Ohkura, T., Yamashita, K., Mishima, Y., & Kobata, A. (1984) *Arch. Biochem. Biophys. 235*, 63–77.
Ringuette, M. J., Sobieski, D. A., Chamow, S. M., & Dean, J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4341–4346.
Shimizu, S., Tsuji, M., & Dean, J. (1983) *J. Biol. Chem. 258*, 5858–5863.
Shur, B. D., & Hall, N. G. (1982) *J. Cell Biol. 95*, 574–579.
Shur, B. D., & Neely, A. G. (1988) *J. Biol. Chem. 263*, 17706–17714.
Stambaugh, R. (1978) *Gamete Res. 1*, 65–85.
Takasaki, S., Mizuochi, T., & Kobata, A. (1982) *Methods Enzymol. 83*, 263–268.
Takasaki, S., Murray, G. J., Furbish, F. S., Brady, R. O., Barranger, J. A., & Kobata, A. (1984) *J. Biol. Chem. 259*, 10112–10117.
Takeuchi, M., Takasaki, S., Miyazaki, H., Kato, T., Hoshi, S., Kochibe, N., & Kobata, A. (1988) *J. Biol. Chem. 263*, 3657–3663.
Topfer-Petersen, E., Friess, A. E., Nguyen, H., & Schill, W. B. (1985) *Histochemistry 83*, 139–145.
Wassarman, P. M. (1987) *Annu. Rev. Cell Biol. 3*, 109–142.
Wassarman, P. M., Florman, H. M., & Greve, J. M. (1985) in *Biology of Fertilization* (Metz, C. B., & Monroy, A. Eds.) Vol. 2, pp 341–360, Academic, New York.
Yamashita, K., Ohkura, T., Yoshima, H., & Kobata, A. (1981) *Biochem. Biophys. Res. Commun. 100*, 226–232.
Yamashita, K., Mizuochi, T., & Kobata, A. (1982) *Methods Enzymol. 83*, 105–126.
Yamashita, K., Kochibe, N., Ohkura, T., Ueda, I., & Kobata, A. (1985) *J. Biol. Chem. 260*, 4688–4693.
Yamashita, K., Totani, K., Ohkura, T., Takasaki, S., Goldstein, I. J., & Kobata, A. (1987) *J. Biol. Chem. 262*, 1602–1607.
Yanagimachi, R. (1988) in *The physiology of reproduction* (Knobil, E., & Neil, J. L., Eds.) Vol. 1, p 135, Raven, New York.
Yazawa, S., Furukawa, K., & Kochibe, N. (1984) *J. Biochem. (Tokyo) 96*, 1737–1742.
Yoshima, H., Takasaki, S., & Kobata, A. (1980) *J. Biol. Chem. 255*, 10793–10804.
Yurewicz, E. C., Sacco, A. G., & Subramanian, M. G. (1987) *J. Biol. Chem. 262*, 564–571.

High Channel-Mediated Water Permeability in Rabbit Erythrocytes: Characterization in Native Cells and Expression in *Xenopus* Oocytes†

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ABSTRACT: Erythrocytes from several mammalian species contain mercurial-sensitive water transporters. By a stopped-flow light scattering technique, osmotic water permeability (P_f) was exceptionally high in rabbit erythrocytes (0.053 ± 0.002 cm/s) and reversibly inhibited by 98% by *p*-(chloromercuri)benzenesulfonate (pCMBS). The activation energy (E_a) was 4.6 kcal/mol (15–37 °C). pCMBS inhibition was half-maximal at 0.1 mM (60-min incubation); at 1 mM pCMBS, half-maximal inhibition occurred in 8 min. P_f was also inhibited by $HgCl_2$ and pCMB with >90% inhibition in 5 min. There was no inhibition by high concentrations of phloretin, DNDS, cytochalasin B, amiloride, ouabain, furosemide, and several proteases. In defolliculated *Xenopus* oocytes microinjected with 50 nL of water or unfractionated mRNA (1 mg/mL) from rabbit reticulocytes, oocyte P_f assayed at 10 °C after 72-h incubation increased from (4 ± 1) × 10^{-4} cm/s (water injected) to (18 ± 2) × 10^{-4} cm/s (mRNA injected). P_f increased linearly with [mRNA] (0–75 ng/oocyte) and was inhibited slowly and reversibly by pCMBS and immediately by $HgCl_2$ but not by cytochalasin B, phloretin, or DNDS. E_a was 9.6 kcal/mol (water injected) and 2.6 kcal/mol (mRNA injected). These results demonstrate that rabbit erythrocytes have the highest P_f and the greatest percentage inhibition of P_f by mercurials of any mammalian erythrocyte studied. The characteristics of the expressed and native water channels were similar, suggesting that the erythrocyte water channel is a membrane protein suitable for expression cloning.

The water permeability of human erythrocytes has been the subject of considerable interest. From biophysical measurements, including (a) high osmotic water permeability (P_f = 0.02 cm/s), (b) a ratio of osmotic-to-diffusional water permeability (P_f/P_d) > 3, (c) a low activation energy (E_a = 4.5 kcal/mol), and (d) 90% inhibition of P_f by mercurials, it has been concluded that the erythrocyte contains a specialized pore or channel for facilitated water transport (Macey, 1984; Solomon et al., 1984). It was proposed that rapid water transport is important for protection of erythrocyte integrity during passage through and return from the hypertonic renal medulla (Macey, 1984).

