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Candidate Inhibitor of the Volume-Sensitive Kinase Regulating K-Cl Cotransport: The Myosin Light Chain Kinase Inhibitor ML-7

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Abstract. K-Cl cotransport, KCC, is activated by swelling in many cells types, and promotes volume regulation by a KCl efflux osmotically coupled to water efflux. KCC is probably activated by swelling-inhibition of a kinase, permitting dephosphorylation, and activation of the cotransporter by a phosphatase. The myosin light chain kinase (MLCK) inhibitor ML-7 inhibits transporters activated by shrinkage. In red blood cells from three mammalian species, ML-7 stimulated KCC in a volume-dependent manner. Relative stimulation was greatest in more shrunken cells. Stimulation was reduced by moderate cell swelling and abolished by further swelling. The half-maximal stimulation is at ~20 μM ML-7, 50-fold greater than the IC_{50} for inhibition of MLCK in vitro. Stimulation of KCC by ML-7 did not require cell Ca, while MLCK does. Therefore the target of ML-7 in stimulating KCC in red cells is probably not MLCK. The evidence favors stimulation of KCC by ML-7 by inhibiting the volume-sensitive kinase. Qualitatively similar effects of ML-7 on KCC in red cells from three mammalian species suggest a general mechanism.

Key words: K-Cl cotransport — Mammalian red blood cells — ML-7 — Cell volume regulation — Signal transduction

Introduction

K-Cl cotransport, when activated by cell swelling, can promote efflux of KCl from cells and an osmotically obliged efflux of water. The cotransporter thereby participates in regulation of cell volume in a number of cell types and also in transcellular salt and water transport in certain epithelia (*see* Lauf et al., 1992, for a review). There is good evidence that the swelling-activation of K-Cl cotransport, KCC, is a consequence of inhibition of a volume-sensitive, swelling-inactivated protein kinase (Jennings & Al-Rohil, 1990; Dunham, Klimczak & Logue, 1993; Jennings, 1999). Inhibition of the kinase leads to dephosphorylation of the cotransporter, or an associated regulatory protein, by a membrane-associated serine-threonine phosphatase (Jennings & Al-Rohil, 1990; Jennings & Schulz, 1991; Kaji & Tsukitani, 1991; Starke & Jennings, 1993; Bize et al., 1998) and activation of cotransport. The identity of the kinase is unknown; it is probably not protein kinase C or adenosine 3′,5′-cyclic monophosphate-dependent kinase (Jennings & Schulz, 1991). The phosphatase is probably type 1 (PP-1) (Starke & Jennings, 1993; Krarup & Dunham, 1996).

In addition to the swelling-inactivated kinase, there is evidence for at least two other kinases playing roles in regulation of KCC in red blood cells. Staurosporine, a high affinity, broad specificity protein kinase inhibitor, stimulated KCC in sheep red cells (Bize & Dunham, 1994). Kinetic studies showed that staurosporine was not inhibiting the swelling-inactivated kinase. Rather, staurosporine stimulates a reaction leading to activation by the phosphatase. Staurosporine stimulates the phosphatase indirectly, probably by inhibiting a kinase that in turn inhibits the phosphatase (Bize & Dunham, 1994). One or perhaps two Src family tyrosine kinases, Fgr and/or Hck, may inhibit the phosphatase in mouse red cells (De Franceschi et al., 1997). Therefore staurosporine probably stimulates cotransport by inhibiting Fgr and/or Hck. PP-1 is known to be inhibited by tyrosine kinases (Johansen & Ingebritsen, 1986), and staurosporine inhibits tyrosine kinases (Ohmichi et al., 1992). The regulatory input to the Src kinase(s) is unknown.

The role of another kinase, myosin light chain kinase (MLCK), in cell volume regulation has been suggested by several studies on two shrinkage-activated

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transport pathways. An inhibitor of MLCK, the naphthalenesulfonamide derivative ML-7 (Saitoh et al., 1987), inhibits shrinkage-induced activation of Na-K-Cl cotransport, NKCC, in endothelial cells (O'Donnell, Martinez & Sun, 1995; Klein & O'Neill, 1995), and Ehrlich ascites tumor cells (Krarup et al., 1998). ML-7 also inhibits activation of Na/H exchange, NHE, in rat astrocytes (Shrode et al., 1995) and C6 glioma cells, a model system for astrocytes (Shrode et al., 1997). MLCK is specific for myosin light chain, MLC, so this kinase does not phosphorylate a transporter. MLCK may play a role in a signal transduction pathway through the cytoskeleton.

A role for MLCK had not previously been sought in the regulation of a swelling-activated transporter. In the present study, we found that ML-7 affects KCC in red cells from three species of mammals, human, sheep, and dog. The effect is more complex than the effects of ML-7 on shrinkage-activated transporters. At lower cell volumes, ML-7 stimulates cotransport, while in swollen cells, ML-7 is inhibitory. Evidence to be presented indicated that the target of ML-7 is not MLCK.

Because the stimulation of cotransport by ML-7 is volume-sensitive, we propose that the target of ML-7 is the elusive swelling-inhibited kinase. The similar effects of ML-7 on KCC in red cells from three species of mammals suggest that the mechanism of regulation of KCC targeted by ML-7 is a general mechanism, at least among red blood cells.

Materials and Methods

Cells and Solutions

Blood was drawn by venipuncture into heparinized containers. Human blood was drawn from healthy adult volunteers. Dog blood was drawn from mongrel dogs maintained at the animal care facility at the University of North Carolina School of Medicine and later from beagles maintained at Marshall Farms USA, Sodus, NY. Sheep blood was obtained from Suffolk breed sheep of the low K (LK) phenotype maintained at the Vinzant Family Farms, Borodino, NY. The red cells were washed free of plasma and white cells by three successive, brief centrifugations and resuspensions in an isotonic, "normal" medium (290 mosmol × kg H_2O^{-1}, measured using a vapor pressure osmometer, Wescor, Logan, UT) containing (in mM): 145 NaCl, 5 KCl, 10 Tris-HCl, 5 glucose, the pH adjusted to 7.4 at 37°C using 1 M HEPES. Blood was used from three mongrel dogs, four beagles, eight sheep, and three human donors; no significant differences in results were observed among donors within each species.

