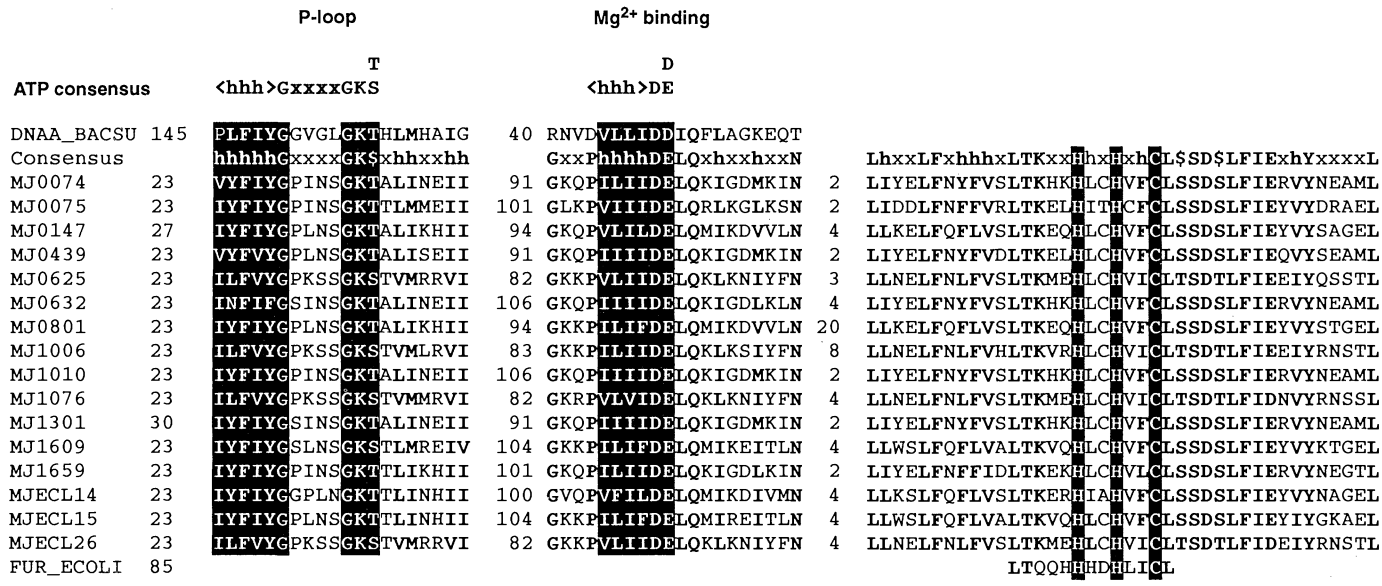

Fig 1. Alignment of the three conserved motifs in the novel family of putative archaeal ATPases. Alignment was constructed using the MACAW program (*11*). Consensus shows amino acid residues conserved in all of the 16 aligned sequences; h indicates a bulky hydrophobic residue (I, L, V, M, F, Y, W); $ indicates serine or threonine. Distances from the protein N-termini and distances between the alignment blocks are indicated by numbers. Fragments of the *Bacillus subtilis* DnaA protein and *Escherichia coli* Fur protein are shown for comparison. Two ATPase motifs and the conserved histidine and cysteine residues in the predicted metal-binding site are shown by reversed type. ATPase motif consensus is from (*4*); <hhh> indicates that three out of five residues preceding the first invariant G in the P-loop and the first D in the Mg^{2+}-binding motif are bulky and hydrophobic. In addition to the proteins shown, open reading frames MJ0819, MJ0820, and MJ0821 appear to represent remnants of a disrupted gene coding for a putative ATPase of the same family.

REFERENCES

1. C. J. Bult *et al.*, *Science* **273**, 1058 (1996).
2. J. E. Walker *et al.*, *EMBO J.* **1**, 945 (1982).
3. M. Saraste *et al.*, *Trends Biochem. Sci.* **15**, 430 (1990).
4. E. V. Koonin, *Nucleic Acids Res.* **21**, 2541 (1993).
5. S. F. Altschul *et al.*, *J. Mol. Biol.* **215**, 403 (1990).
6. S. F. Altschul and W. Gish, *Methods Enzymol.* **266**, 460 (1996).
7. F. Confalonieri and M. Duguet, *Bioessays* **17**, 639 (1995).
8. M. S. Lam *et al.*, *J. Bacteriol.* **176**, 5108 (1994).
9. T. Tomoyasu *et al.*, *EMBO J.* **14**, 2551 (1995).
10. A. R. Mushegian and E. V. Koonin, unpublished observations.
11. G. D. Schuler *et al.*, *Proteins Struct. Funct. Genet.* **9**, 180 (1991).

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Aquaporins and Ion Conductance

A. J. Yool *et al.* studied membrane conductance in *Xenopus laevis* oocytes injected with aquaporin1 complementary RNA (*AQP1* cRNA) and concluded that the conductance observed represented an intrinsic property of the protein (*1*). We have reevaluated our own studies of the AQP1 protein and have conducted new experiments; our results support our earlier conclusion that AQP1 transports water, but does not conduct ions (*2*).

