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Regulation of potassium transport in rat mesangial cells : a fluorescent analysis using the potassium sensitive dye, PBFI

Scott Eric Kasner
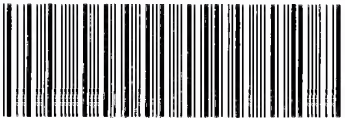*Yale University*

REGULATION OF POTASSIUM TRANSPORT
IN RAT MESANGIAL CELLS:
A FLUORESCENT ANALYSIS USING
THE POTASSIUM-SENSITIVE DYE, PBFI

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REGULATION OF POTASSIUM TRANSPORT IN RAT

MESANGIAL CELLS: A FLUORESCENT ANALYSIS USING

THE POTASSIUM-SENSITIVE DYE, PBFI

A Thesis Submitted to the Yale University School of Medicine

in Partial Fulfillment of the Requirements for the Degree of

Doctor of Medicine
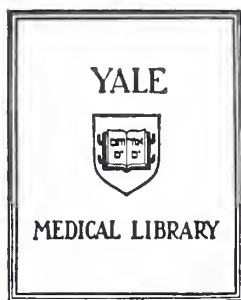
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ABSTRACT

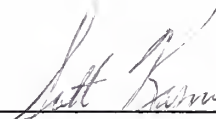REGULATION OF POTASSIUM TRANSPORT IN RAT MESANGIAL CELLS: A FLUORESCENT ANALYSIS USING THE POTASSIUM-SENSITIVE DYE, PBFI. Scott E. Kasner and Micha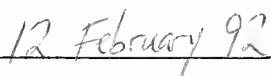el B. Ganz. Section of Nephrology, Department of Medicine, and Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, CT. 1992.

We investigated the regulatory transport processes that maintain potassium homeostasis in cultured rat glomerular mesangial cells (MCs). Intracellular potassium concentration ($[K^+]_i$) of MCs was measured by spectrofluorometry using the potassium-sensitive dye, potassium-binding benzofuran isophthalate (PBFI). Ionophores valinomycin and nigericin were used to clamp $[K^+]_i$ to known $[K^+]_o$ and thereby obtain an intracellular calibration of the dye. Normal resting $[K^+]_i$ in MCs was 102 ± 7 mM. When MCs were exposed to ouabain, $[K^+]_i$ fell to 48 ± 6 mM and did not recover, suggesting the presence of the the Na^+/K^+-ATPase. When MCs were exposed to furosemide, $[K^+]_i$ transiently declined to 58 ± 11 mM that was followed by rapid recovery to near steady-state, indicating the additional presence of the $Na^+/K^+/Cl^-$ cotransporter. Recovery was completely abolished when MCs were exposed to ouabain. Exposure to barium led to an immediate increase in $[K^+]_i$ to 124 ± 8 mM followed by a rapid return to steady-state $[K^+]_i$. Resting $[K^+]_i$ was not altered by angiotensin II (ANG II), serotonin (5-HT), bradykinin (BK), or atrial natriuretic peptide (ANP). ANG II, 5-HT, and BK stimulated the Na^+/K^+-ATPase by 28, 41, and 24%, respectively, as measured during recovery of $[K^+]_i$ toward normal following a diuretic-induced fall in $[K^+]_i$. ANG II and 5-HT also significantly stimulated the activity of the $Na^+/K^+/Cl^-$ cotransporter, by 55 and 47%, respectively. BK had no significant effect on the cotransporter, and ANP had no significant effect on either the ATPase

or the cotransporter. We conclude: 1) MCs possess the ouabain-sensitive Na^+/K^+-ATPase, the loop diuretic-sensitive $Na^+/K^+/Cl^-$ cotransporter, and barium-sensitive K^+ channels. 2) ANG II, 5-HT, and BK stimulate the Na^+/K^+-ATPase, but only ANG II and 5-HT stimulate the $Na^+/K^+/Cl^-$ cotransporter in MCs. ANP had no significant influence on either. 3) Continuous measurement of $[K^+]_i$ and an examination of its regulatory mechanisms in MCs can be achieved through the use of the fluorescent dye, PBFI.

ACKNOWLEDGEMENTS

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INTRODUCTION

Overview

Vasoactive agents have been shown to regulate glomerular filtration, presumably by eliciting changes in glomerular surface area via mesangial cell contraction and relaxation. Moreover, many of these agents have been demonstrated to influence ion transport mechanisms in contractile cells. Potassium transport mechanisms have been proposed to play a key part in mesangial cell volume and shape regulation, and they may be modulated by vasoactive agents. The mechanisms of potassium transport and their roles in determination of membrane potential and regulation of cell volume will be briefly discussed in the following sections. The effects of vasoactive agents on these cellular functions will also be reviewed. We sought to evaluate the effects of vasoactive agents on mesangial cell potassium transport during hormonally-induced alterations in mesangial cell shape. This was accomplished through the use of the novel potassium-sensitive fluorescent dye, PBFI.

Mesangial Cells and Glomerular Filtration

The mammalian renal glomerulus is a microvascular network which serves as the site of ultrafiltration of plasma. This capillary network is comprised of epithelial, endothelial, and mesangial cells, as

well as an unique basement membrane. The process of ultrafiltration appears to be dependent on the interactions among these three cell types, in addition to regulatory systemic and local factors. Recent evidence has emerged indicating that the glomerulus is both a target for, and a site of synthesis of, endogenous vasoactive agents. These hormones are thought to play an important role in the regulation of the glomerular filtration rate (GFR).

The filtration rate for a single glomerulus (single nephron glomerular filtration rate, SNGFR) is determined by the relationship between the hydraulic pressure (P) and oncotic pressure (Π) gradients across the glomerulus, as well as the ultrafiltration coefficient (K_f):

$$SNGFR = K_f(\Delta P - \Delta\Pi)$$

K_f is a composite parameter which is defined as the product of the effective hydraulic permeability of the capillary wall and the capillary surface area available for filtration (15). Consequently, SNGFR can be changed by a perturbation in any one or more of these factors. Hydraulic and oncotic pressure alterations may occur at systemic, whole kidney, or local levels and will not be discussed here. The effective hydraulic permeability appears to maintain a relatively constant value in mammals, and it has been suggested that differences in K_f between and within species are predominantly a function of variations in capillary surface area (16,65).

A wide variety of humoral agents have been shown to regulate K_f: angiotensin II (ANG II), arginine vasopressin (AVP), parathyroid hormone (PTH), norepinephrine, acetylcholine (ACh), bradykinin (BK), histamine (HIS), and prostaglandins (PG) E_2 and I_2 (4,32,67). Although the glomerular response to ANG II is most extensively studied, it has been observed that the changes in glomerular filtration caused by PTH, HIS, PGI_2, PGE_2, and dibutyryl cyclic AMP (DBcAMP) are strikingly similar to those caused by ANG II (67). However, the decline in K_f caused by all of the aforementioned agents can be completely blocked by the specific ANG II antagonist, saralasin (67). This implies that these hormones exert their effect on the glomerulus by an ANG II-dependent pathway. It has thus been suggested that these vasoactive agents act in part by stimulating local synthesis and release of ANG II. Further work confirmed this hypothesis; the juxtaglomerular epithelial cells contain all of the enzymes required for the synthesis of ANG II, in addition to those used in the synthesis of renin (9). Therefore, within the glomerulus itself lies the machinery to regulate filtration.

Renal glomerular mesangial cells (MCs) are smooth muscle-like cells that occupy the highly specialized interstitium of the glomerulus. Their interposition between endothelial and epithelial cells, combined with their abundant intracellular contractile myofilaments, make them particularly well-suited for a key role in the modulation of GFR via their ability to alter their shape and surface area (66). MCs also appear to have an immunological function, and proliferation of these cells has been implicated in the inflammatory response to glomerular injury (35,76).

MCs in culture have been demonstrated to have binding sites for the vasoactive peptide, ANG II, and will contract in response to this agent (3). In addition, cultured MCs exhibit contractile responses to other vasoactive substances, such as AVP and norepinephrine, as well as to mediators of inflammation including HIS and serotonin (3,16,38,39,46). Atrial natriuretic peptide (ANP) appears to inhibit the contractile response (2). To date, only ANG II, AVP, and norepinephrine have been demonstrated to stimulate specific receptors on MCs leading to contraction (3,46). The ANG II-dependent agents described above have no contractile effect on isolated cultures of MCs (3,39), thus providing further support for ANG II as a mediator in the regulation of K_f and SNGFR.

Many stimuli for contraction are also known to influence specific ion transport systems in contractile cells (6,8,19,31,53,62,78). Ion transport is of major importance in the regulation of volume in all eukaryotic cells, and is likely to be of particular significance in cells that are frequently changing their shape and surface area. Vascular tone is clearly dependent on membrane potential, which in turn is a function of the transmembrane ion gradients and conductivity. In vascular smooth muscle, regulation of potassium (K^+) transport mechanisms is thought to play a critical role in the control of vascular tone at the cellular level (13,26,51). Vasoactive agents have been recently found to regulate K^+ flux pathways in vascular smooth muscle cells, and evidence is emerging that these pathways are also closely regulated in MCs. Thus, identification of the mechanisms of K^+ homeostasis in MCs and investi-

gation of the behavior of the K^+ transporters during hormonally-induced MC contraction and relaxation is crucial for understanding the role of the MC in glomerular filtration.