K Influxes

Unidirectional influxes of K were measured using ^{86}Rb as a tracer (Rb is a good congener of K in KCC). In experiments on sheep and human red cells, the media contained 0.1 mM ouabain to inhibit the Na/K pump. For human red cells, the media also contained 2 μM bumetanide, which inhibits >95% of NKCC and none of KCC (Lim, Gasson & Kaji, 1995). Dog and sheep red blood cells lack NKCC; dog red cells lack the Na/K pump. KCC was defined as the Cl-dependent ^{86}Rb influx, the difference between fluxes in Cl-media and Cl-free media, with NO_3 the substitute anion. All fluxes were measured in triplicate. The methods for measuring and calculating the influxes were slight modifications of earlier methods (Sachs et al., 1974). Fluxes are expressed as mmol × 1 cells^{-1} × hr^{-1} when carried out in normal media. When cells were shrunken or swollen with respect to physiological volume, fluxes were corrected to physiological, or original cell volume using the hemoglobin concentrations of the flux samples. These fluxes are expressed as mmol × 1 original cells^{-1} × hr^{-1}. Preincubation with pharmacological agents was for 20 min; the drugs were also present while measuring the fluxes.

Reduction of Cellular Divalent Cation Concentrations

The method of Bergh, Kelley and Dunham (1990) was used. Briefly, cells were incubated in a nominally Ca, Mg-free medium containing A23187 (10 μM) and EGTA (1 mM) for 10 min. The cells were then washed by centrifugation and resuspension and incubated for another 10 min in the A23187-EGTA medium. ^{86}Rb influxes were measured in the same medium; A23187 has no effect on Rb permeability.

Chemicals

Inorganic salts, ouabain, glucose, HEPES, EGTA, Tris-HCl, and trifluoperazine were from Sigma (St. Louis, MO). ML-7, ML-9, wortmannin, staurosporine, and A23187 were from Calbiochem (La Jolla, CA). KT5926 was from Biomol (Plymouth Meeting, PA).

Abbreviations

KCC: K-Cl cotransport; NKCC: Na-K-Cl cotransport; NHE: Na/H exchange; MLC: myosin light chain; MLCK: myosin light chain kinase; PP-1: protein phosphatase type 1; IC_{50}: concentration of an agent at half-maximal inhibition of transport; LK: red blood cells from the phenotype of sheep with low cell [K] red cells; ML-7: 1-(5-iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine; ML-9: 1-(5-chloronaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine; KT5926: (8R*,9S*,11S*)-(_)-9-hydroxycarbonyl-8-methyl-14-*n*-propoxy-2,3,9,10 tetrahydro-8,11-epoxy,1*H*,8*H*,11*H*-2,7b,11a-triazadibenzo[a,g] cycloocta[cde] trinden-1-one; HEPES: N-(2-hydroxy-ethyl) piperazine-N′-2-ethanesulfonic acid; Tris: tris(hydroxy methyl) aminomethane; EGTA: ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′ tetracetic acid; TFP: trifluoperazine.

Results

Concentration-Dependence of Stimulation of K-Cl Cotransport by ML-7 in Dog, Human, and Sheep Red Blood Cells

Figure 1 shows stimulation of K-Cl cotransport (KCC) in red cells from the three species over a range of concentrations of ML-7. As will be shown below, ML-7-stimulation of KCC is sensitive to changes in cell volume. In sheep cells ML-7-stimulates KCC only in shrunken cells, while in dog cells, stimulation was enhanced in slightly swollen cells. Therefore in the experi-

