for the resting membrane potential² (made at room temperature in standard APS), we calculate a resting membrane permeability to K⁺ of \( 2.88 \times 10^{-8} \ \text{cm/s} \) (see Appendix for details). This is quite similar to the resting permeability to K⁺ observed in frog stomach muscle \( (4.04 \times 10^{-8} \ \text{cm/s}) \), corrected to 22°C using the author's \( Q_{10} \), Stephenson, 1976), and to that observed in guinea pig taenia coli \( (6.71 \times 10^{-8} \ \text{cm/s at 37°C}) \), Brading, 1971) and vas deferens \( (8.9 \times 10^{-8} \ \text{cm/s at 35°C}) \), Casteels, 1969) if corrections are made for temperature effects. This estimate of \( P_k⁺ \) in turn can be used to calculate \( g_{K⁺} \) (see Appendix for details). We estimate the resting potassium conductance of the smooth muscle membrane at room temperature to be \( 2.7 \ \mu\text{mho/cm}² \), a value similar to that observed in guinea pig taenia coli \( (5.7 \ \mu\text{mho/cm}² \), Brading, 1971; 9.0 \ \mu\text{mho/cm}² \), Casteels, 1969) but considerably lower than that observed in most skeletal muscles (detubulated frog sartorius, \( g_{K⁺} = 28 \ \mu\text{mho/cm}² \), Eisenberg and Gage, 1969) and in nerve \( (\text{Loligo axon} \ g_{K⁺} = 230 \ \mu\text{mho/cm}² \), Hodgkin and Huxley, 1952; \text{Sepia axon} \ g_{K⁺} = 50 \ \mu\text{mho/cm}² \), Hodgkin and Keynes, 1955)². Our calculated \( g_{K⁺} \) constitutes only a small fraction of the total membrane conductance \( (g_{\text{total}} \sim 15 \ \mu\text{mho/cm}² \) as estimated from electrophysiological data³) suggesting that the smooth muscle cell membrane at rest is permeable to ions in addition to K⁺. Similarly, in skeletal muscle \( g_{K⁺} \) constitutes less than half of the total ionic conductance of the membrane (Eisenberg and Gage, 1969). In the latter tissue chloride conductance appears to contribute a large fraction of the total ionic conductance. Chloride conductance may also constitute a large fraction of \( g_{\text{total}} \) in our isolated smooth muscle cells. Chloride permeability in other types of smooth muscle is high, similar in magnitude to \( P_{K⁺} \) (Casteels, 1969), and the transmembrane chloride gradient appears to affect the resting membrane potential (Kuriyama, 1963). In addition to chloride, sodium conductance could also contribute significantly to \( g_{\text{total}} \) in our isolated smooth muscle cells. Sodium permeability in these cells is considerably higher than that of skeletal muscle or nerve³ and

² Yamaguchi, H. Unpublished observations.
³ Scheid, C. R., and F. S. Fay. Manuscript in preparation.
quite similar to that for potassium. In addition, the resting membrane potential in these cells shows a considerable Na⁺ dependence (Singer and Walsh, 1977).

Besides providing some insight into the passive membrane properties of smooth muscle, the present studies provide evidence supporting the existence of a carrier-mediated transport system for K⁺. ⁴²K influx in these cells was greater than could be accounted for by passive processes, and the nonpassive component of K⁺ influx exhibited saturation kinetics as would be expected for a carrier-mediated process. Several lines of evidence suggest that this transport reflects activity of a Na⁺/K⁺ pump. For one, ⁴²K influx was markedly inhibited by ouabain, a known inhibitor of Na⁺/K⁺-coupled transport. In addition, studies of enzyme activity in membrane fragments from these isolated smooth muscle cells have demonstrated a Na⁺- and K⁺-dependent ATPase which exhibits many characteristics of the sodium-potassium “pump” of other systems (Table I of Scheid et al., 1979). Furthermore, the apparent Michaelis constant for this transport process in the isolated smooth muscle cells (0.6 mM) is quite similar to that of the Na⁺/K⁺ transport system in red blood cells (Km = 1.4 mM, Garrahan and Glynn, 1967), and frog stomach muscle (Km = 0.3 mM, Stephenson, 1976). Moreover, the apparent Vmax for this transport process at 22°C (1.3 pmol·cm⁻²·s⁻¹) is virtually identical to the Vmax of the Na⁺/K⁺ transport system in frog stomach muscle (0.9 pmol·cm⁻²·s⁻¹ corrected to 20°C using the author’s Qjo, Stephenson, 1976). This estimate of Vmax observed under basal conditions probably does not accurately assess the maximum transport capacity of the K⁺ transport system, however, since agents such as isoproterenol can elicit a 3.6-fold increase in ouabain-sensitive K⁺ uptake (Scheid et al., 1979).