Mechanisms of Cell Potassium Homeostasis

The maintenance of a high intracellular potassium concentration is essential for many processes including cell growth, enzyme activity, cell volume regulation, and determination of membrane potential. Cell potassium concentration is determined by the balance of influx and efflux across the cell membrane, including processes of active and passive transport. The cell membrane may be conceived as a barrier to ionic species. Spanning this lipid bilayer are integral membrane proteins that transport non-lipid-soluble substances through the barrier (61).

Three categories of membrane transport proteins are described. The first major type of transport mechanism works passively, moving ions down their electrochemical gradients without dependence on other ions. Passive transport commonly occurs through ion-specific channels. Primary active transport mechanisms utilize the chemical energy from ATP hydrolysis in order to move ions across a membrane. In general, ions are moved against either a concentration gradient or an electrical gradient by this system. Secondary active transport, or coupled transport, does not require the chemical energy of hydrolysis. Instead, it makes use of the potential energy of the electrical or concentration

gradient of one ion to drive another ion against its own gradient. This can occur with the solutes being simultaneously transported in the same direction or opposite directions. Such mechanisms are known as cotransporters and exchangers (or antiporters), respectively.

Membrane transport of potassium is generally performed by one or more of four major pathways. The sodium/potassium ATPase (Na^+/K^+-ATPase) pumps sodium (Na^+) out of the cell and K^+ into the cell, hydrolyzing ATP in order to transport both ions against their concentration gradients. The inward Na^+ gradient can drive simultaneous transport of Na^+, K^+, and two chloride (Cl^-) ions into the cell via a secondary active transporter, the $Na^+/K^+/Cl^-$ cotransporter (also known as the $Na^+/K^+/2Cl^-$ cotransporter). K^+ efflux may occur via K^+ channels or a K^+/Cl^- cotransporter. The function of each cell type will determine which of these K^+ transporters will be present, as well as the number of transport molecules, and the appropriate mechanisms for regulation. In most cases, intracellular K^+ concentration ($[K^+]_i$) is maintained at a steady-state value under normal conditions by a dynamic equilibrium among the multiple K^+ transporters (61). In contractile cells, at least three of the major K^+ transport mechanisms have been demonstrated to be involved in K^+ homeostasis: the Na^+/K^+-ATPase, the $Na^+/K^+/Cl^-$ cotransporter, and K^+ channels (17,62). The coordinated interaction of these mechanisms thus keeps $[K^+]_i$ relatively constant at a level well above the extracellular K^+ concentration ($[K^+]_o$).

Potassium and Membrane Potential

Potassium is an important factor in the determination of membrane potential. The large K^+ gradient across the membrane is maintained by the balanced interactions of the many ion transporters. Further, resting cell membranes are generally more permeable to K^+ than to Na^+, Cl^-, or other ions. Membrane potential depends on both the magnitude of the gradient and the permeability for each ion, and therefore is determined predominantly by K^+. The equilibrium potential for a particular ion is defined as the potential at which there is no net ion transfer across the membrane, i.e., when the forces on the ion are equal on both sides of the membrane. In the case of K^+, the chemical potential of the concentration gradient drives K^+ outward because $[K^+]_i$ is greater than $[K^+]_o$, and the electrical potential pulls K^+ inward since the cell is electronegative inside and attracts positively-charged ions. No net potassium current flows when these forces are balanced, and K^+ is at equilibrium across the membrane. This equilibrium potential for K^+ (E_K) is also known as the Nernst potential and is calculated as:

$$E_K = \frac{RT}{zF} \ln \frac{[K^+]_o}{[K^+]_i}$$

where R is the gas constant, T is the absolute temperature, z is the charge of the ion, and F is the Faraday constant. For example, a hypothetical cell with a K^+ concentration of 140 mM inside and 4 mM outside would have an E_K of approximately -90 mV. In a living cell, the measured membrane potential (E_m) is usually near E_K, and is

generated not only by K^+, but by other ions to which the membrane may also be permeable (11).

In arterial smooth muscle cells, E_m ranges between -35 mV and -75 mV (26,51). These cells have a relatively lower permeability to K^+ than other excitable tissues and a somewhat higher ratio of Na^+ to K^+ permeability (26). Moreover, vascular smooth muscle can alter its K^+ conductance in response to environmental changes (29). Small changes in $[K^+]_0$ therefore do not lead to dramatic alterations of E_m. Nonetheless, small graded potential changes in E_m do lead to significant changes in intracellular calcium concentration, which is the major regulator of the smooth muscle contractile apparatus. Thus, slight depolarization may cause a marked increase in tone and slight hyperpolarization can lead to relaxation (8,33,70). It is also well documented that depolarization of smooth muscle will lead to a significant increase in tension induced by norepinephrine, whereas hyperpolarization results in a diminished contractile response to several vasoactive agents, particularly norepinephrine (8,25,27). The actual relationship between the effect of vasoactive agents and E_m is unclear, however. In many studies, norepinephrine-induced contraction of smooth muscle was found to occur in the absence of membrane depolarization; this phenomenon is referred to as pharmacomechanical coupling (75). It appears that the value of E_m is an important factor in the development of tension in contractile tissue, but the initiating event, either electro- or pharmacomechanical, seems to depend on the specific cell type (26). Further, the influence of vasoactive agents on K^+ conductance and other K^+ transport mechanisms is likely to be a critical factor in the regula-

tion of membrane potential and vascular tone. This accentuates the biological importance of understanding K^+ homeostasis in cells. These issues have not been explored with regard to mesangial cell contractility.

Potassium and Cell Volume Regulation

Potassium is the predominant intracellular cation in most eukaryotic cells, and as such, its transport is of major importance in the control of volume in both isotonic and anisotonic media. In the steady state, ion transporters maintain transmembrane ion gradients while water flows freely across membranes to balance intra- and extracellular osmolality. This state can be disrupted by cellular injury such as hypoxia, or by substances which interfere with specific transport mechanisms.

An osmotic "crisis" can be created by inhibition of the Na^+/K^+-ATPase with ouabain. As soon as the ATPase is inactivated, the cell loses its ability to balance K^+ efflux (i.e., via channels) with an equal influx, and also cannot oppose the inward Na^+ gradient. Na^+ flows inward as K^+ exits in order to maintain electroneutrality. The loss of intracellular K^+ reduces the equilibrium potential for K^+ and the cell depolarizes. Depolarization then allows small anions, predominantly Cl^-, to move into the cell along their electrochemical gradients. The relative number of anionic molecules in the cell (including both the polyvalent macromolecules and Cl^-) increases, and thus a greater

amount of Na^+ is drawn inward. The net result of ATPase inhibition is thus an accumulation of osmotically active particles within the cell. Water moves into the cell to equilibrate the osmotic pressure across the membrane and the cell swells (24).

Many cell types have mechanisms to limit osmotic swelling. The degree to which a cell will take up solute during ATPase inhibition depends on the permeability of the membrane to Na^+, K^+, and Cl^-. In toad bladder epithelial cells, for example, inhibition of the Na^+/K^+-ATPase will lead to a secondary block of Na^+ permeability which prevents severe osmotic overload and cell rupture (45). Similarly, the $Na^+/K^+/Cl^-$ cotransporter may function as an alternative pathway for solute influx, and its inhibition may decrease the effect of ATPase inactivation. This may be accomplished by an intracellular mechanism, or pharmacologically by loop diuretics such as furosemide, bumetanide, or ethacrynic acid (45). Regulation of one transporter in response to the altered functioning of another may thus serve as a homeostatic volume control mechanism in cells that may experience changes in their osmotic environment. This has been best characterized in the diluting segment cells of the thick ascending limb of the loop of Henle. These cells rapidly transport ions from their apical to basolateral surfaces without significant volume changes. Blockade of the $Na^+/K^+/Cl^-$ cotransporter by loop diuretics should lead to an abrupt decrease in cell volume, assuming that K^+ continues to leak out of the cell while influx is halted, but actually no dramatic change in volume was observed (24). The efflux of K^+ must therefore have been suddenly reduced to maintain a high $[K^+]_i$ and a constant volume (24). The

complex series of interactions which adjusts and coordinates the transporters is not understood, but it is clearly of great importance for these epithelial cells to regulate their volume, and it is likely to be a key regulator in many other tissues, especially contractile cells.

Vasoactive Agents and Potassium Transport

The regulation of potassium transport certainly plays a pivotal role in the maintenance of high $[K^+]_i$, determination of membrane potential, and control of cell volume. A vast amount of literature has attempted to demonstrate that vasoactive agents influence K^+ transport mechanisms in many cell types. In mesangial cells, vasoactive agents may, via the modulation of K^+ transporters, modify cellular tension and volume, thereby alter glomerular filtration, and ultimately affect systemic blood pressure, serum electrolyte balance, and urine output.

Clinically important regulation of sodium and potassium balance occurs in response to the mineralocorticoid, aldosterone. This hormone stimulates the Na^+/K^+-ATPase, as well as Na^+ and K^+ channels, in the cells of the distal nephron (80). Alternatively, atrial natriuretic peptide has been shown to inhibit the ATPase in several cell types (52,68), and appears to oppose the effect of aldosterone *in vivo*. AVP, ANG II, and norepinephrine have been shown to cause indirect stimulation of the ATPase in vascular smooth muscle cells (7,55,71). The stimulatory effect of ANG II on the ATPase was demonstrated in renal cortical tubules (56), and AVP stimulated the ATPase in rat medullary thick

ascending limb cells as well (10). It is unclear whether these agents have a direct effect on the Na^+/K^+-ATPase or if they cause an increase primarily in intracellular Na^+ concentration which then secondarily stimulates the ATPase (71). Modulation of the ATPase also appears to have an effect on the cell's response to these vasoactive agents. Inhibition of the ATPase enhances the vascular contraction induced by AVP, ANG II, and norepinephrine in smooth muscle (55,71), and the effect of bradykinin on atrial endothelial cells was also augmented by ATPase inhibition (42). Although there is a vast literature on the Na^+/K^+-ATPase, relatively little is known about its regulation by vasoactive agents in mesangial cells.