The molecular identity of the erythrocyte water channel is uncertain. Studies of [^{203}Hg]pCMBS binding have raised the possibility that the anion transport protein band 3 is the water channel (Benga et al., 1986); however, the lack of water transport inhibition by anion transport inhibitors and the

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nonspecific nature of pCMBS binding raise doubts. It has been proposed that glucose transporters can be water channels (Fischbarg et al., 1990); however, water transport in human erythrocytes is not inhibited by the glucose transport inhibitors phloretin and cytochalasin B. Similarly, the lack of inhibition of water transport by urea transport inhibitors makes it unlikely that a common water and urea transporter exists. In addition, Macey has pointed out that duck erythrocytes have high water and low urea permeability, whereas amphiuma erythrocytes have low water and high urea permeability. There is a rare human erythrocyte blood type associated with normal water transport but low urea transport (Frohlich et al., 1988). Finally, it has been proposed that the erythrocyte water channel may be in part composed of lipids on the basis of the inability to inhibit water transport by radiation inactivation (Dix et al., 1985) and proteolysis (Benga et al., 1983).

Although the kidney and amphibian urinary bladder also contain water channels [recent reviews: Handler (1988) and Verkman (1989)], the erythrocyte is probably the best tissue to obtain molecular information about water transporters because of its abundance, purity, and independence of water transport on hormonal factors. We have characterized water transport in rabbit erythrocytes, a cell found to have significantly higher P_f than other mammalian erythrocytes; P_f in rabbit erythrocytes was dramatically (>98%) inhibited by mercurial sulfhydryl reagents. Using a transport assay for expressed water channels in *Xenopus* oocytes, we find that water transport in oocytes microinjected with poly(A+) mRNA from rabbit reticulocytes is similar to that in the native erythrocyte. The rabbit erythrocyte thus provides an excellent tissue source for biochemical and molecular analysis of biological water channels.

Materials and Methods

Cells and Ghost Preparation. Fresh erythrocytes from rabbits or a human donor (A. S. Verkman) were obtained by venipuncture and anticoagulated with heparin. Cells were washed twice, and the erythrocyte pellet was suspended in 140 mM NaCl–5 mM potassium phosphate, pH 7.4 (PBS), for stopped-flow measurements. Red cell ghosts were prepared from washed erythrocytes by a modification of the procedure of Steck and Kant (1974). Cells were lysed and washed three times in 5 mM sodium phosphate (pH 8.0), suspended in PBS, and sealed by a 60-min incubation at 37 °C.

Stopped-Flow Measurements. Osmotic water permeability was measured by stopped-flow 90° light scattering (510 nm) in a Hi-Tech SF-51 apparatus (Wiltshire, England). A suspension of erythrocytes (~0.25% hematocrit) or sealed ghost membranes (~0.5 mg of protein/mL) was mixed with an equal volume of hyperosmotic PBS containing sucrose to cause osmotic water efflux and increased light scattering. P_f was calculated from the time course of light scattering and erythrocyte surface-to-volume ratio as described previously (Dix et al., 1985). In some experiments water transport inhibitors were incubated with erythrocytes for specified times prior to stopped-flow measurements. The mercurials pCMBS, pCMB, and $HgCl_2$ were prepared immediately before use and shielded from light during incubations. Amiloride and phloretin were added from stock solutions in ethanol.

Oocyte Expression Studies. mRNA was prepared from rabbit reticulocytes as described previously (Zhang et al., 1990). Reticulocytosis (25–30%) was induced in 1.5–2-kg New Zealand white rabbits by venipuncture-induced anemia (Hct <25%). Rabbits were exsanguinated by cardiac puncture, and the cell layer containing reticulocytes and erythrocytes was homogenized in 4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% Sarcosyl, and 0.1 M mercaptoethanol, pH 7, for phenol extraction, CsCl gradient centrifugation, and oligo(dT)–cellulose affinity chromatography (Sambrook et al., 1989); ~2 mg of total RNA and 40 μg of poly(A+) RNA was prepared from 100 mL of rabbit blood.