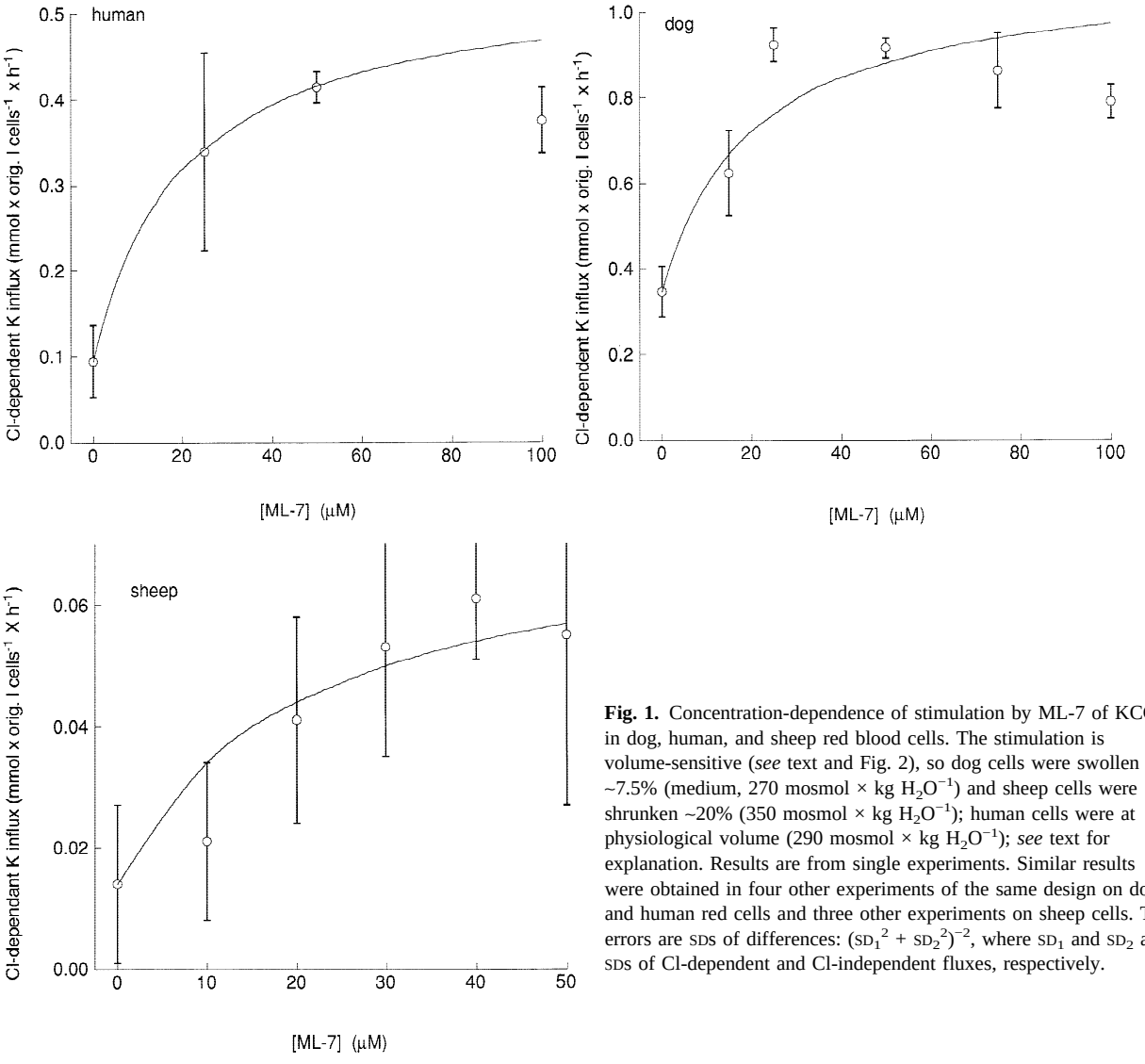

Fig. 1. Concentration-dependence of stimulation by ML-7 of KCC in dog, human, and sheep red blood cells. The stimulation is volume-sensitive (*see* text and Fig. 2), so dog cells were swollen ~7.5% (medium, 270 mosmol × kg H_2O^{-1}) and sheep cells were shrunken ~20% (350 mosmol × kg H_2O^{-1}); human cells were at physiological volume (290 mosmol × kg H_2O^{-1}); *see* text for explanation. Results are from single experiments. Similar results were obtained in four other experiments of the same design on dog and human red cells and three other experiments on sheep cells. The errors are SDs of differences: $(SD_1^2 + SD_2^2)^{-2}$, where SD_1 and SD_2 are SDs of Cl-dependent and Cl-independent fluxes, respectively.

ments in Fig. 1, the dog red cells had been swollen ~7.5% in a hypotonic medium (270 mosmol × kg H_2O^{-1}) and sheep red cells had been shrunken ~20% in a hypertonic medium (375 mosmol × kg H_2O^{-1}). The human red cells were in an isotonic medium (290 mosmol × kg H_2O^{-1}). The KCC fluxes in the sheep cells were particularly low because the cells were shrunken. The errors for sheep cells are large because a small fraction of total K influx in the shrunken cells was Cl-dependent.

ML-7 stimulated KCC 3-fold or more in cells of all three species. Half-maximal stimulation was at about 20 μM in all three. There was slight inhibition at higher concentrations of ML-7 in dog and human cells. Inhibition by ML-7 is examined further below.

Volume-Dependence of Effects of ML-7 on K-Cl Cotransport

The effects of ML-7 on KCC as a function of osmolality of the media, and therefore of cell volume, were tested on red cells from the three species. The results of a number of experiments are shown in Fig. 2. Effects of varying external osmolality on KCC in control cells, determined at the same time, are also shown. The concentration of ML-7, 50 μM, gave near maximal stimulation at the osmolalities tested in Fig. 1. In cells at the highest osmolalities tested, ML-7 stimulated KCC. As the cells were swollen, absolute stimulation by ML-7 increased to maxima at 275 mosmol × kg H_2O^{-1} in dog cells, and between 190 and 230 mosmol × kg H_2O^{-1} in human cells. Stimulation was maximal at 350 mosmol × kg H_2O^{-1} in sheep cells, the lowest osmolality tested. With further swelling, the stimulation by ML-7 declined as the curves for control cells increased in slope. The two curves then intersect, and KCC was lower in ML-7-treated cells than in controls. The crossover points, shown in Table 1, were at swollen cell volumes for dog and human cells, and near or below physiological cell volume for sheep cells, with some uncertainty in this