The effect of vasoactive agents on the $Na^+/K^+/Cl^-$ cotransporter seems to vary according to each individual tissue or cell type. In vascular smooth muscle cells, ANG II and ANP have both been demonstrated to stimulate the cotransporter (54,59,72). In endothelial cells, however, cotransport is increased by ANG II and decreased by ANP (52). Furthermore, the intracellular signals involved in the coupling of receptor-binding to modulation of transport also appear to differ among various cell types. Elevation of cyclic adenosine monophosphate (cAMP) will stimulate cotransport in avian erythrocytes (60) but inhibit it in mammalian erythrocytes (23). Cyclic guanosine monophosphate (cGMP), calcium, and protein kinase C (PKC) have also been implicated in signal transduction for $Na^+/K^+/Cl^-$ cotransporter regulation, and appear to exert different effects in different cell types (17,54,57,59). Serotonin (5-hydroxytryptamine, 5-HT) has been noted to activate adenylate cyclase and therefore increase cAMP levels in the guinea pig

hippocampus and in blowfly salivary gland tissue, but to inhibit the cyclase in rat hippocampus (64). Multiple receptor subtypes are known for this neurotransmitter and may explain the diversity of its effects on cAMP levels (64), but its effects on the $Na^+/K^+/Cl^-$ cotransporter are not established.

Homma *et al.* (31) have recently demonstrated the presence of the loop diuretic-sensitive $Na^+/K^+/Cl^-$ cotransporter in rat MCs using radioactive rubidium flux measurements. Their experiments demonstrated further that the cotransporter was stimulated by ANG II, AVP, and ANP (31). Increased intracellular cAMP levels inhibited the $Na^+/K^+/Cl^-$ cotransporter in MCs, while PKC caused transient stimulation of cotransport followed by significant inhibition (30). Homma and Harris (30) speculate that this biphasic response of the cotransporter may reflect undetected changes in the intracellular ionic environment, such as a response to redistribution of transported ions or alteration of cell volume. The $^{86}Rb^+$ flux method, however, does not provide a method for continuous monitoring of intracellular ion concentrations, and the precise sequence of events cannot be determined.

The effect of vasoactive agents on K^+ channels and conductance is not well understood, nor is the relationship between E_m and vasoactive agent-induced contraction of smooth muscle cells. In arterial smooth muscle, norepinephrine will induce an increase in tension without a change in E_m at low doses, but at higher doses will cause depolarization with increasing tension (8). The threshold dose for depolarization varies among different species (26). In addition, the permeability of the

membrane to ions such as Na^+, K^+, and Cl^- may be altered by either direct or indirect effects of norepinephrine (8,14). Total ionic conductance of the cell to potassium might then be increased to prevent any significant net depolarization. This is further confounded by the observations that 5-HT, a known stimulus for smooth muscle contraction, has been observed to either increase or decrease K^+ conductance in various cell types (18,64). Bradykinin, a potent vasodilator, is consistent in that it increases the K^+ permeability and hyperpolarizes renal epitheloid cells (41), as well as aortic endothelial cells (12). ANP also relaxes the vasculature, yet it has been found to hypopolarize renal medullary collecting duct cells by inhibition of Na^+ channels rather than altering K^+ conductance (43). Ultimately, the effect of a vasoactive agent on mechanical coupling by either an electrical or pharmacological mechanism appears to depend primarily on the particular contractile tissue studied and the species from which it was derived (26). This again points to the complexity of the regulation of ion transport, and the need to evaluate these mechanisms in each unique cell type.

Previous studies in mesangial cells have investigated the effects of vasoactive agents on membrane potential and chloride conductance. E_m was found to range from about -45 mV to -53 mV (40,47,58). ANG II and AVP were demonstrated to stimulate a calcium-activated Cl^- conductance and depolarize the MC membrane (40,58). However, the influence of other vasoactive agents on this and other ion transport mechanisms remains to be investigated.

Measurement of Intracellular Potassium

Few methods exist for evaluating K^+ transport in intact living cells over a continuous time period. Many studies have employed methods which require destruction of the cells at particular timed intervals, such as flame photometry, atomic absorption, and $^{86}Rb^+$ flux analysis. The loss of temporal resolution presents a major drawback to these methods when rapid changes (on the order of seconds) occur in the ionic environment. Furthermore, $^{86}Rb^+$ may not substitute exactly one-to-one for potassium in all transport pathways [73]. Nuclear magnetic resonance studies are nondestructive but require large quantities of tissue to be placed in a magnet cavity which may limit access for manipulation, and are also extremely costly [49]. Impalement with K^+-sensitive micro-electrodes has thus far been of limited applicability in many types of small eukaryotic cells because of technical difficulty [1].

The recent development of the K^+-sensitive fluorescent dye, potassium-binding benzofuran isophthalate (PBFI), has provided a non-invasive technique for determining $[K^+]_i$ in MCs under both steady-state and transient conditions with excellent temporal resolution. As synthesized by Minta and Tsien, PBFI fluoresces differentially in the presence of varied $[K^+]$ but is less affected by other ions such as Na^+ and H^+ [49]. Fluorescent dyes have been used extensively to measure intracellular pH and calcium concentration in MCs [5,19,20,22,78,79], as well as membrane voltage and intracellular concentrations of sodium and magnesium in other cells [28,49,50,56].

We examined $[K^+]_i$ using the fluorescent technique to further our understanding of mesangial cell ion transport processes. The purposes of this study were: 1) to examine the spectral properties of PBFI, 2) to obtain an intracellular calibration of $[K^+]_i$ with the dye in mesangial cells, and 3) to investigate the mechanisms of potassium transport in these cells. We sought to establish that by using this fluorescent technique, fluctuations in $[K^+]_i$ during specific experimental maneuvers could be measured in MCs, and would be useful in showing the presence of multiple transport pathways for K^+ and their interrelation. We measured $[K^+]_i$ continuously under control conditions and during selective inhibition of putative K^+-cotransporters, exchangers, and channels (36). The effect of several vasoactive agents on K^+ flux in these unique cells was also examined.

METHODS

Culture of Mesangial Cells

Isolated glomeruli from young male rat kidneys were obtained by differential sieving and harvesting from a wire mesh. Glomeruli were then digested with collagenase to remove epithelial cells, leaving cores which consisted predominantly of mesangial cells, matrix, and capillary loops (44,48). The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL selenous acid, 10 mM L-glutamine, 25 mM glucose, 400 ng/mL penicillin, 500 ng/mL strepto-mycin, and 25 mM HCO_3^-. In this medium, MCs grew readily while other glomerular cells did not survive (44,48). MCs proliferated to form confluent monolayers (*i.e.*, one-cell-thick layers, with each cell in contact with neighboring cells) and were subsequently suitable for passage of subcultures.

Routine examination of the cultured cells, to confirm their identity as MCs, was performed by indirect immunofluorescence microscopy using rabbit immunoglobulin G (IgG) directed against vascular smooth muscle myosin and fluorescein isothiocyanate (FITC)-conjugated mouse IgG directed to rabbit IgG. Cells showed uniformly strong positive staining of longitudinal filaments, a pattern that is characteristic of MCs (77). In addition, cultured cells stained uniformly with anti-Thy 1.1, which is also considered to be indicative of MCs (22).

Our studies employed subcultures of the third to eighth passage of MCs grown on glass cover slips at 37°C in 5% CO_2-95% air. The cells were suitable for experimentation after 10-14 days of growth in culture on the the cover slips, shortly before reaching confluence. Twenty-four hours prior to potassium fluorescence measurements, the medium was changed from 10% to 0.5% FBS to halt cell growth and facilitate subsequent dye-loading.

Fluorescent Measurement of Intracellular Potassium

$[K^+]_i$ was determined by use of the K^+-sensitive dye potassium-binding benzofuran isophthalate (PBFI). The cell-permeant acetoxy-methyl ester (AM) form of the dye was dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 5 mM and then diluted to 5 μM in saline. The detergent Pluronic F-127 enhances dye-loading, and was added to the dye mixture to make a 0.04% (w/v) solution. Serum-starved MCs on cover slips were incubated with the dye mixture for 90 min at 37°C. The cells were then washed with standard saline solution and placed in a thermostatically controlled (37°C) polystyrene cuvette in a Perkin-Elmer LS-5B spectrofluorometer (Norwalk, CT). The AM ester is hydrolyzed within the cell to yield the impermeant, polyanionic, K^+-sensitive PBFI free acid form, which exhibited an excitation maximum at 340 nm and an emission maximum at 500 nm (excitation slit width was 3 mm, emission slit width was 5 mm). The fluorescent signal recorded was the summation of luminescences from all cells in the light

path. Continuous perfusion of the cuvette with the standard saline solution removed extracellular PBFI and minimized any interference by dye leakage into the extracellular space.