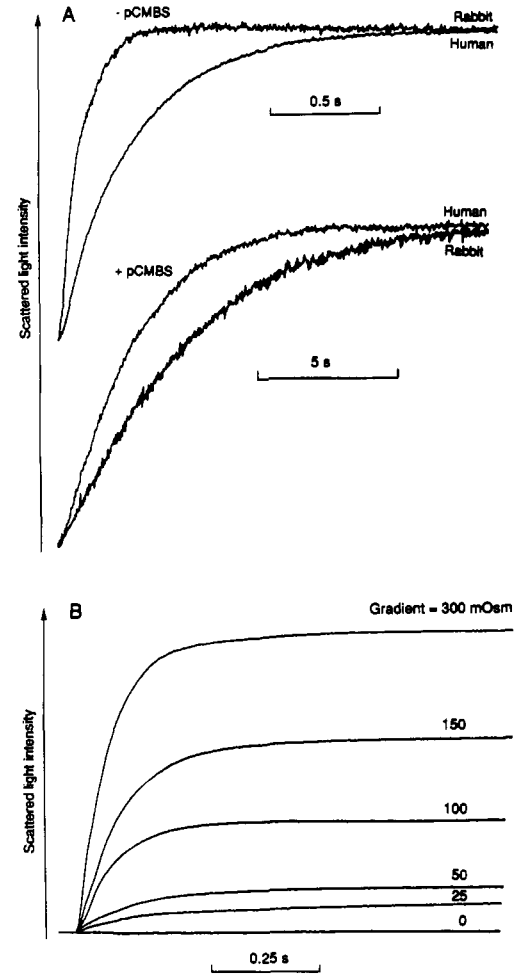

FIGURE 1: (A) Osmotic water transport in human and rabbit erythrocytes. 0.075 mL of an erythrocyte suspension in PBS at 23 °C was mixed with an equal volume of PBS containing 100 mM sucrose in a stopped-flow apparatus (see Materials and Methods). The time course of 90° light scattering is shown. Where indicated, 1 mM pCMBS was incubated for 60 min prior to the stopped-flow experiment. (B) Dependence of the light scattering time course on osmotic gradient size for rabbit erythrocytes. The osmotic gradient corresponds to the extracellular sucrose concentration after mixing of the erythrocyte suspension with the hyperosmotic buffer.

Defolliculated *Xenopus* oocytes obtained by collagenase digestion were microinjected with 50 nL of water or 25–75 nL of reticulocyte mRNA (1 mg/mL in water) by high-pressure superficial injection with an ~1 μm diameter micropipet (Eppendorf). Oocytes were incubated for 72 h at 18 °C in Barth's buffer (200 mOsm) with daily buffer changes. P_f was measured from the initial time course of oocyte swelling when oocytes were immersed in a 1:20 dilution of Barth's buffer with distilled water. Oocyte volume was monitored continuously by a real-time imaging method developed by Fischbarg et al. (1990) and extended by Zhang and Verkman (1991). In some experiments inhibitors were added before and during the osmotic swelling measurement.

Results

Figure 1A shows the time course of light scattering in human and rabbit erythrocytes in response to an inwardly di-