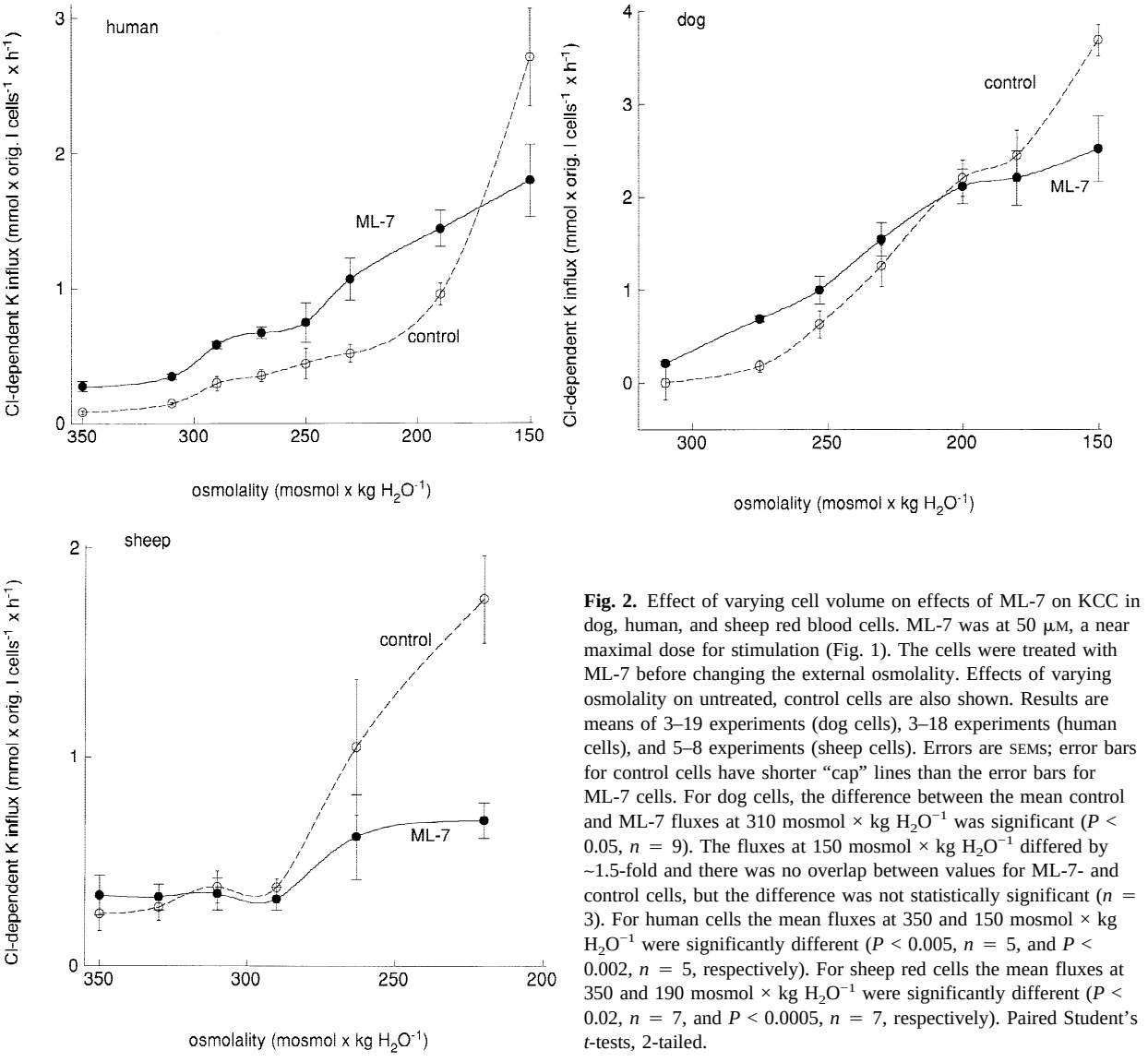

Fig. 2. Effect of varying cell volume on effects of ML-7 on KCC in dog, human, and sheep red blood cells. ML-7 was at 50 μM, a near maximal dose for stimulation (Fig. 1). The cells were treated with ML-7 before changing the external osmolality. Effects of varying osmolality on untreated, control cells are also shown. Results are means of 3–19 experiments (dog cells), 3–18 experiments (human cells), and 5–8 experiments (sheep cells). Errors are SEMS; error bars for control cells have shorter "cap" lines than the error bars for ML-7 cells. For dog cells, the difference between the mean control and ML-7 fluxes at 310 mosmol × kg H_2O^{-1} was significant ($P < 0.05$, $n = 9$). The fluxes at 150 mosmol × kg H_2O^{-1} differed by ~1.5-fold and there was no overlap between values for ML-7- and control cells, but the difference was not statistically significant ($n = 3$). For human cells the mean fluxes at 350 and 150 mosmol × kg H_2O^{-1} were significantly different ($P < 0.005$, $n = 5$, and $P < 0.002$, $n = 5$, respectively). For sheep red cells the mean fluxes at 350 and 190 mosmol × kg H_2O^{-1} were significantly different ($P < 0.02$, $n = 7$, and $P < 0.0005$, $n = 7$, respectively). Paired Student's *t*-tests, 2-tailed.

estimate. The crossover probably means that ML-7 decreases the sensitivity of KCC to activation by swelling.

The set points for KCC can be estimated from the curves for control cells. The set point of a volume-sensitive transporter is the cell volume at the threshold for activation. At cell volumes on one side of the set point, there is relatively little effect of changes in volume on rate of transport. On the other side of the set point, there is a marked increase in transport with changes in volume. The set point is the maximum in the second derivative of a plot of transport *vs.* cell volume. Estimates of the set points for KCC for the three cell types, shown in Table 1, were made from the control curves in Fig. 2 from the intersections of the low slope portions of the curves at higher osmolalities and the steep portions of the curves at lower osmolalities. [Set points from the literature for KCC are at slightly higher osmolalities than those reported here for dog, human, and sheep red cells (Parker, Dunham & Minton, 1995, Figs. 2, 3; Kaji, 1986, Fig. 3; Dunham et al., 1993, Fig. 11).]

The crossover points and set points differ among the three cell types. They are at the most swollen volumes in human cells and the most shrunken volumes in sheep cells. The possible significance of these differences among species is considered below.

Concentration-Dependence of Effect of ML-7 on K-Cl Cotransport in Swollen Human, Dog, and Sheep Red Blood Cells

Figure 3 shows the effect of a range of concentrations of ML-7 on cotransport in human and dog red cells swollen

45% and sheep red cells swollen 30%. Concentrations of ML-7 higher than 50 μM inhibited KCC in dog and human cells. KCC in sheep cells was inhibited at all ML-7 concentrations and the IC_{50} was ~20 μM, the same as the concentration for half-maximal stimulation of KCC in shrunken sheep cells (Fig. 1). ML-7 at 25 μM stimulated K-Cl cotransport in human cells. In dog cells 25 μM ML-7 neither inhibited nor stimulated. The superimposition of the curves for these effects on the curves for inhibition makes the IC_{50}s difficult to estimate in dog and human cells.

Effects of Three Other Inhibitors of MLCK on K-Cl Cotransport in Human Red Cells

Three other inhibitors of MLCK were tested: ML-9 (a derivative of ML-7; Saitoh, Naka & Hidaka, 1986), KT5926 (a derivative of the broad specificity kinase inhibitor K252a; Nakanishi et al., 1990), and wortmannin (Nakanishi et al., 1992). [In the nM range, wortmannin is a specific inhibitor of phosphatidylinositol 3-kinase (Powis et al., 1994), but in the μM range, it inhibits MLCK (Nakanishi et al., 1992).] Table 2 compares the effect of ML-7 with those of the other three inhibitors at concentrations used in the literature cited to inhibit MLCK in intact cells. Both ML-7 and ML-9 stimulated KCC, but KT5926 and wortmannin were without effect.

The dose/response of the stimulation by ML-9 was similar to that of ML-7 (*results not shown*). The stimulatory effects of ML-7 and ML-9 at their maximal doses were not additive. Therefore their targets are probably the same, not surprising since their structures are nearly identical (the only difference is that ML-7 is iodinated at a position where ML-9 is chlorinated).

Comparison of the Effects of ML-7 and Staurosporine in Stimulating K-Cl Cotransport in Human Red Blood Cells

Staurosporine, a protein kinase inhibitor with high affinity and broad specificity, also stimulates KCC in sheep red cells. It stimulates indirectly by promoting the phosphatase reaction (Bize & Dunham, 1994). It was important to determine if ML-7 stimulates by the same mechanism as staurosporine. The first comparison of the two inhibitors was their stimulation as a function of cell volume. Figure 4 shows KCC in media of varying osmolality (390, 290, and 190 mosmol × kg H_2O^{-1}) in control human red cells and in cells treated with 50 μM ML-7 or 5 μM staurosporine, concentrations giving maximal stimulation. The stimulation by ML-7 was greatly increased by cell swelling (as in human cells in Fig. 2), whereas there was modest stimulation of KCC by swelling in staurosporine-treated cells. At physiological cell volume (290 mosmol × kg H_2O^{-1}), the stimulation by staurosporine was much greater than by ML-7. These results indicate that the stimulatory effects of the two kinase inhibitors are exerted on different targets.