While these studies on the isolated muscle cells circumvent several difficulties inherent to work on intact tissue, not all uncertainty could be eliminated from our estimates of Pk⁺, gK⁺, gK⁺/gtotal nor from our estimates of the Vmax and Km of the Na⁺/K⁺ pump. There are several possible sources of error. For one, we can not be absolutely certain of the membrane potential and input resistance of our cells under the conditions where flux measurements were made. The flux studies were carried out on large populations of cells using noninvasive techniques, whereas the electrophysiological studies utilized intracellular microelectrode recordings from a much smaller group of cells. The latter recordings appear to provide a reasonable estimate of Vm in the isolated cells since our estimate of Vm is similar to those obtained in intact smooth muscle from various amphibian tissues (frog stomach: -60 mV, Suenaga and Nishi, 1956; frog stomach: -20 to -70 mV, Kolodny and Van der Kloot, 1961; salamander stomach: -30 to -70 mV, Greven, 1953). However, uncertainties in the electrophysiological measurements cannot be fully ruled out since there may be leakage paths around the microelectrodes which could lead to an underestimate of Vm and an overestimate of gtotal. An overestimate of gtotal would affect our estimate of the contribution of gK⁺ to gtotal. An underestimate of Vm would affect our estimate of the passive component of influx and thus would affect our assessment of the Km and Vmax for carrier-
mediated $^{42}\text{K}$ influx. In addition, our assessment of the $K_m$ for this process is compromised by the possibility that changes in $[K^+]_{OUT}$ bring about secondary changes in $[K^+]_{IN}$ and/or $[Na^+]_{IN}$, which could alter the activity of the $Na^+/K^+$ transport system. These are not problems unique to isolated cells, however; similar uncertainties have complicated the analysis of ion permeation across cells within intact tissue. Despite these remaining problems, it is important to note that studies of ion movements in isolated smooth muscle cells do seem to eliminate many of the uncertainties associated with analysis of transmembrane ion flux. We believe that this approach may aid in understanding the mechanisms controlling the resting distribution of ions in addition to $K^+$ and may prove useful in attempts to determine the mechanism of action of a whole range of agents known to be excitatory and inhibitory on smooth muscle.

APPENDIX

The efflux rate for $K^+$ was calculated using the equation of Keynes and Lewis (1951):

$$M_{OUT} = (k_{OUT})([K^+]_{IN})(V/A),$$

where $k_{OUT}$ is the slope of the line relating the logarithm of the intracellular specific activity to time. In these experiments, washout was initiated by diluting the extracellular specific activity rather than by transferring the cells to an unlabeled buffer. Thus, the specific activity of the intracellular fluid declined asymptotically towards a small but nonzero level, the specific activity of the extracellular fluid ($SA_{ICF}$); and the washout data were plotted as

$$\log\frac{(SA_{ICF} - SA_{ICF_0})}{(SA_{ICF} - SA_{ICF_0})}$$

vs. time.

The intracellular $K^+$ concentration, $[K^+]_{IN}$, used in calculating the $K^+$ efflux rate, was obtained from atomic emission analysis of the total $K^+$ contents of the cell pellets; the $K^+$ content data was then corrected for extracellular $K^+$ (that distributed in the $[^3\text{H}]$inulin space) and the intracellular $K^+$ concentration was calculated using an estimate of the ICF associated with the cell pellet ($[^{14}\text{C}]$urea space minus $[^3\text{H}]$inulin space). The average ratio of volume: surface area for these cells, $2 \times 10^{-4}$ cm, was obtained from morphometric analysis as described in Methods.

The influx rate for $K^+$ was calculated as:

$$M_{IN} = \frac{ICF_{^{42}\text{K}}_{t_2} - ICF_{^{42}\text{K}}_{t_1}}{SA_{ECF}},$$

$$\frac{(t_2 - t_1)(ICF \text{ vol})(A/V)}{SA_{ECF}}$$

where the $ICF_{^{42}\text{K}}$ = total $^{42}\text{K}$ in the cell pellet minus the $^{42}\text{K}$ assumed to be distributed in the trapped extracellular fluid (the $[^3\text{H}]$inulin space); the $SA$
ECF is the specific activity of the bathing medium; the ICF volume is that measured as the difference between the \(^{14}\)Curea space and the \(^{3}\)Hinulin space of the cell pellet; and \(A/V = 0.5 \times 10^4 \text{ cm}^{-1}\) is the average ratio of surface area/volume obtained from morphometric analysis. This estimate of surface area/volume does not take into account the extra membrane area associated with caveolae. Preliminary observations\(^1\) on smooth muscle cells within strips of toad stomach suggest that caveolae may increase the membrane area by 56\%. Note that the apparent radioactivity at each time \(t\) (ICF \(^{42}\)K) was corrected for the backflux of isotope from the cell using the correction factor \(kt (1-e^{-kt})\) as described by Keynes (1954), where \(k\) is the rate constant obtained in the efflux experiments as described above. Note also that the extracellular fluid compartment (10 ml/ aliquot) was much larger than the intracellular fluid compartment (\(<10 \mu l\)), and thus we assumed that the extracellular specific activity remained constant with time. The influx of \(^{42}\)K was also expressed in terms of a fractional equilibrium, i.e., the fraction of the intracellular potassium which equilibrated within a given time \(t\). This fraction ICF \(^{42}\)K/ICF \(^{40}\)K, was calculated for each of three to four replicate cell aliquots assuming all of the cellular \(^{40}\)K to be exchangeable. The mean fraction at each time point was converted to a percentage and plotted as (100 − percent equilibrium) vs. time as shown in Figs. 2 and 3.