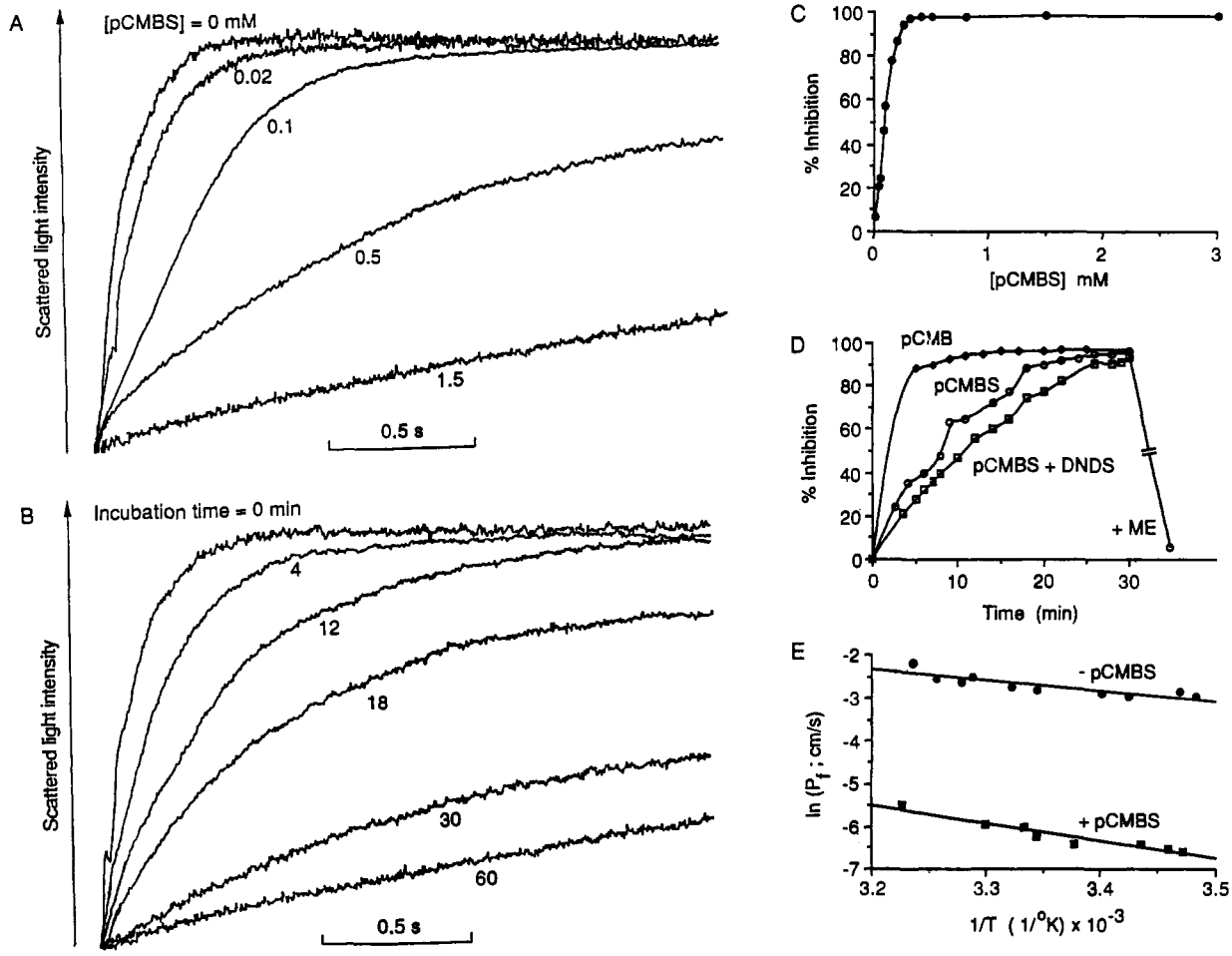

FIGURE 2: Inhibition and temperature dependence of osmotic water permeability in rabbit erythrocytes. (A) Concentration dependence of pCMBS inhibition. Erythrocytes (0.25% Hct) were incubated with specified concentrations of pCMBS for 60 min at 23 °C prior to stopped-flow measurements. P_f was measured with a 50 mM inwardly directed sucrose gradient. (B) Time course of P_f inhibition by 1 mM pCMBS. Erythrocytes were incubated with pCMBS for specified times prior to stopped-flow measurements. (C) Summary of pCMBS concentration dependence. Each point is the mean of 5–10 measurements; the average SD was ~1.5%. The K_I was 0.13 mM. (D) Summary of time course of inhibition of P_f by pCMB (1 mM) and pCMBS (1 mM) in the absence and presence of 0.2 mM DNDS. (E) Temperature dependence of P_f measured in the absence of pCMBS and after a 60-min incubation with 1 mM pCMBS at 23 °C. Each point is the mean of three to five determinations. Fitted activation energies are 4.6 ± 0.8 (-pCMBS) and 8.3 ± 1 (+pCMBS).

rected gradient of sucrose. The 50 mM sucrose gradient caused a decrease in cell volume to ~86% of its initial volume, resulting in an increase in light scattering. The apparent rate of cell shrinkage was significantly greater in rabbit than in human erythrocytes. pCMBS (under maximal inhibitory conditions, see below) inhibited water permeability by ~90% in human erythrocytes and >98% in rabbit erythrocytes. In sealed rabbit erythrocyte ghosts prepared as described under Materials and Methods and subjected to a 50 mM inwardly directed sucrose gradient, the increase in light scattering was multiexponential; ~60% of the total amplitude had a rapid time course, similar to that in intact erythrocytes, and was inhibited by >95% by pCMBS (not shown).

To determine P_f quantitatively, the dependence of scattered light intensity upon osmotic gradient size was established (Figure 1B). There was little change in light scattering with zero osmotic gradient, indicating absence of mixing artifact. As osmotic gradient size increased, the total amplitude and the initial slope of the curves increased. The initial slope, proportional to the transmembrane osmotic flux, increased linearly with osmotic gradient size as predicted by nonequilibrium thermodynamics. From the surface-to-volume ratio of 26 300 cm^{-1} estimated for rabbit erythrocytes (Albritton, 1972), P_f was calculated to be 0.053 ± 0.002 cm/s (SE, six separate preparations, 23 °C), ~2.5 times greater than P_f in human erythrocytes (0.02 cm/s; Macey, 1984). In the presence of maximal pCMBS, rabbit erythrocyte P_f was 0.0011 ± 0.0001 cm/s (n = 6), similar to human erythrocyte P_f (0.0018 cm/s). These low values are typical of P_f in lipid bilayers (Fettiplace & Haydon, 1980).

The concentration dependence and kinetics of pCMBS inhibition of P_f in rabbit erythrocytes were examined (Figure 2A,B). At a 60-min incubation to achieve maximal response, pCMBS inhibited P_f by 98% with K_I for a single-site inhibition model of 0.13 mM (Figure 2C). At 1 mM pCMBS, inhibition developed slowly with a halftime of 8 min (Figure 2D). After 98% inhibition was achieved, addition of 5 mM mercaptoethanol reversed the pCMBS inhibition almost completely.

The slow development of pCMBS inhibition may be due to restricted access to its inhibitory site because of steric factors or because it must cross the membrane to bind to a site at a cytoplasmic surface of the water channel. To distinguish between these possibilities, the kinetics of inhibition by membrane-permeant mercurials with small ($HgCl_2$) and large (pCMB) molecular size were examined. $HgCl_2$ at 0.1 mM inhibited P_f by 97 ± 1% (SE, three preparations) in under 2 min; inhibition was reversed by mercaptoethanol. pCMB at 1 mM inhibited P_f with a remarkably faster time course than pCMBS (Figure 2D); inhibition was ~90% in 5 min. These results suggest that mercurial inhibitors must cross the membrane to reach the water transport inhibition site. Because pCMBS is anionic, membrane transport might be facilitated