Table 1. Crossover points between control and ML-7 curves and set points of control curves of KCC for human, dog, and sheep red cells

Species	Crossover point	Set point
	(mosmol × kg H_2O^{-1})	
Human	175	200
Dog	210	270
Sheep	290–330	290

Values were estimated from the results in Fig. 2. The crossover point is the osmolality at which the curves for control cells and ML-7-treated cells intersect. There was uncertainty in the estimate of the crossover point for sheep cells. The set point is the threshold for swelling-activation of KCC (*see* text for further explanation).

The additivity of the effects of two agents was investigated. As shown in Table 3, the effects are not additive when tested at concentrations giving maximal stimulation. (KCC is not maximal at 5 μM staurosporine at normal cell volume since swelling stimulated further.) Since the targets of ML-7 and staurosporine are not the same (Fig. 4), the lack of additivity indicates that the two agents are acting on the same step in the signal transduction pathway, most likely the phosphorylation/dephosphorylation of the cotransporter.

Roles of Cell Mg and Ca in Stimulation of K-Cl Cotransport by ML-7

MLCK has an obligatory dependence on Ca/calmodulin for its activity (Trybus, 1996). If the target of ML-7 in stimulating KCC is MLCK, then stimulation should require Ca.

To test this, the effects of reducing and restoring intracellular Mg and Ca on KCC and its stimulation by ML-7 in human red cells were determined. Cells were treated with A23187 to deplete them of divalent cations. Table 4 summarizes the results of six experiments. Treatment with A23187 stimulated cotransport in control cells. This effect was reported earlier, and was shown to be a consequence of reducing cell Mg, not cell Ca (Bergh et al., 1990; Dunham et al., 1993). Adding Mg (125–300 μM; free [Mg], ~10 μM) to suspensions of red cells with A23187 reduced the flux to near the control flux, while adding Ca (5–25 μM Ca; free [Ca], ~1 μM) had little effect on the A23187-stimulated flux. The effect of lowering cell Mg is presumably due to reducing the concentration of Mg-ATP, the substrate for the swelling-inhibited kinase.

Table 4 also shows the effects of varying Ca and Mg on cotransport in ML-7-treated cells. ML-7 stimulated cotransport nearly 3-fold compared to untreated control

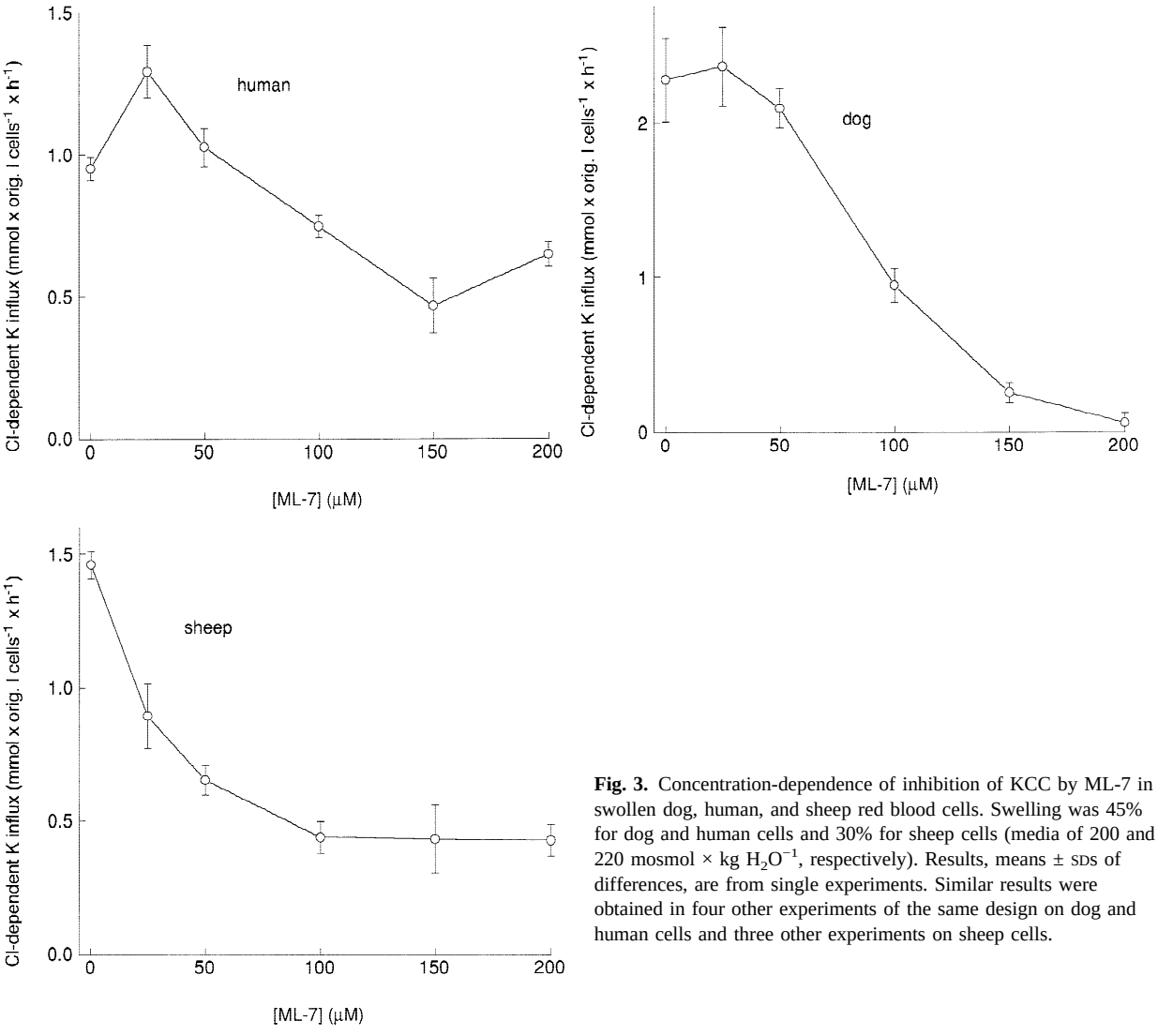

Fig. 3. Concentration-dependence of inhibition of KCC by ML-7 in swollen dog, human, and sheep red blood cells. Swelling was 45% for dog and human cells and 30% for sheep cells (media of 200 and 220 mosmol × kg H_2O^{-1}, respectively). Results, means ± SDs of differences, are from single experiments. Similar results were obtained in four other experiments of the same design on dog and human cells and three other experiments on sheep cells.