As shown in the series of excitation spectra (Figure 1), the fluorescence of the dye was particularly sensitive to $[K^+]_i$ when excited at 340 nm, but was relatively unaffected by varied $[K^+]_i$ upon excitation at the isosbestic point near 380 nm. The ratio of the luminescences, obtained by exciting the cells at these wavelengths (340 nm/380 nm) while measuring at a constant emission at 500 nm, was thus indicative of $[K^+]_i$ yet undisturbed by other parameters such as number of cells in the light path and dye leakage from the cells. Background noise contributed to less than 10% of the signal in dye-loaded cells, and agonists and antagonists contributed to less than 10% of the total fluorescence throughout all experiments. Autofluorescence of cells, solutions, and antagonists was corrected for in all experiments. Furthermore, luminescences obtained from excitation at both 340 and 380 nm declined minimally (by less than 15%) during an average 10-20 minute experiment, yet the ratio remained constant despite these changes. This most likely represents photobleaching and did not influence determinations of $[K^+]_i$ since the ratio was not affected.

Prior analysis of PBFI by the manufacturer has demonstrated that dye is not completely selective for K^+, but also has affinity for Na^+ and H^+ ions (49). In order to ascertain the contribution of Na^+ to fluorescence, we preincubated MCs with monensin and nigericin and then exposed the cells to varying concentrations of Na^+ (replaced with N-

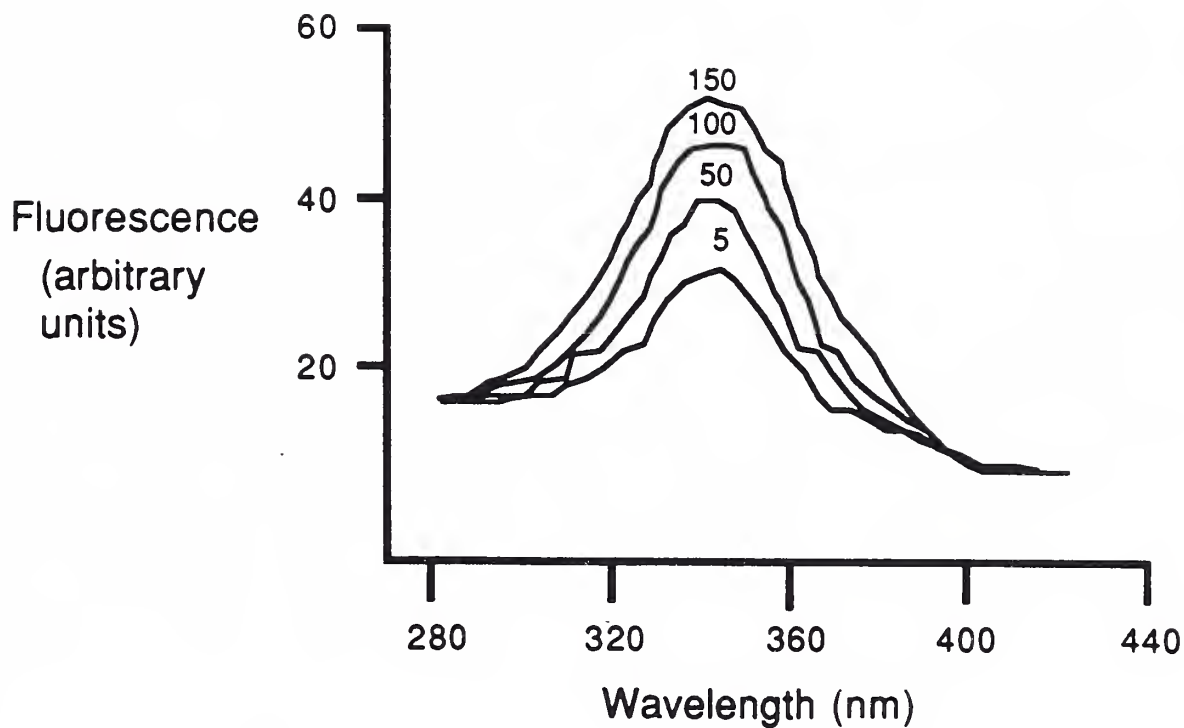Figure 1. Fluorescence excitation spectra of PBFI in MCs as a function of $[K^+]_i$. These spectra were obtained in various calibration solutions as described in Methods. Emission wavelength was set at 500 nm. Excitation maximum occurred near 340 nm, and a relatively $[K^+]_i$-insensitive isosbestic point occurred near 380 nm. The values of extracellular $[K^+]$ (in mM) are indicated above their respective spectra.

methyl-D-glucamine as necessary to maintain a constant osmolarity) and recorded the fluorescent intensity of the dye. This method, like the calibration which follows, should clamp intracellular Na^+ concentration ($[Na^+]_i$) to the known extracellular Na^+ concentration (28). The fluorescence of the dye was not significantly affected by $[Na^+]_i$ of 5, 50, and 75 mM (n=3 each, data not shown). At higher levels of $[Na^+]_i$, PBFI fluorescence did increase by 27% when $[Na^+]_i$ was 100 mM, and by 34% when $[Na^+]_i$ was 150 mM (n=3 each, p<0.05). These results suggest that the sensitivity of PBFI for changes in $[K^+]_i$ are only likely to be significantly and adversely affected by an extraordinarily high $[Na^+]_i$.

In order to confirm that PBFI was not affected by intracellular pH (pH_i), MCs were subjected to an acid load by ammonium pulsing (NH_4^+/NH_3) and allowed to recover spontaneously (as described previously (5,19)). No significant changes in fluorescence were detected during these acute acid-base perturbations at pH_i values of 6.6, 7.0, 7.2, and 7.8 (n=3 for each value of pH_i, data not shown) PBFI therefore retains its sensitivity to K^+ in the face of wide variations in pH_i.

Experiments involving manipulation of ion transporters and $[K^+]_i$ may also cause transient changes in cell volume, which may consequently alter the properties of the dye. To assess the effect of large and rapid volume changes on PBFI fluorescence, we altered extracellular osmolarity of the MC bathing solution from 200 mM to 500 mM by the addition of sucrose. Within this range, no significant changes in fluorescence were noted (n=3 for each). At higher extracellular osmolarity, however, the fluorescence ratio of the dye increased, by 8% at 550

mM, 15% at 600 mM, and 20% at 650 mM (n=3 each, p<0.05). These osmolarities represent near doubling of the likely physiological range for the cells, and these data indicate that the calibration of the dye may yield unreliable values of $[K^+]_i$ when cells experience severe osmotic disturbances. In other words, factors which modify $[K^+]_i$ by a direct mechanism on the K^+ transporters may also alter cell volume, secondarily resulting in a change in $[K^+]_i$. In this situation, it would not be possible to distinguish between the direct and volume-related effects on $[K^+]_i$.

Calibration of PBFI

PBFI was calibrated intracellularly by clamping $[K^+]_i$ to known values of $[K^+]_o$ using the ionophores valinomycin and nigericin. The cyclic peptide antibiotic valinomycin, a K^+-ionophore and mitochondrial uncoupler, increased the permeability of the membrane to K^+ and depleted intracellular energy stores (74) while nigericin, a K^+/H^+ exchanger, equilibrated extracellular pH with intracellular pH (79). All active ion transport was inhibited and K^+ became distributed equally on both sides of the membrane. Valinomycin and nigericin were initially dissolved into ethanol to 1 mM stock and then diluted 1:1000 and 1:200 in the calibration saline solutions, respectively. Cells were first dye-loaded with PBFI and then incubated for 15 minutes in the presence of 1 μM valinomycin, 5 μM nigericin, and varied $[K^+]_o$ in a calibration solution. Assuming that $[K^+]_i$ equalized to $[K^+]_o$ under these conditions, a standard curve was generated by plotting the fluorescence ratio against $[K^+]_i$. Whole cell patch-clamp experiments support the validity of this

method. They confirm that the membrane potential approaches zero when MCs are incubated with valinomycin and nigericin (unpublished observations by R.M. Henderson and R. Unwin at the Royal Hammersmith Hospital, U.K.).

As the fluorescence intensity of the dye depends on $[K^+]_i$ in the same manner as the activity of an enzyme does on substrate concentration, a nonlinear least squares fit of the data to the form of the Michaelis-Menten equation was performed:

$$\frac{I_{340}}{I_{380}} = I_{max} \frac{[K^+]_i}{[K^+]_i + K_M} + I_0$$

where I_{340}/I_{380} is the measured fluorescence ratio, I_0 indicates the minimal ratio (as $[K^+]_i$ goes to zero), the sum of I_{max} and I_0 represents the maximal ratio (as $[K^+]_i$ approaches infinity), and K_M is the Michaelis-Menten constant that reflects the affinity of the dye for K^+. The curve fitting analysis determined the values of $I_0 = 2.29$ and $I_{max} = 3.93$ with a K_M equal to 113 mM (n=40, r=0.998, Statistical Analysis System, Yale Computer Center) (See Figure 2). The calibration curve thus generated was used in all experiments to determine the values of $[K^+]_i$ from the measured 340 nm/380 nm fluorescence ratios in PBFI-loaded glomerular mesangial cells.
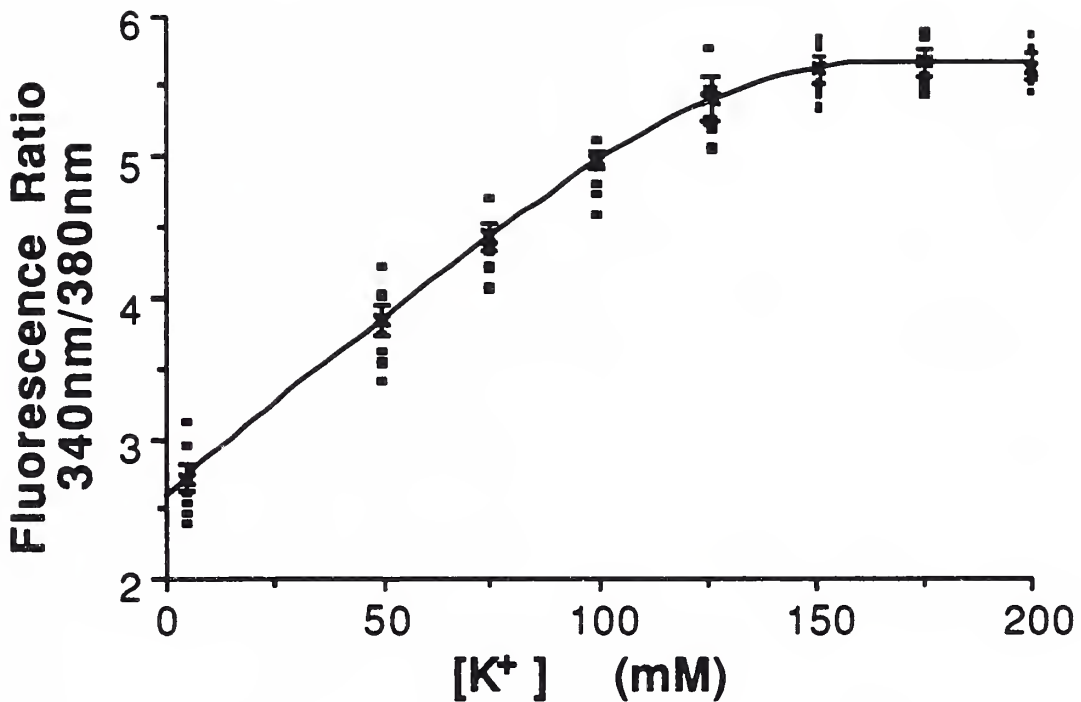
Figure 2. Calibration Curve. The dependence of the fluorescence ratio (340 nm/380 nm) on [K$^+$] was fit to the Michaelis-Menten equation (nonlinear least squares fit, r=0.998, n=40) with K_M determined to be 113 mM. Five samples were measured for each value of [K$^+$]$_o$, and standard error bars are indicated about the means (x).

Data Analysis

Tracings of $[K^+]_i$ (Figures 3-6) are actual recordings of fluorescence when the cells are excited at 340 nm, but data were evaluated via the calibration curve (Figure 2). $[K^+]_i$ is reported as a mean ± standard deviation, and statistical significance was determined by paired t test or analysis of variance. Results were considered to be significant when p<0.05.

Solutions

The standard saline solution contained (in mM) 145 Na^+, 5 K^+, 150 Cl^-, 1.0 Mg^{2+}, 1.8 Ca^{2+}, 1.0 PO_4^{3-}, 10.0 glucose, and 32.2 N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and was titrated to a pH of 7.40. Calibrating solutions containing valinomycin and nigericin were prepared in the same standard saline buffer, but K^+ and Na^+ were varied such that the sum of $[K^+]_o$ and $[Na^+]_o$ was maintained at 150 mM. Ouabain was used to inhibit the Na^+/K^+-ATPase and was dissolved in standard saline to a final concentration of 0.5 mM. Furosemide and ethacrynic acid antagonize the activity of the $Na^+/K^+/Cl^-$ cotransporter, and were made up as 10 mM stock solutions in DMSO and were diluted in standard saline to 10 μM for application to the cells. Barium (0.5 mM) and TEA (10 μM) were used to block K^+ conductance and were dissolved in the standard solution, except the barium solution was made without phosphate. These substances were used alone and in combinations with each other in these studies to

separate the roles of each transporter on $[K^+]_i$ homeostasis. Angiotensin II (1 µM), serotonin (10 µM), atrial natriuretic peptide (100 nM), and bradykinin (1 µM) were all dissolved in the standard saline solution on the day of the experiment. The fluorescence of each of these agents in solution, if significant, was subtracted from the fluorescence measurements of the PBFI-loaded MCs. The solvents, DMSO and ethanol, had no effect on fluorescence measurements or determinations of $[K^+]_i$.

Materials

PBFI/AM, Pluronic F-127, and valinomycin were obtained from Molecular Probes (Eugene, OR). Furosemide, ethacrynic acid, angiotensin II, bradykinin, serotonin, barium, nigericin, ouabain, monensin and plastic cuvettes were purchased from Sigma (St. Louis, MO). Atrial natriuretic peptide (human 1-28) was purchased from Peninsula Laboratories (Belmont, CA). DMEM, FBS, penicillin, streptomycin, were obtained from GIBCO Laboratories (Grand Island, NY). Anti-Thy 1.1 was purchased from Chemicon (Temecula, CA).

Personnel/Technical Assistance

The sacrifice of rats, isolation of glomeruli, and preparation of primary MC cultures were performed by M.B. Ganz and M.C. Perfetto. MCs were stained by P. Dann for the purposes of identification. Pre-

paration of passages 2 through 8, plating of MCs on cover slips, and routine maintenance of MCs were done by S.E. Kasner. All culture media were prepared by M.C. Perfetto.

The protocol for PBFI dye-loading, method for calibration, and experimental design for all fluorescence measurements were developed by S.E. Kasner. All determinations of fluorescence ratios and $[K^+]_i$ were performed by S.E. Kasner. M.B. Ganz assisted in the acid-loading experiments used in the assessment of the effect of pH on PBFI.

Statistical analysis was performed by S.E. Kasner. The calibration data were analyzed for curve fitting on the Statistical Analysis System (Yale Computer Center) with the assistance of J. Goffinett.

All solutions used in experiments were made by either S.E. Kasner or M.B. Ganz. The continuous perfusion apparatus was designed and constructed by S.E. Kasner.

RESULTS

Mechanisms of Potassium Homeostasis

$[K^+]_i$ in MCs. The resting level of $[K^+]_i$ in MCs, pooled from all experiments, was determined to be 102 ± 7 mM (n=81). The 340/380 nm fluorescence ratio, and therefore $[K^+]_i$, remained relatively constant for at least 30 min during all static experiments, regardless of any photo-bleaching or leakage of dye from the cells. None of the experiments in this study required a time period longer than 30 minutes.

Na^+/K^+ ATPase. This laboratory has previously demonstrated that rat MCs express the alpha subunit for the Na^+/K^+ ATPase (63). The physiological activity of the Na^+/K^+ ATPase was assessed here by inhibiting with ouabain. Concentrations as low as 10 µM ouabain led to a significant decrease in $[K^+]_i$ in MCs without evidence of return toward initial $[K^+]_i$. A maximal fall in $[K^+]_i$ occurred at ouabain concentrations of 0.5 mM or greater. In MCs exposed to 0.5 mM ouabain, $[K^+]_i$ declined to 48 ± 6 mM (n=8, p<0.01) within 2.9 ± 0.9 min (Figure 3). At no time in the continued presence of ouabain was any recovery of $[K^+]_i$ back toward the initial steady-state value observed, implying that there was not an ouabain-insensitive mechanism that could maintain $[K^+]_i$ homeostasis when the Na^+/K^+-ATPase was disabled.

$Na^+/K^+/Cl^-$ cotransporter. We determined the activity of the $Na^+/K^+/Cl^-$ cotransporter through the use of two antagonists, the loop-

diuretics furosemide and ethacrynic acid. As shown in Figure 4A, when MCs were exposed to 10 μM furosemide, there was a rapid drop in baseline $[K^+]_i$ to 58 ± 11 mM, a 43% decrease (n=16, p<0.01) within 1.2 ± 0.5 min (Figure 4A). The decrease in $[K^+]_i$ was followed by a return to near baseline $[K^+]_i$ within 5.0 ± 1.2 min in the continued presence of furosemide. Furthermore, when MCs were exposed acutely to furosemide and then to 0.5 mM ouabain, the recovery was completely abolished. These experiments suggest that the Na^+/K^+ ATPase plays a major role in the recovery of $[K^+]_i$ to near steady-state levels in the presence of furosemide. We sought to additionally confirm the presence of the $Na^+/K^+/Cl^-$ cotransporter in MCs using ethacrynic acid, since this antagonist to the cotransporter is minimally fluorescent at these same wavelengths and thus required no mathematical correction. In a manner similar to that of furosemide, MCs exposed to 10 μM ethacrynic acid exhibited a fall from initial $[K^+]_i$ to 63 ± 9 mM, a 38% decline (n=13, p<0.01) in 1.9 ± 0.6 min, that was followed by a return to near baseline $[K^+]_i$ in 4.1 ± 0.9 min (Figure 4B). In the continued presence of ethacrynic acid, the application of ouabain completely prevented the recovery of $[K^+]_i$ to original levels. The difference between the effects of furosemide and ethacrynic acid was not significant.