Table I: Inhibition of P_f in Rabbit Erythrocytes[a]

inhibitor	concn	incubation time (min)	% inhibiton −pCMBS	% inhibiton +pCMBS
none			0	98 ± 1
N-ethyl-maleimide	1 mM	30	3 ± 5	97 ± 1
$HgCl_2$	0.4 mM	5	97 ± 1	
DNDS	0.2 mM	5	−6 ± 7	96 ± 1
cytochalasin B	10 μM	30	8 ± 5	97 ± 1
phloretin	0.25 mM	5	1 ± 6	97 ± 1
ouabain	0.1 mM	30	−3 ± 4	98 ± 1
furosemide	0.5 mM	5	0 ± 3	98 ± 1
amiloride	0.1 mM	5	8 ± 9	97 ± 1
neuraminidase	20 milliunits/mL	120	12 ± 10	
chymotrypsin	1 mg/mL	60	4 ± 9	96 ± 2
trypsin	1 mg/mL	60	6 ± 4	98 ± 1
pepsin	1 mg/mL	60	2 ± 8	97 ± 1

[a] P_f was measured in rabbit erythrocytes at 23 °C in response to a 50 mM inwardly directed sucrose gradient. Erythrocytes were incubated with inhibitors at 23 °C for indicated times prior to stopped-flow measurements. P_f was measured in the absence and presence of 1 mM pCMBS (30-min incubation). Data are mean ± SD of four to six measurements.

by a stilbene-inhibitable pathway. Figure 2D shows that a high concentration of the potent anion transport inhibitor DNDS (0.2 mM) had a small influence on the time course of pCMBS inhibition, suggesting that passage of pCMBS across band 3 is not the rate-limiting step for water transport inhibition.

The temperature dependence of P_f was measured to calculate E_a from the Arrhenius relation (Figure 2E). In the absence of pCMBS, a single E_a of 4.6 ± 0.8 kcal/mol was measured in the temperature range 14–37 °C. This low value of E_a supports the conclusion that water transport is channel mediated in rabbit erythrocytes. In the presence of pCMBS, E_a was higher, 8.3 ± 1 kcal/mol, suggesting the closure of water channels.

Table I summarizes the effects of a series of transport inhibitors on P_f in rabbit erythrocytes. Whereas the mercurials pCMBS, pCMB, and $HgCl_2$ strongly inhibited P_f, the nonmercurial sulfhydryl reagent NEM had no effect, nor did pretreatment with NEM affect the pCMBS inhibitor potency. The potent inhibitors of erythrocyte anion transport (DNDS), glucose transport (cytochalasin B), urea transport (phloretin), Na/K pump (ouabain), K/Cl symport (furosemide), and Na/H exchange (amiloride) did not inhibit P_f significantly, nor did they influence the pCMBS inhibitory potency. Cleavage of sialic acid by neuraminidase did not affect water transport. Incubation of erythrocytes with a series of proteases also had little effect on P_f.

The next group of experiments were carried out in *Xenopus* oocytes microinjected with mRNA from rabbit reticulocytes or with water as control. It was shown previously that P_f of water-injected oocytes was not different from that of oocytes injected with 50 ng of mRNA from brain or muscle, tissues which are not expected to contain water channels (Zhang et al., 1990). Figure 3A shows the time course of oocyte swelling in response to an osmotic gradient (inside 200 mOsm, external 20 mOsm). The rate of swelling was 4–5 times greater in mRNA-injected oocytes than in control oocytes. The increased swelling due to expression of mRNA was inhibited by pCMBS; inhibition by pCMBS was reversed by mercaptoethanol.

To evaluate further whether the increase in oocyte P_f was due to expression of mRNA coding for the erythrocyte water channel, effects of mRNA concentration, temperature, and inhibitors were studied. Figure 3B shows that the rate of oocyte swelling increased with increasing amounts of micro-