Table 2. Comparison of effects of four inhibitors of MLCK on KCC in human red cells

Condition	Cl-dependent K influx
	(mmol × 1 cells^{-1} × hr^{-1})
Control	0.32 ± 0.04
ML-7 (50 μM)	0.73 ± 0.01
ML-9 (50 μM)	0.90 ± 0.04
KT5926 (10 μM)	0.24 ± 0.01
Wortmannin (30 μM)	0.31 ± 0.09

Cells were suspended in media of physiological osmolality. Results are means ± SDs of differences from one experiment. Stimulation by ML-7 and ML-9 was demonstrated numerous other times. Similar results were seen with KT5926 in four other experiments and with wortmannin in one other experiment of the same design.

cells. Reducing cell Ca or cell Mg reduced KCC slightly. Adding Ca had little effect. Addition of Mg resulted in a flux ~30% greater than that in cells treated with ML-7 alone. This effect was unexpected. What seems clear is that stimulation by ML-7 is sensitive to cell [Mg], but not to cell [Ca].

The effect of a calmodulin antagonist should provide further evidence on the dependence of the effects of ML-7 on Ca. The effect of trifluoperazine (TFP) was tested on stimulation of KCC by ML-7 in human red cells. TFP remains a cell permeant calmodulin antagonist of choice (Li, Joyal & Sacks, 2000). Table 5 shows the effect of ML-7 on control fluxes and fluxes in TFP-treated cells. ML-7 stimulated KCC only 2-fold in this experiment. ML-7 also stimulated KCC in TFP-treated cells. Therefore the calmodulin-antagonist did not prevent ML-7's effect. The possible slight stimulation by TFP alone was not a consistent finding.

Discussion

The MLCK inhibitor ML-7 stimulated KCC in red blood cells of three mammalian species. The stimulation was

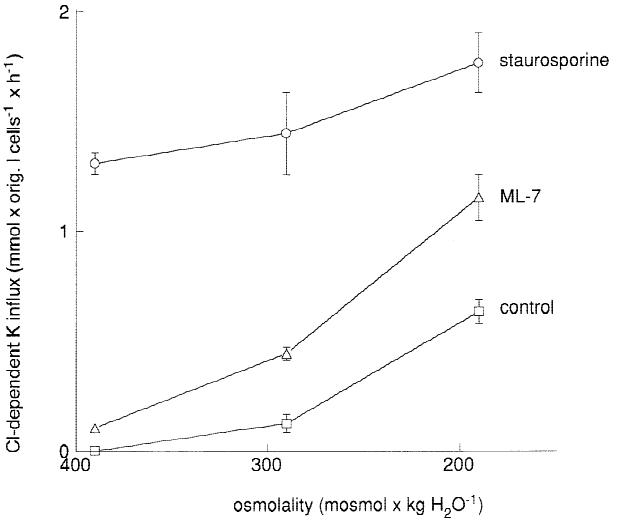

Fig. 4. Cell volume-dependence of stimulation of KCC by staurosporine (5 μM) and ML-7 (50 μM) in human red blood cells. Fluxes are also shown for untreated, control cells. Osmolalities of the media were 390, 290, and 190 mosmol × kg H_2O^{-1}. Shown are means ± SDs of differences from one experiment. Error bars not shown when smaller than the symbols. Similar results were obtained in three other experiments of the same design.

Table 3. Test for additivity of stimulatory effects of ML-7 and staurosporine on KCC in human red blood cells

Condition	Cl-dependent K influx
	(mmol × 1 cells^{-1} × hr^{-1})
Control	0.21 ± 0.04
ML-7	0.50 ± 0.02
Staurosporine	1.63 ± 0.05
ML-7 → staurosporine	1.78 ± 0.17
Staurosporine → ML-7	1.51 ± 0.11

Cells were suspended in media of physiological osmolality. ML-7 was 50 μM, staurosporine 5 μM. For treatment with two agents in sequence, cells were preincubated with the first agent for 20 min; the second agent was added without washing the cells for an additional 20 min. Results are means ± SDs of differences from one experiment. Similar results were obtained in three other experiments of the same design.

observed in cells at near physiological volume. Cell swelling reduced stimulation; in the most swollen cells, ML-7 inhibited cotransport. Other evidence supports the view that MLCK is not the target of ML-7 in modulating KCC in red cells. The argument will be made that ML-7's target is the swelling-inhibited kinase that inactivates KCC.

MLCK is not the Target of ML-7 in Red Cells

Three observations indicate that MLCK is not the target of ML-7 in the regulatory pathway for KCC in mammalian red cells. (i) The concentration for half-maximal

Table 4. Effects of depleting and restoring cellular divalent cations on KCC in control and ML-7-stimulated human red cells

Condition	Cl-dependent K influx	
	(mmol × 1 cells^{-1} × hr^{-1})	
	Control	ML-7
Control	0.16 ± 0.03	0.47 ± 0.03
A23187	0.43 ± 0.05	0.32 ± 0.03
A23187 + Mg	0.17 ± 0.03	0.61 ± 0.08
A23187 + Ca	0.34 ± 0.03	0.38 ± 0.04

Cells were suspended in media of physiological osmolality. Media with A23187 (10 μM) also contained 1 mM EGTA. Added concentrations of Mg and Ca were 125–200 μM and 5–50 μM, respectively. ML-7 concentration was 50 μM. Results are means ± SEMs of differences from six experiments.