K^+ conductance pathways. In order to determine whether the presence of a K^+ conductance can be shown using PBFI, known blockers of K^+ channels, barium and TEA, were employed. In MCs exposed to barium, $[K^+]_i$ rapidly increased from the baseline value to 124 ± 8 mM (n=9, p<0.01) (Figure 5). The sudden increase in $[K^+]_i$ was followed by a prompt return to near baseline within 3.9 ± 0.7 min. This barium-
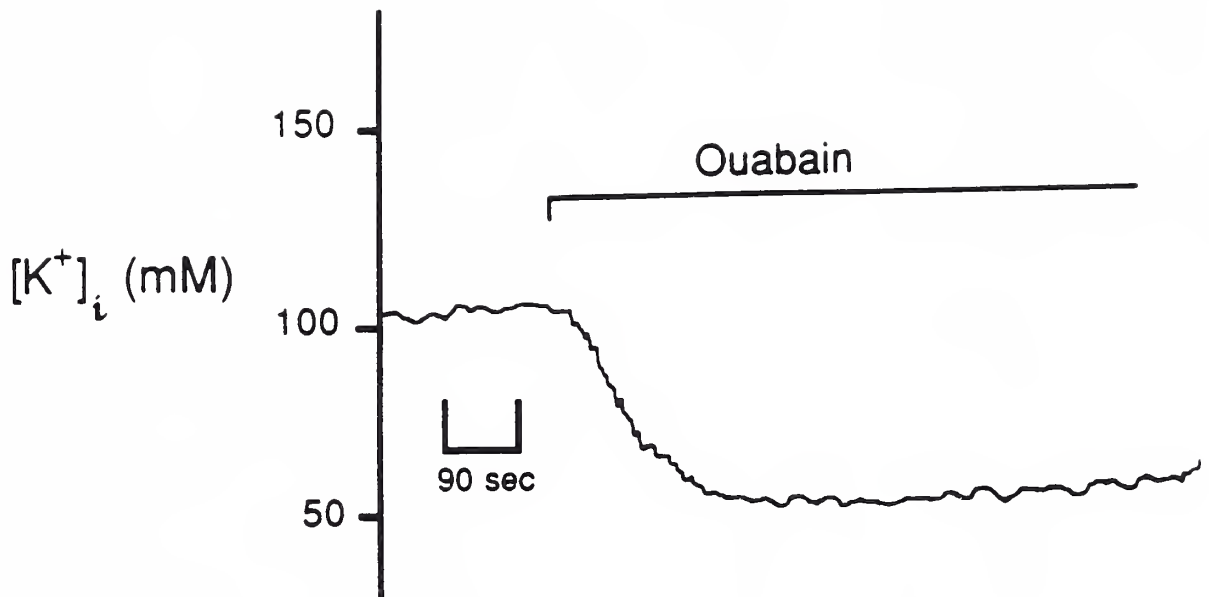
Figure 3. Effect of ouabain on $[K^+]_i$. Ouabain (0.5 mM) was added directly to the cuvette. $[K^+]_i$ fell to an average of 48 ± 6 mM ($p<0.01$). No recovery was observed in the continued presence of ouabain. This tracing is a representative of 8 experiments.

A.

B.
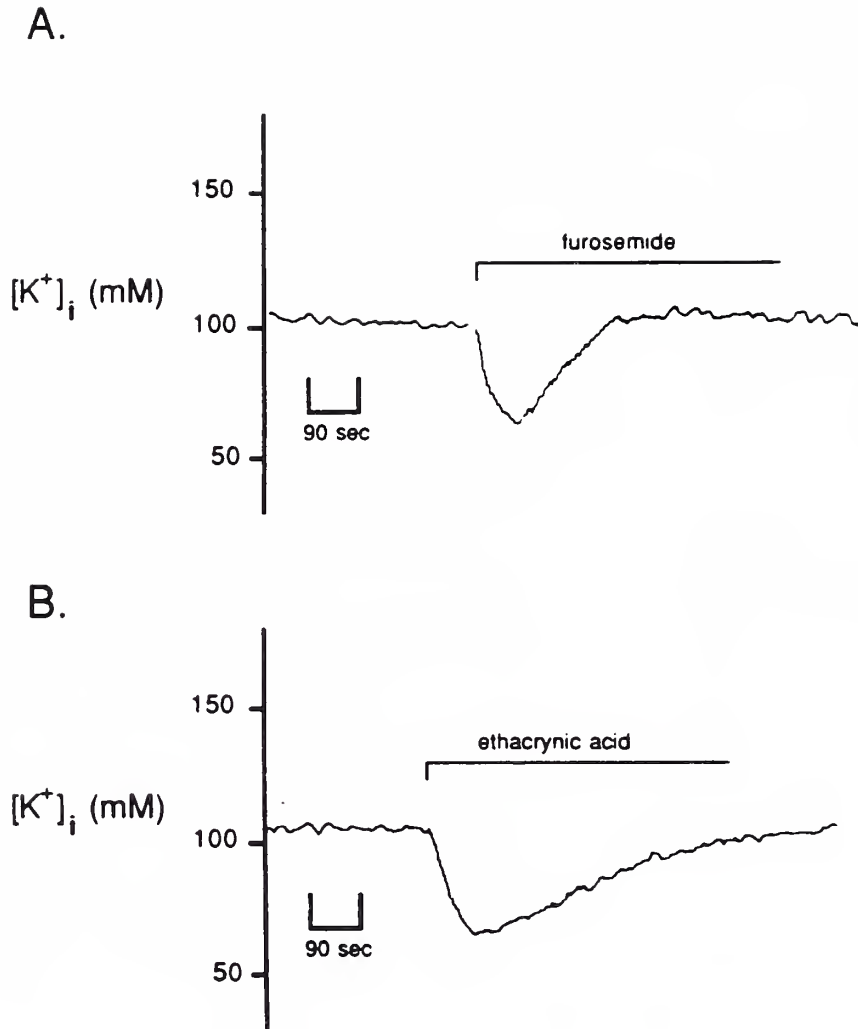
Figure 4. Effect of diuretics on $[K^+]_i$. A: Effect of furosemide. $[K^+]_i$ declined to an average of 58 ± 11 mM ($p<0.01$). In the continued presence of furosemide, $[K^+]_i$ returned toward near baseline levels. Exposure to ouabain completely prevented the recovery. This tracing is a representative of 16 experiments. B: Effect of ethacrynic acid. $[K^+]_i$ declined to an average of 63 ± 9 mM ($p<0.01$). In the continued presence of ethacrynic acid, $[K^+]_i$ returned toward near baseline levels. Exposure to ouabain completely prevented the recovery. This tracing is a representative of 13 experiments.

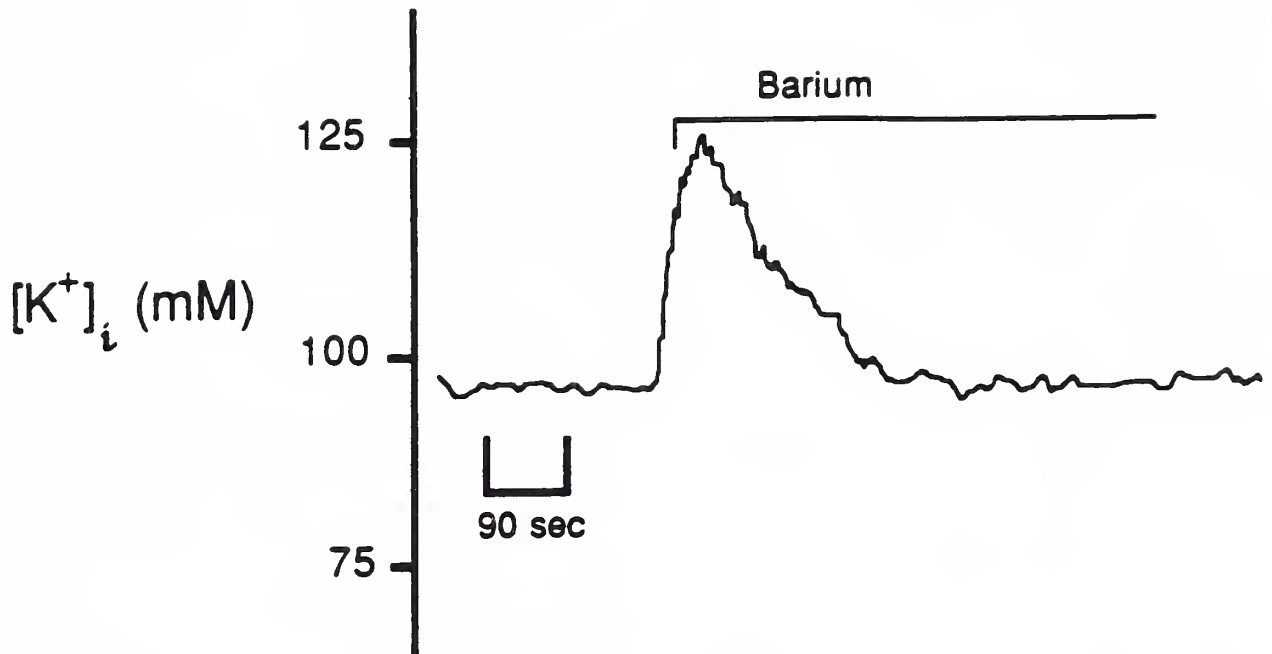Figure 5. Effect of barium (Ba^{2+}) on $[K^+]_i$. $[K^+]_i$ increased to an average of 124 ± 8 mM (p<0.01). In the continued presence of Ba^{2+}, $[K^+]_i$ returned toward near baseline levels. This tracing is a representative of 9 experiments.

induced increase in $[K^+]_i$ was prevented when the cells were preincubated with both ouabain and furosemide (n=5, non-significant difference from baseline, data not shown). When MCs were exposed to TEA there was a similar increase in $[K^+]_i$ to 123 ± 10 (n=8, $p<0.01$), which was followed by a return to near baseline (data not shown).