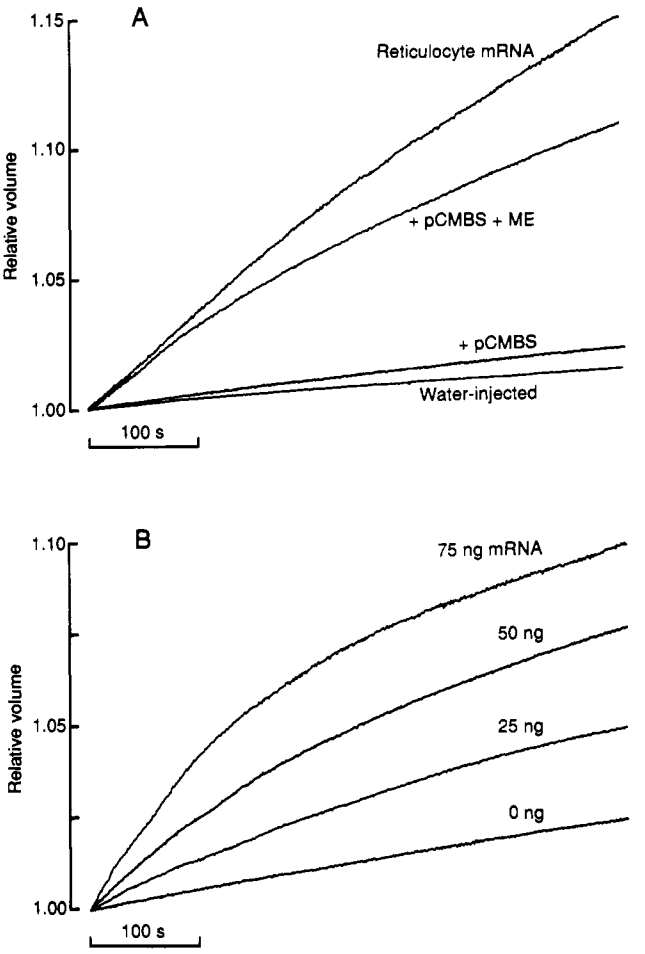

FIGURE 3: Expression of mRNA from rabbit reticulocytes in *Xenopus* oocytes. (A) Time course of oocyte swelling measured at 10 °C when oocytes in Barth's buffer were immersed in a 20-fold dilution of Barth's buffer with water. Oocytes were microinjected with 50 nL of water or unfractionated reticulocyte mRNA (1 mg/mL). Where indicated, the mRNA-injected oocyte was assayed after a 30-min incubation with 1 mM pCMBS or a 30-min incubation with 1 mM pCMBS followed by a 5-min incubation with 5 mM mercaptoethanol. (B) Time course of oocyte swelling in oocytes microinjected with 0, 25, 50, and 75 ng of reticulocyte mRNA.

injected mRNA. The summary of data for a series of oocytes shows a linear dose–response relationship between the amount of microinjected mRNA and oocyte P_f assayed 72 h later (Figure 4A). When assayed within 4 h after microinjection of reticulocyte mRNA (50 ng), P_f did not increase significantly (not shown). Figure 4B shows the time course of pCMBS inhibition of P_f in mRNA-injected oocytes. pCMBS inhibition occurred with a halftime of ~10 min, and inhibition was reversed after 30 min by mercaptoethanol. pCMBS did not alter P_f in water-injected oocytes (not shown). Figure 4C shows an Arrhenius plot for the temperature dependence of P_f in water- and mRNA-injected oocytes. E_a for mRNA-injected oocytes (2.6 kcal/mol) was remarkably lower than E_a for water-injected oocytes (9.6 kcal/mol).

Figure 4D summarizes P_f data for a series of inhibitors in reticulocyte mRNA-injected oocytes. The assay temperature was 10 °C in order to maximize the fraction of total P_f (lipid plus channel-mediated transport) due to the expressed water channel. None of the inhibitors altered P_f significantly in water-injected oocytes (not shown). pCMBS strongly inhibited P_f in mRNA-injected oocytes to a value near that in water-injected oocytes. $HgCl_2$ also inhibited P_f to a value of (6.2 ± 0.8) × 10^{-4} cm/s (n = 9, not shown); however, the $HgCl_2$ effect did not require preincubation. The inhibitors cyto-

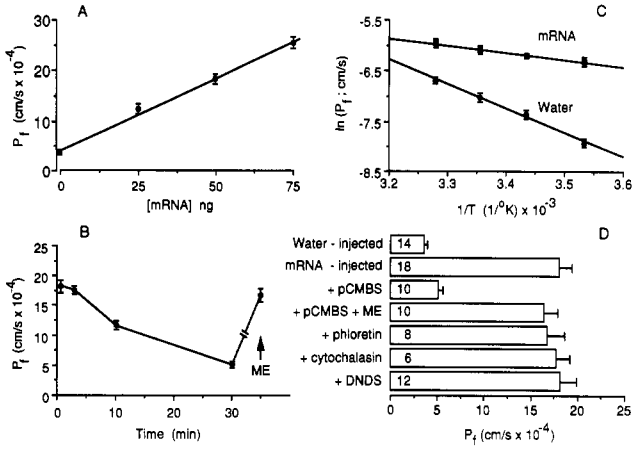

FIGURE 4: Characteristics of water transport in *Xenopus* oocytes microinjected with mRNA from rabbit reticulocytes. (A) mRNA dose–response relation. Mean ± SE of P_f in swelling measurements performed on 8–10 oocytes at each [mRNA]. (B) Inhibition of P_f in mRNA-injected oocytes by incubation for various times with 1 mM pCMBS. Each point is the mean ± SE of six to eight measurements. Where indicated, 5 mM mercaptoethanol was incubated with oocytes for 5 min after the 30-min incubation with pCMBS. (C) Temperature dependence of P_f in oocytes injected with water or 50 ng of reticulocyte mRNA. Each point in the mean ± SE of five to eight measurements. Fitted activation energies are 2.6 ± 0.7 kcal/mol (mRNA injected) and 9.6 ± 0.8 kcal/mol (water injected). (D) Summary of inhibitor effects on P_f for oocytes microinjected with 50 ng of reticulocyte mRNA. All measurements were carried out at 10 °C at 72 h after microinjection. Error bars are SE; the number of oocytes is given in the bars.

chalasin B, phloretin, and DNDS did not inhibit P_f in the mRNA-injected oocytes.