Analysis of \(^{42}\)K movements using the Ussing flux ratio (Ussing, 1949) suggested that a large portion of \(^{42}\)K influx was not mediated by passive diffusion. A minimum estimate of the nonpassive component of \(^{42}\)K influx, 49\% nonpassive, was obtained from studies of ouabain inhibition of \(^{42}\)K influx. However, it was not clear whether ouabain at 5 \(\times\) 10\(^{-4}\) M was capable of fully inhibiting the \(\text{Na}^+/\text{K}^+\) pump in these cells. Thus the nonpassive component of influx was estimated using the following equation:

\[
M_{\text{IN passive}} = M_{\text{OUT}} \cdot \beta /\alpha \cdot [K^+]_{\text{OUT}}/[K^+]_{\text{IN}},
\]

where

\[
\beta = \frac{+V_mF/RT}{1 - e^{+V_mF/RT}},
\]

and

\[
\alpha = \frac{-V_mF/RT}{1 - e^{-V_mF/RT}}.
\]

In the latter terms describing the electrical forces on ion movement \(V_m\), the resting membrane potential, was assumed to be \(-50 \text{ mV}\). \(F\) is the Faraday, \(R\) the gas constant, and \(T\) the absolute temperature (295°K in these experiments).

Eq. 1 was derived from the expression of Hodgkin and Keynes (1955 b)
relating permeability and efflux rate:

\[ P_{K^+} = \frac{M_{OUT}}{[K^+]_{IN}\alpha}, \]

an expression describing steady state ion fluxes: \( M_{OUT} = M_{IN}^{\text{passive}} + M_{IN}^{\text{nonpassive}} \) and an expression relating membrane permeability to passive influx of \( K^+ \):

\[ P_{K^+} = \frac{M_{IN}^{\text{passive}}}{[K^+]_{OUT}\beta}. \]

The passive component of \( ^{42}\text{K} \) influx at each value of \( [K^+]_{OUT} \) was calculated and subtracted from \( M_{IN}^{\text{total}} \) to estimate the nonpassive component of \( ^{42}\text{K} \) influx. It should be noted that the value for \( V_m \) was assumed to remain constant at \(-50 \text{ mV} \) over the range of extracellular \( K^+ \) concentrations tested (0.25–9 mM). Electrophysiological studies of the dependence of \( V_m \) on \( [K^+]_{OUT} \) in other smooth muscle have indicated a 5-mV change in resting membrane potential over this range (Kuriyama and Suzuki, 1978). Similarly, in isolated smooth muscle cells there is only a slight dependence of \( V_m \) on \( [K^+]_{OUT} \) over this concentration range.\(^4\) If indeed the membrane potential depolarized by 5 mV in the presence of 9 mM \( [K^+]_{OUT} \), we would overestimate the passive component in influx by 21% and thus underestimate the nonpassive component by \( \sim 17\% \). The effect of \( V_m \) on the estimate of \( M_{IN}^{\text{passive}} \) is smaller at lower values of \( [K^+]_{OUT} \) since this component constitutes a smaller fraction of the total \( K^+ \) influx. Thus the estimate of \( K_m \) could be underestimated by \( \sim 15\% \) and \( V_{max} \) by \( \sim 9\% \).

\( g_{K^+} \) was calculated from the difference in the potassium current at \(-50 \) and \(-70 \text{ mV} \) using the equations of Hodgkin and Katz (1949):

\[ I_{K^+} = (P_{K^+}) \left( \frac{F^2}{RT} \right) (V_m) \frac{[K^+]_o - [K^+]_i e^{V_m F/RT}}{1 - e^{V_m F/RT}}, \]

in which \( P_{K^+} \), \( F \), \( R \), and \( T \) have their usual meanings, \( E_{K^+} \) is the Nernst equilibrium potential for potassium and \( [K^+]_o \) and \( [K^+]_i \) represent the extracellular and intracellular \( K^+ \) concentration, respectively. Values of \(-50 \) and \(-70 \) for \( V_m \) were chosen since these voltages were utilized to determine the total membrane conductance in the electrophysiological experiments.

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