Table 5. Effects of trifluoperazine and ML-7 on KCC in human red blood cells

	Cl-dependent K influx	
	(mmol × 1 cells^{-1} × hr^{-1})	
	Control cells	TFP-treated cells
Control	0.28 ± 0.05	0.42 ± 0.02
ML-7-treated	0.56 ± 0.10	0.67 ± 0.03

Cells were suspended in media of physiological osmolality. Pretreatment with TFP was for 20 min followed by ML-7, as indicated, for 20 min with TFP present. Both agents were at 50 μM. Results are means ± SDs from one experiment. Similar results were obtained in two other experiments of the same design.

stimulation by ML-7, ~20 μM, is nearly two orders of magnitude greater than the IC_{50} for the inhibition of MLCK (Saitoh et al., 1987). (ii) Two other inhibitors of MLCK, wortmannin and KT5296, had no effect on KCC. (iii) Reducing cell [Ca] and treatment with a calmodulin antagonist had no effect on the stimulation of cotransport by ML-7; MLCK requires Ca/calmodulin.

Is MLCK the Target of ML-7 in Shrinkage-Activated Transporters?

There are reports of inhibition by ML-7 of shrinkage-activated transport pathways. It was concluded that ML-7's target is MLCK, but not all of the evidence is supportive. Very different concentrations of ML-7 were required in different systems. For NKCC in Ehrlich ascites cells, the IC_{50} for ML-7 was 0.38 μM (Krarup et al., 1998), consistent with MLCK being ML-7's target in these cells. In bovine aortic endothelial cells, the IC_{50} for inhibition of NKCC was 0.3 μM (O'Donnell et al., 1995). In calf aortic endothelium, the IC_{50} for inhibition was ~40 μM (Klein & O'Neill, 1995). There is no obvious explanation for this 100-fold difference between these two very similar systems. Shrinkage-activated

NHE in astrocytes was inhibited by ML-7 with an IC_{50} of 91 μM (Shrode et al., 1995).

The signal for changes in MLCK activity is likely to be changes in cell Ca activity; MLCK has an obligatory requirement for Ca/calmodulin. Loading Ehrlich ascites cells with the Ca chelator BAPTA inhibited shrinkage-activation of NKCC (Krarup et al., 1998), consistent with the target of ML-7 being MLCK in these cells. However there was only a modest increase in cell Ca activity induced by shrinkage (90 → 100 nM) (Jensen, Jessen & Hoffmann, 1993). O'Donnell et al. (1995) found no shrinkage-induced increase in Ca activity in bovine aortic endothelial cells. In these cells, reduction of Ca activity by BAPTA-loading had little effect on shrinkage-activation of NKCC measured as unidirectional K influx, but had a large effect on shrinkage-activation of NKCC measured as net K influx (O'Donnell et al., 1995). In astrocytes there was no increase in Ca activity following shrinkage; furthermore shrinkage induced NHE in BAPTA-loaded cells (Shrode et al., 1995). These studies indicate that the target of ML-7 is not Ca-dependent in all cases. While MLCK may participate in the regulation of some shrinkage-activated transporters, it may not be a part of a general mechanism.

There is a skeletal muscle form of MLCK with a 10-fold lower sensitivity to ML-9 and ML-7 than the smooth muscle and platelet enzymes (Saitoh et al., 1987). MLCK from mammalian smooth muscle and non-muscle cells are the same protein and this enzyme is distinct from skeletal muscle MLCK (Stull, Nunnally & Michnoff, 1986; Gallagher et al., 1991). It is probable that the form of MLCK in the cell types in the studies just reviewed is of the smooth muscle/non-muscle type. The skeletal muscle type has been found only in skeletal muscle and the other type of MLCK in every other tissue in which it has been sought.

Smooth muscle MLCK can undergo Ca-independent autophosphorylation (Tokui, Ando & Ikebe, 1995). The activity of the Ca/calmodulin-independent autophosphorylated MLCK is much less than the Ca/calmodulin-dependent activity. It is not clear if autophosphorylated MLCK has a role in smooth muscle. It is not known if it is present in other cell types.

Other Targets of ML-7

A number of targets for ML-7 have been shown with relatively high IC_{50}s. Fibronectin-binding to fibroblast membranes is inhibited by ML-7 with an IC_{50} of 49 μM, an effect attributed to inhibition of protein kinase C, PKC (Somers & Mosher, 1993). ML-7 inhibited shape changes in lymphocytes and in U937 cells (motile variants of the monocytoid cell line); the IC_{50}s were 10 and 40 μM respectively (Thorp, Southern & Matthews, 1994). A serine/threonine kinase was implicated, possibly PKC. Gap junctional intercellular communication in two cell lines was inhibited by ML-7 (Jansen, de Vrije & Jongen, 1996), perhaps due to inhibition of calmodulin-dependent kinase II. ML-7 inhibited the mammalian small heat-shock protein (HSP25) kinase with a particularly low affinity, $IC_{50} > 200$ μM (Hayess & Benndorf, 1997). Finally, ML-9 and ML-7 inhibited cAMP-dependent kinase, PKC, and Ca/calmodulin-dependent phosphodiesterase with IC_{50}s of 32, 54, and 50 μM, respectively (Saitoh et al., 1987). So there are precedents for targets of ML-7 other than MLCK.

Volume-Sensitivity of Stimulation of K-Cl Cotransport by ML-7 in Red Cells

A striking feature of the effect of ML-7 on KCC in mammalian red cells was its sensitivity to cell volume. At the lowest volumes tested, where control KCC was the lowest, there was significant stimulation by ML-7 in red cells from all three species (Fig. 2). As cells were swollen, relative stimulation by ML-7 was reduced as absolute stimulation increased. With further swelling, the control and ML-7 curves crossed (Fig. 2) and KCC was less in ML-7-treated cells than in the controls. Therefore in addition to stimulating KCC, ML-7 appears to reduce the sensitivity of KCC to activation by swelling. The stimulation can be understood if the target for stimulation by ML-7 is the volume-sensitive kinase, as discussed below. The kinase may also be the target for the reduction of sensitivity to swelling-activation and for inhibition of KCC in the most swollen cells. The explanations for these last two effects of ML-7 are not clear. Roles of the kinase in both seem likely because of the similarity in the apparent affinities of ML-7 as an stimulator and inhibitor of KCC in cells of different volumes (Figs. 1 and 3).