Discussion

The purpose of these studies was to characterize water transport in rabbit erythrocytes for use as a tissue source for water channel identification and cloning. Rabbit erythrocytes and reticulocytes are readily available in large quantities. Compared to human erythrocytes, P_f in rabbit erythrocytes was remarkably higher (0.053 vs 0.02 cm/s), and the percentage inhibition by mercurials was dramatically better (98% vs 90%). The residual water permeability after maximal inhibition by pCMBS was similar for rabbit and human erythrocytes. The differences in channel-mediated water permeability may be related to the number and/or activity of individual water channels. From biophysical calculations based on hydrodynamic equations for water pores (Solomon et al., 1983) or from estimates of single-channel P_f based on gramicidin data (Rosenberg & Finkelstein, 1978), it is estimated that the rabbit erythrocyte should contain a minimum of 5 × 10^5 copies of a water channel. The water channel may thus be a significant membrane protein that is encoded by a significant fraction of reticulocyte mRNA (0.05–0.1%).

Reticulocytes were used to obtain mRNA encoding the erythrocyte water channel because the erythrocyte has little mRNA. Reticulocyte mRNA conferred a large increase in oocyte P_f which was inhibited reversibly by mercurials. An important advantage of expression of reticulocyte rather than kidney water transporters is the likelihood that the expressed reticulocyte mRNA encodes the plasma membrane water transporter. In multiple nephron segments of the kidney, there are water channels on apical and basolateral membranes, and in intracellular vesicles (Handler, 1988; Kuwahara et al., 1988; Verkman, 1989; Verkman et al., 1989). In addition, in the vasopressin-responsive collecting duct, an increase in apical membrane water permeability requires cAMP-dependent cytoskeletal activation for exocytic insertion of water channels. Erythrocyte water transporters are not compartmentalized or subject to complex regulatory mechanisms.

An important purpose of this study was to determine whether the increase in oocyte P_f with microinjection of reticulocyte mRNA represented expression of the erythrocyte water channel or of other protein(s) which might trigger the activation of endogenous oocyte water transporters. The expressed water channel was inhibited reversibly and slowly by pCMBS and rapidly by $HgCl_2$, similar to results in native erythrocytes. In addition, E_a was low, and P_f was not inhibited by cytochalasin B, phloretin, or DNDS. The low E_a cannot be interpreted rigorously in terms of a water channel or pore because of the complexity of the oocyte system. The linearity between oocyte P_f and the amount of injected mRNA suggests that the number of expressed water channels is the rate-limiting determinant of P_f, rather than a saturable intracellular translation/targeting process. Similar linearity between functional expression and [mRNA] has been demonstrated for the Na-independent glucose transporter (Gould & Lienhard, 1989). Linearity between activity and [mRNA] is also consistent with, but does not prove, the hypothesis that the functional subunit structure of the water channel is nonoligomeric; if two identical subunits in a dimer configuration were required for activity and if there were a dynamic equilibrium between monomers and dimers, then activity would increase in proportion to the square of mRNA concentration. Taken together, these results support the conclusion that a proteinaceous erythrocyte water channel has been expressed in oocytes.

P_f in rabbit erythrocytes is considerably higher than that in erythrocytes from human (0.02 cm/s), dog (0.027 cm/s), cat (0.034), cow (0.016 cm/s), and horse (0.012 cm/s) (Rich et al., 1967). Although transport of water in rabbit erythrocytes has not been measured previously, the rabbit erythrocyte has been used as a model system for a number of biochemical and transport studies (Jennings et al., 1986; Poole & Halestrap, 1988; Lawrence et al., 1989). Compared with human erythrocytes, the rabbit erythrocyte has a different pattern of protein glycosylation and remarkably less sialic acid at the external membrane surface (Durocher et al., 1975; Fukuda et al., 1987; Clark et al., 1987). However, the presence of sialic acid has little effect on water transport because removal by neuraminidase did not affect P_f in rabbit and human erythrocytes. The other major biochemical difference between human and rabbit erythrocytes is a 10-fold lesser amount of glycophorin A in rabbit erythrocytes (Fukuda et al., 1987). Comparison of polyacrylamide gels of human and rabbit erythrocyte ghost membranes stained by Coomassie Blue [Ballas (1986) and unpublished observations] reveals small differences in the regions of bands 4.1, 4.2, and 5; however, there is no suggestion about the identity of the water channel.

In summary, the rabbit erythrocyte has exceptionally high water permeability that is inhibited by mercurial sulfhydryl reagents. The properties of the erythrocyte water channel expressed in *Xenopus* oocytes are similar to those in the native erythrocyte, providing an approach for expression cloning.

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Registry No. Water, 7732-18-5.

References

Albritton, E. C. (1982) *Standard Values in Blood*, pp 42–43, W. B. Saunders, Philadelphia, PA.