Effect of Vasoactive Agents

$[K^+]_i$ in MCs. MCs were treated with the vasoactive substances, angiotensin II, serotonin (5-hydroxytryptamine), bradykinin, and atrial natriuretic peptide at the concentrations stated above as a single pulsed dose. These agonists did not induce any significant change in the resting level of $[K^+]_i$ in MCs, either acutely or during the 10 min following initial exposure (data not shown); $[K^+]_i$ remained at the preagonist level of 103 ± 6 mM (n=16, four experiments for each agonist tested).

Na^+/K^+-ATPase. The recovery of $[K^+]_i$ following treatment of MCs with furosemide or ethacrynic acid is attributed to continued functioning of the Na^+/K^+-ATPase. As a means of assessing the kinetics of the Na^+/K^+-ATPase, we determined a recovery rate in the presence of ethacrynic acid defined as the rate of increase in $[K^+]_i$ over a 10 s interval, measured at the point of reference when $[K^+]_i$ was 70 mM. The influence of ANG II, 5-HT, BK, and ANP on the activity of the Na^+/K^+-ATPase was evaluated by comparison of these rates. In the absence of other modifiers, ethacrynic acid transiently caused $[K^+]_i$ to fall as

described above, and subsequently returned toward original levels at a rate of 2.9 ± 0.4 mM/10 s (n=6). Pretreatment with ANG II, 5-HT, and BK significantly stimulated recovery velocities when compared to this control, but no significant differences between any two of these agents were noted: ANG II increased the rate to 3.7 ± 0.4 mM/10 s (n=6, p<0.01, Figure 6A), 5-HT to 4.1 ± 0.7 mM/10 s (n=6, p<0.01), and BK to 3.6 ± 0.4 mM/10 s (n=6, p<0.01). ANP exhibited no effect on the recovery phase (rate was 3.0 ± 0.2 mM/10 s, n=6, NS, Figure 6B) (See Table 1).

$Na^+/K^+/Cl^-$ cotransporter. The influence of these vasoactive agents on the $Na^+/K^+/Cl^-$ cotransporter was more difficult to assess. MCs were not able to restore K^+ homeostasis in the presence of ouabain in a manner analogous to their recovery from loop diuretics. This precluded the direct evaluation of the kinetics of the cotransporter in response to a fall in $[K^+]_i$. As an alternative means of evaluation, we sought to compare the effects of vasoactive factors on the fraction of K^+ uptake that could be attributed to the $Na^+/K^+/Cl^-$ cotransporter. As described above, ethacrynic acid applied to MCs caused a precipitous fall in $[K^+]_i$ to 63 ± 9 mM, and thus we inferred that 38% of steady-state $[K^+]_i$ was maintained by the cotransporter. In MCs preexposed to ANG II, ethacrynic acid caused a significantly greater decline to 42 ± 6 mM (n=6, p<0.01). MCs treated with 5-HT demonstrated an increased sensitivity to ethacrynic acid of similar magnitude, as $[K^+]_i$ fell to 45 ± 8 mM (n=7, p<0.01). The effect of BK was not significant, with $[K^+]_i$ decreasing to 56 ± 7 mM (n=6, NS), and ANP also appeared to have no influence on the cotransporter ($[K^+]_i$ was 60 ± 4 mM, n=7, NS) (See Table 2).
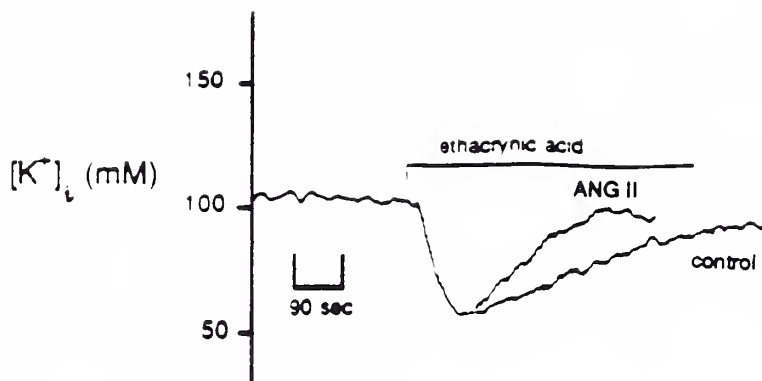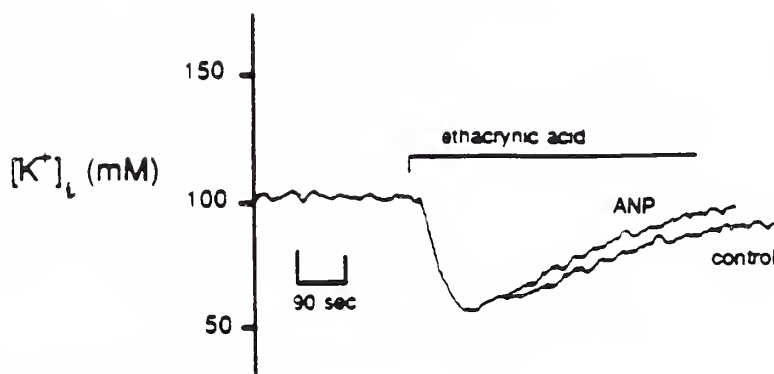
Figure 6. Effect of vasoactive agents on recovery from an ethacrynic acid-induced fall in $[K^+]_i$. A: Effect of angiotensin II (ANG II). Pretreatment with ANG II increased the rate of recovery to 3.7 ± 0.4 mM/10 s, a 28% increase compared to the control rate of 2.9 ± 0.4 mM/10s ($p<0.01$). B: Effect of atrial natriuretic peptide (ANP). Pretreatment with ANP caused no significant change in the rate of recovery (3.0 ± 0.2 mM/10s) when compared to control. Each tracing is a representative of 6 experiments.

EFFECT OF VASOACTIVE AGENTS ON THE Na$^+$/K$^+$-ATPase

	Rate of Recovery (mM/10s)	Percent Increase
control	2.9 ± 0.4	--
ANG II	3.7 ± 0.4 *	28 % *
5-HT	4.1 ± 0.7 *	41 % *
BK	3.6 ± 0.5 *	24 % *
ANP	3.0 ± 0.2	3 %

Table 1. Effect of the vasoactive agents on the Na$^+$/K$^+$-ATPase. This was determined as the rate of recovery of $[K^+]_i$ in the presence of ethacrynic acid. Significant differences (p<0.01 by analysis of variance) from control rates are indicated with an asterisk (*).

EFFECT OF VASOACTIVE AGENTS ON THE $Na^+/K^+/Cl^-$ COTRANSPORTER

	$[K^+]_i$ after EA (mM)	Fractional Contribution to $[K^+]_i$	Percent Increase
control	63 ± 9	38 %	--
ANG II	42 ± 6 *	59 % *	55 % *
5-HT	45 ± 8 *	56 % *	47 % *
BK	56 ± 7	45 %	18 %
ANP	60 ± 4	41 %	8 %

Table 2. Effect of the vasoactive agents on the $Na^+/K^+/Cl^-$ cotransporter. This was calculated as the fractional contribution to $[K^+]_i$ maintenance that was inhibited with ethacrynic acid (EA) (i.e. the maximal EA-induced change in $[K^+]_i$ from the initial value, divided by the initial value of $[K^+]_i$). Significant differences ($p<0.01$ by analysis of variance) from control rates are indicated with an asterisk (*).

DISCUSSION

Analysis of PBFI

This is the first cellular application of which we are aware for the new fluorescent potassium indicator, PBFI. In a previous report, this dye had been used in liposomes, and the calibration method did not utilize fluorescence excitation ratios (34). We were able to characterize the properties of the dye in cells, as well as examine and discern some of the major K^+ transport mechanisms in mesangial cells and their response to several vasoactive agents.

Our experiments demonstrated that fluorescence of PBFI was not significantly affected by acute changes in pH_i from 6.6 to 7.8, alterations in intracellular Na^+ concentrations when $[Na^+]_i$ was less than 75 mM, nor changes in osmolarity from 200 to 500 mM. The use of the ionophores, valinomycin and nigericin, proved to be a valid method for calibration of dye fluorescence ratios in the determination of $[K^+]_i$. Our results further indicate that PBFI is reasonably well-suited for the continuous monitoring of rapid changes in K^+ homeostasis in whole cells. We have also shown that the net effect of various K^+ transporters on $[K^+]_i$ may be assayed both individually and collectively.

The fluorescent technique offers a number of distinct advantages over other methods in the examination of ion transport. It provides a continuous rather than interval record of the changes in $[K^+]_i$, elimi-

nates the need for radioisotopes, and is extremely sensitive within the likely physiological range of $[K^+]_i$ (50-120 mM). It is a technically simple and inexpensive method that does not require the extensive apparatus or skill such as that necessary for intracellular electrophysiological measurements.