Comparison of the Effects of Staurosporine and ML-7

KCC in ML-7-treated cells is highly sensitive to swelling-activation (Figs. 2, 4). In contrast, swelling of staurosporine-treated cells provokes modest activation (Fig. 4). This is consistent with the earlier conclusion that staurosporine exerts its stimulatory effect upstream to the phosphatase which directly activates KCC (Bize & Dunham, 1994); the phosphatase has been argued to be insensitive to volume changes (Jennings & Al-Rohil, 1990; Dunham et al., 1993; Jennings, 1999; *cf.* Bize et al., 1999). Therefore staurosporine and ML-7 stimulate at different targets, the former volume-insensitive and the latter volume-sensitive. However the effects of the two agents are not additive (Table 3). This result suggests that the two agents affect the same step in the signal transduction process, the phosphorylation/dephos-

phorylation of the transporter. Staurosporine promotes, indirectly, the activating phosphatase reaction. ML-7 may stimulate by inhibiting the volume-sensitive, inhibitory kinase reaction.

The Volume-Sensitive Kinase

The putative volume-sensitive kinase inhibits KCC by phosphorylating the cotransporter (or an associated regulatory protein). The kinase is probably in equilibrium between free in the cytoplasm and bound. The kinase may not bind directly to the cotransporter but rather to a nearby "anchoring" protein (Mochly-Rosen, 1995). An anchoring or scaffold protein regulating the N-methyl-D-aspartate receptor binds both a phosphatase and a kinase (Westphal et al., 1999). This type of arrangement is envisioned to regulate KCC: an anchor protein with a phosphatase, PP-1, constitutively bound, and a kinase whose binding is sensitive to changes in cell volume. The fraction of total kinase bound to the anchor is highest in cells of lowest volume, accounting for the low KCC activity in shrunken cells. The bound kinase phosphorylates the cotransporter. With swelling, binding of the kinase decreases and KCC increases due to the phosphatase reaction.

The swelling-induced decrease in binding of the kinase to the anchor is hypothesized to be a consequence of the reduced thermodynamic activity of the kinase (and all other cytoplasmic proteins) due to reduced macromolecular crowding (Zimmerman & Minton, 1993; Minton, 1994; Parker et al., 1995). The high concentrations of proteins in the cytoplasm of cells at physiological volume (300 g/l in red cells) result in thermodynamic activities of all cytoplasmic proteins far in excess of those in dilute solution. Small changes in protein concentrations due to cell volume changes result in large changes in thermodynamic activities of proteins (Zimmerman & Minton, 1993; Minton, 1994). Decreased cell volume and the resultant increased crowding promote binding of the kinase to the anchor, phosphorylation of the cotransporter, and inhibition of cotransport.

With moderate swelling, a large reduction in kinase binding is expected due to reduced crowding. The rate constant of the reaction promoted by the kinase, a function of the fraction of total kinase which is bound, has been proposed to decrease 100-fold with 50% swelling in sheep red cells (Dunham et al., 1993).

Does ML-7 Inhibit the Volume-Sensitive Kinase?

The results presented here are consistent with ML-7 stimulating cotransport by inhibiting the volume-sensitive kinase. As discussed above, ML-7 inhibits several kinases in the concentration range in which it stimulates KCC, and ML-7 appears not to act by inhibiting MLCK. The pertinent data are the sensitivity of stimulation by ML-7 to cell volume. The volume-sensitive stimulation can be understood in terms of the following hypothesis. ML-7, a nucleotide analogue that binds to nucleotide binding sites on kinases (Saitoh et al., 1987), binds to the kinase, both that free in the cytoplasm and bound to the anchor. ML-7-kinase complex bound to the anchor will not phosphorylate the cotransporter. Therefore when a fraction of the kinase bound is the ML-7-kinase complex, cotransport will be correspondingly enhanced. In shrunken cells, with most of the anchors occupied by kinase, the relative stimulation of KCC by ML-7 will be larger than in swollen cells with fewer of the anchors occupied by kinase, either the ML-7-kinase complex or the uninhibited kinase.

In cells swollen ~30% above physiological volume, control KCC was stimulated an order of magnitude or more. At this volume, there was small but measurable stimulation by ML-7 in dog and human cells. Further swelling of the cells to ~65% above normal volume caused a further increase in control cotransport but ML-7 no longer stimulated (Fig. 2). According to our hypothesis, this is because kinase occupied few of the anchors, and swelling-activation at these higher volumes is independent of the kinase.

In these terms it can be understood how ML-7 stimulates KCC in mammalian red cells by inhibiting the volume-sensitive kinase and why stimulation by ML-7 is volume-sensitive.

The reduction of sensitivity to swelling-activation caused by ML-7 may also be a consequence of interaction of the drug with the volume-sensitive kinase. However no straight forward explanation is apparent. The same is true of the inhibition by ML-7 in swollen cells. The IC_{50}s are similar to the concentrations for half-maximal stimulation in cells nearer physiological volume, but it is not clear why binding to the kinase inhibits KCC in swollen cells.

This is the first report of a kinase inhibitor inhibiting the volume-sensitive kinase. Further studies with ML-7 could lead to the identification of the kinase.

The Differences Among Species

The quantitative difference among species in volume-sensitivity of the response to ML-7 is apparent in the differences in crossover points (Table 1), the osmolalities at which the curves for control and ML-7 cells intersect. The crossover is likely to be a consequence of the reduced sensitivity to swelling-activation caused by ML-7. With no clear explanation for this reduced sensitivity, it is difficult to explain the differences in crossover points.

Another difference among the species is in the set points for KCC (Table 1). The set point of a volume-sensitive transporter is the cell volume of the threshold

for activation. The crossover points and set points are correlated: they are at the lowest osmolalities (most swollen cells) for human cells and at the highest osmolalities (most shrunken cells) for sheep cells. This may be the first report of differences in set points for KCC among species (*cf.* Romero et al., 1997).

The set point for KCC can be understood as a critical concentration of the volume-sensitive kinase, the concentration at the set point (Parker et al., 1995). The near-discontinuity at the set point in the curve of transport *vs.* osmolality is a consequence of the highly nonlinear dependence of thermodynamic activities of cytoplasmic proteins, including the kinase, on their concentrations and therefore on cell volume (Zimmerman & Minton, 1993; Minton, 1994). Since crossover points and set points are correlated among the three cell types, both parameters are probably determined by a property of the kinase, a property that differs among species. The kinases in the three cell types may vary in affinity for the anchoring protein, in molecular mass, or in total concentration.

The physiological significance of this difference in set points is also unclear. There may be none in red cells, but there could be in other cell types. One can imagine a functional significance of a set point at a particular cell volume in one cell type and at a different cell volume in another, the significance being related to varying patterns of cell volume regulation.

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