Ballas, S. K. (1987) *Comp. Biochem. Physiol. 4*, 837-842.
Benga, G., Popescu, O., Borza, V., & Pop, V. I. (1983) *Cell Biol. Int. Rep. 7*, 807-818.
Benga, G., Popescu, O., Borza, V., Pop, V. I., Muresan, A., Mocsy, I., Brain, A., & Wiggleworth, J. M. (1986) *Eur. J. Cell Biol. 41*, 252-262.
Clark, G. F., Krivan, H. C., Wilkins, T. D., & Smith, D. F. (1987) *Arch. Biochem. Biophys. 257*, 217-229.
Dix, J. A., Ausiello, D. A., Jung, C. Y., & Verkman, A. S. (1985) *Biochim. Biophys. Acta 821*, 243-252.
Durocher, J. R., Payne, R. C., & Conrad, M. E. (1975) *Blood 45*, 11-20.
Fettiplace, R., & Haydon, D. A. (1980) *Physiol. Rev. 60*, 510-550.
Fischbarg, J., Kuang, K., Vera, J. C., Arant, S., Silverstein, S. C., Loike, J., & Rosen, M. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3244-3247.
Frohlich, O., Gunn, R. B., Gargus, J. J., & Rizzolo, L. J. (1988) *Biophys. J. 53*, 531a (Abstract).
Fukuda, K., Honma, K., Manabe, H., Utsumi, H., & Hamada, A. (1987) *Biochim. Biophys. Acta 926*, 132-138.
Gould, G. W., & Leinhard, G. E. (1989) *Biochemistry 28*, 9447-9452.
Handler, J. S. (1988) *Am. J. Physiol. 255*, F375-F382.
Jennings, M. L., Douglas, S. M., & McAndrew, P. E. (1986) *Am. J. Physiol. 251*, C32-C40.
Kuwahara, M., Berry, C. A., & Verkman, A. S. (1988) *Biophys. J. 54*, 595-602.
Lawrence, W. D., Schoenl, M., & Davis, P. J. (1989) *J. Biol. Chem. 264*, 4766-4768.
Macey, R. I. (1984) *Am. J. Physiol. 246*, C195-C203.
Mlekoday, H. J., Moore, R., & Levitt, D. G. (1983) *J. Gen. Physiol. 81*, 213-220.
Poole, R. C., & Halestrap, A. P. (1988) *Biochem. J. 254*, 385-390.
Rich, G. T., Sch'afi, R. I., Barton, T. C., & Solomon, A. K. (1967) *J. Gen. Physiol. 50*, 2391-2405.
Rosenberg, P. A., & Finkelstein, A. (1978) *J. Gen. Physiol. 72*, 341-350.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., p 7.19, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Shi, L.-B., & Verkman, A. S. (1989) *J. Gen. Physiol. 94*, 1101-1115.
Solomon, A. K., Chasan, B., Dix, J. A., Lukacovic, M. F., Toon, M. R., & Verkman, A. S. (1984) *Ann. N.Y. Acad. Sci. 414*, 79-124.
Steck, T. L., & Kant, J. A. (1974) *Methods Enzymol. 31*, 172-180.
Verkman, A. S. (1989) *Am. J. Physiol. 257*, C837-C850.
Verkman, A. S., Lencer, W., Brown, D., & Ausiello, D. A. (1988) *Nature 333*, 268-269.
Verkman, A. S., Weyer, P., Ausiello, D. A., & Brown, D. (1989) *J. Biol. Chem. 264*, 20608-20613.
Zhang, R., & Verkman, A. S. (1991) *Am. J. Physiol. 260*, 26-34.
Zhang, R., Logee, K., & Verkman, A. S. (1990) *J. Biol. Chem. 265*, 15375-15378.

Action of Insulin in Rat Adipocytes and Membrane Properties†

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ABSTRACT: Several small peptides inhibit insulin-promoted glucose uptake in rat adipocytes. At 10 μM peptide concentration, the extent of their inhibition of the insulin effect is related to the ability of these peptides to raise the bilayer- to hexagonal-phase transition temperature in model membranes. Hexane and DL-*threo*-dihydrosphingosine lower this phase transition temperature in model membranes, and they promote glucose uptake in adipocytes. There is thus an empirical relationship between the action of membrane additives on glucose uptake in adipocytes and their effect on the hexagonal-phase-forming tendency in model membranes. The most potent of the bilayer-stabilizing peptides tested in this work is carbobenzoxy-D-Phe-L-Phe-Gly. This peptide also inhibits insulin-stimulated protein synthesis in adipocytes. In contrast, DL-*threo*-dihydrosphingosine stimulates protein synthesis. The uptake of [^{125}I]iodoinsulin by adipocytes is inhibited by carbobenzoxy-D-Phe-L-Phe-Gly. The mechanism of action of the bilayer-stabilizing peptides includes inhibition of insulin-dependent protein phosphorylation in adipocytes. The peptides are not specific inhibitors of a single function but are suggested to cause their effects by altering the physical properties of the membrane in a nonspecific manner. These results demonstrate that insulin-dependent functions of rat adipocytes can be modified by membrane additives in a manner predictable from the properties of these additives in model membranes.

Several membrane functions are modulated by the bulk biophysical properties of the membrane (Shinitzky, 1984). There are many such properties that may affect function. Much attention has been given to the modulation of membrane function by changes in the microviscosity or "fluidity" of the membrane. However, other physical properties that may be related to function are the molecular arrangement, stability, and polarity of the membrane–water interface. Subtle changes in these properties, which may have profound effects on function, are difficult to measure directly. However, the bilayer- to hexagonal-phase transition temperature in model

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