The usefulness of the dye may be limited by several parameters. 1) Many of the agents used to inhibit the K^+ transporters are fluorescent to some degree, and although interfering fluorescence has been subtracted from all experiments, some extremely transient alterations in $[K^+]_i$ may be masked by extraneous changes in fluorescence. 2) The resolution of the dye for $[K^+]_i$ above the physiological range is greatly diminished. At levels of $[K^+]_i$ greater than 130 mM, the calibration curve (Figure 2) flattens, and thus the sensitivity of the dye decreases dramatically: a small error in the measurement of a fluorescence ratio would result in a large error in the determination of $[K^+]_i$. Measurements of high $[K^+]_i$ are therefore likely to be inaccurate. In our experiments, we did not measure increases in $[K^+]_i$ above 130 mM in MCs. 3) The effect of alteration in cell volume on the calibration of PBFI is ill-defined. While the dye does not appear to exhibit significant change in its fluorescent properties when osmolarity is changed, this does not negate the possibility that an acute volume shift may cause a secondary effect on the measured value of $[K^+]_i$.

PBFI is clearly not optimal for all investigations of $[K^+]_i$. A more quantitatively precise K^+-sensitive dye must demonstrate greater selectivity for K^+ over Na^+. It should have longer excitation maximum

and isosbestic wavelengths in order to minimize the effects of cell and reagent fluorescence, thereby increasing the signal-to-noise ratio. Finally, it should have a higher value of K_M to improve the resolution of $[K^+]_i$ at levels greater than 130 mM. Nonetheless, PBFI provides a vast improvement in noninvasive continuous potassium measurement in cultured cells, and promises to be useful for physiological measurements in many systems.

Regulation of $[K^+]_i$ in Mesangial Cells

The properties of the PBFI allow us to examine $[K^+]_i$ under basal and stimulated conditions. Moreover, these experiments demonstrate that real time changes in intracellular potassium concentrations in mesangial and other cells may be measured using PBFI. Our determination of resting $[K^+]_i$ to be 102 ± 7 mM in MCs concurs with results obtained recently by preliminary patch-clamping experiments in MCs (R.M. Henderson and R. Unwin, unpublished observations). The results also confirm ongoing work by Homma *et al.* (31), demonstrating that MCs possess at least two mechanisms for K^+ uptake, the Na^+/K^+-ATPase and the $Na^+/K^+/Cl^-$ cotransporter. As measured in intact quiescent MCs *in vitro*, we found that the ouabain-sensitive component of K^+ uptake is responsible for maintaining 53% of $[K^+]_i$ and the furosemide-sensitive component for 43%. Thus, it appears that these two transport mechanisms can account for nearly all of K^+ uptake in MCs. These findings are also consistent with those of Homma *et al.* (31), in which 46% of $^{86}Rb^+$ uptake was shown to be mediated by the Na^+/K^+-

ATPase and 24% by the furosemide-sensitive $Na^+/K^+/Cl^-$ cotransporter. Furthermore, it is clear that MCs can recover from the inhibitory effect of furosemide on the cotransporter, via the activity of uptake pathways, i.e., the Na^+/K^+-ATPase, but they are unable to maintain $[K^+]_i$ homeostasis if the ATPase is inhibited by ouabain. The operation of the Na^+/K^+-ATPase, as a primary active transport mechanism, is therefore deemed to be a prerequisite for K^+ transport in MCs, but its quantitative role in K^+ uptake appears to be interrelated with the activity of the $Na^+/K^+/Cl^-$ cotransporter.

The effects of the vasoactive agents on K^+ transport mechanisms in MCs defy easy categorization. The Na^+/K^+-ATPase is shown to be stimulated by the vasoconstrictors, ANG II and 5-HT, in MCs recovering from an acute (diuretic-induced) drop in $[K^+]_i$. However, the ATPase is also stimulated by BK, a vasodilator, but not by ANP. ANP is often considered to be an inhibitor of the ATPase (52,68), but here no significant effect was recorded. The physiological importance of these data are somewhat unclear, given the observation that both vasoconstrictive and one of the vasorelaxant substances led to stimulation of the Na^+/K^+-ATPase. However, the ATPase is generally not thought to play a major part in volume regulation (69). Rather, the activity of the Na^+/K^+-ATPase has been shown to be secondarily augmented in many volume regulatory responses to osmotic stimuli, presumably in response to increased $[Na^+]_i$ from other pathways, and may serve only a supportive role in $[K^+]_i$ and $[Na^+]_i$ homeostasis (17,55,69,71). We therefore needed to examine the influence of the vasoactive agents on the other K^+ flux mechanisms.

The activity of the $Na^+/K^+/Cl^-$ cotransporter in MCs was dramatically increased by ANG II and 5-HT. Thus, the vasoconstrictors stimulated both the ATPase and the cotransporter, suggesting a close relationship between these transporters in maintaining $[K^+]_i$ and in regulating cell shape and volume. BK stimulated the Na^+/K^+-ATPase while ANP had no effect, but neither of these vasodilators exerted any significant influence on the $Na^+/K^+/Cl^-$ cotransporter in MCs. A difference thus exists between the effects of constrictors and dilators on the cotransporter. This suggests that the cellular mechanisms for contraction and relaxation in response to these vasoactive agents may be coupled to their effects on the $Na^+/K^+/Cl^-$ cotransporter, but perhaps not to the Na^+/K^+-ATPase. In addition, a distinction can be made between the effects of the two vasodilators examined here. BK stimulated the Na^+/K^+-ATPase but not the $Na^+/K^+/Cl^-$ cotransporter, whereas ANP had no significant effect on either, implying that peptide-induced relaxation of MCs may be mediated through more than one biochemical pathway. These results imply greater complexity of K^+ transport regulation in MCs than had been previously realized.

Previous studies have utilized $^{86}Rb^+$ flux measurements to show that ANG II stimulates the $Na^+/K^+/Cl^-$ cotransporter in MCs (31) and in vascular smooth muscle cells (53,54,59,72), but BK has also been demonstrated to increase the activity of the cotransporter in endothelial cells (6). In addition, some of these flux studies have also shown ANP to exert a stimulatory effect on the cotransporter in smooth muscle (53,54), but not in endothelial cells (52). The discrepancy between our results in

mesangial cells compared to those in vascular smooth muscle and endothelial cells is at present unclear. Mesangial cells are unique, however, in that they share several morphologic features with both smooth muscle and endothelial cells, and they lie in a position of direct contact with the endothelium at particular sites in the glomerular capillary loop (48). The data presented here suggest that MCs manifest a composite of the properties of these two cell types with regard to their potassium transport regulatory processes as well.

PBFI also allows us to demonstrate the presence of K^+ channels in MCs. K^+ conductance pathways in MCs were blocked by treating the cells with barium or TEA. The excellent time resolution of the dye revealed a rapid 23% rise in $[K^+]_i$ when K^+ channel blockers were added. The effects of barium and TEA were indistinguishable from each other. The abrupt and transient increase in $[K^+]_i$ appears to be a consequence of continued functioning of the two major K^+ influx transporters during channel inhibition, since it was readily inhibited by a combination of ouabain and furosemide. In addition, this rise in $[K^+]_i$ was halted and reversed spontaneously, such that it may be presumed that some currently undefined feedback mechanisms must slow K^+ influx and/or activate other efflux pathways in MCs. The presence of other mechanisms for K^+ transport is suggested by indirect evidence, as MCs are capable of restoring $[K^+]_i$ to initial levels when channels are blocked by barium and TEA. This may represent alternative regulation via barium/TEA-insensitive K^+ channels, activation of K^+/Cl^- cotransport (K^+ exit), opening of Cl^- channels (57), and/or additional modulation of the Na^+/K^+-ATPase and the $Na^+/K^+/Cl^-$ cotransporter. Both

barium and TEA are known to be nonspecific K^+ channel blockers, particularly at high concentrations, and examination of the properties of the specific types of K^+ channels in MCs is a necessary part of future examinations. However, this is likely to require a more direct channel evaluation by an alternative method such as patch-clamping, since determinations of whole cell $[K^+]_i$ are likely to be obscured by simultaneous fluctuations induced by the other K^+ transporters. The role of these K^+ channels in MC contraction and relaxation in response to vasoactive agents also awaits further investigation.

The concerted regulation of K^+ flux pathways appears to be of vital importance in MCs. Under steady-state conditions, MCs closely maintain $[K^+]_i$ at a constant level with significant contributions from the Na^+/K^+-ATPase, the $Na^+/K^+/Cl^-$ cotransporter, and K^+ conductance pathways. While categorization of the effects of the vasoactive agents on K^+ transport is difficult, certain conclusions can be reached. $[K^+]_i$ is held at a constant level despite modulation of the K^+ transporters by the vasoactive agents. ANG II, 5-HT, and BK stimulated the Na^+/K^+-ATPase, but only ANG II and 5-HT stimulated the $Na^+/K^+/Cl^-$ cotransporter. Since no change in $[K^+]_i$ was recorded during vasoactive agent-induced stimulation of the K^+ influx pathways, it is likely that feedback and regulation of K^+ efflux pathways occurs as well. In the response to these substances, the Na^+/K^+-ATPase does not appear to be a primary modulator of cellular shape change, however, since it fails to react oppositely to vasoconstrictors and vasodilators. The $Na^+/K^+/Cl^-$ cotransporter, on the other hand, seems to be stimulated by vasoconstrictors and is unaffected by vasodilators, suggesting a potential direct

role for this transporter in MC contraction. The central mechanism for the contraction or relaxation of MCs under the influence of these agents may additionally be related to the regulation of K^+ conductance via K^+ channels. However, the specific regulation of K^+ channels in MCs requires further study. The intracellular biochemical signals that coordinate and selectively activate or inhibit the individual K^+ transporters in MCs also remain to be identified. These studies may be facilitated by the use of the potassium-sensitive fluorescent dye, PBFI.

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