As measured in our laboratory (*2*), oocytes injected with water (as a control) or with *AQP1* cRNA exhibited similar, low conductances, while their permeabilities to water differed greatly [coefficient of osmotic water permeability (P_f)~10 μm/s and ~200 μm/s, respectively]. Because we had not previously investigated effects of forskolin on membrane-conductance, we used a two-electrode voltage clamp. Yool *et al.* (*1*) describe a rising scale of currents (measured in 100 mM NaCl, 2 mM KCl, 4.3 mM $MgCl_2$, and 5 mM Hepes; pH 7.3): water-injected oocytes rose 2.9 μA/V; AQP1 oocytes, 8.6 μA/V; and AQP1 oocytes with forskolin, 63 μA/V. In contrast, using standard conditions (frog Ringers solution: 115 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM Hepes; pH 7.4), we did not see any significant difference in membrane conductance when water-injected oocytes (n = 7) or AQP1 oocytes (n = 20) were compared before or after forskolin treatment (<1 μA/V for all measurements). We obtained similar results when $CaCl_2$ was omitted or when 0.5 mM 4.4′-diisothiocyanato-stilbene-2,2′-disulfonate (DIDS, Sigma, St. Louis, MO) was added to the solutions. Because AQP1 does not contain a classic cyclic AMP (cAMP)–dependent protein kinase A (PKA) phosphorylation consensus site, we performed experiments to evaluate oocytes that express AQP5 (which contains a classic PKA motif), but found that forskolin treatment also did not increase membrane currents of AQP5 oocytes (<1 μA/V in all measurements, n = 16).

The differences in membrane behaviors observed in our laboratories are not a result of the *AQP1* cDNA, because the construct used by Yool *et al.* came from our laboratory. Recently, we provided oocytes injected with *AQP1* cRNA in our laboratory to Yool *et al.*; when they analyzed these oocytes in their laboratory using their technique, forskolin-induced ion currents were observed (*3*). The explanation for this discrepancy is not known.

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REFERENCES

1. A. J. Yool *et al.*, *Science* **273**, 1216 (1996).
2. G. M. Preston *et al.*, *ibid.* **256**, 385 (1992).
3. J. W. Regan, personal communication.

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The description by Yool *et al.* (*1*) of the stimulation of cation permeability in AQP1

by forskolin suggests that aquaporins in general might have an ion channel function, an activity that is unmasked by some stimulation, such as phosphorylation by PKA.

We have observed that PKA directly phosphorylates another aquaporin, AQP2, and stimulates its water channel function by approximately 50% (*2*). We did not, however, examine the ionic conductance of AQP2 after cAMP treatment (*2*), although negligible ionic conductance of AQP2 in the basal condition is well demonstrated. If cAMP evokes ionic (especially cationic) conductance of AQP2, it would be important in the light of physiological data that vasopressin through the cAMP/PKA system stimulates both Na^+ and K^+ conductance across the apical membrane of collecting duct cells (*3*).

We measured ionic conductance of AQP2 expressed in *Xenopus* oocytes before and 15 min after forskolin treatment (10 μM). Our two-electrode voltage clamp studies, performed in isotonic NaCl saline solution without Ca^{2+} (*1*), showed currents from whole oocyte of 210 ± 15 and 221 ± 20 nA (at +60 mV, mean ± SE, n = 5) before and after forskolin treatment, respectively. These values were not significantly different from those of water-injected oocytes. There was no shift in the reversal potentials after the treatment. Thus, in our hands, AQP2 is not an ion channel, even after the forskolin treatment.

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REFERENCES

1. A. J. Yool *et al.*, *Science* **273**, 1216 (1996).
2. M. Kuwahara, *J. Biol. Chem.* **270**, 10384 (1995).
3. J. A. Schafer *et al.*, *Kidney Int.* **41**, 255 (1992).

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We have been unable to confirm the finding of Yool *et al.* (*1*) of cAMP-dependent osmotic water permeability in oocytes that express AQP1 or in erythrocytes that natively express AQP1, nor do we detect cAMP-induced cation leak in erythrocytes.

We microinjected *Xenopus* oocytes according to protocols given by Yool *et al.*, with cRNA (0.1 and 5 ng) that encodes the human and rat isoforms of AQP1. Standard Barth's buffer with 0.78 mM Ca was used. After oocytes were incubated for 24 hours at 18°C, P_f values were (in centimeters per second × 10^{-4}): 6 (water-injected oocytes, as a control), 22 (0.1 ng of human AQP1), 118 (5 ng of human AQP1), 18 (0.1 ng of rat AQP1), and 87 (5 ng of rat AQP1). In oocyte swelling measurements having less than 5% standard error, incubation of oocytes with forskolin (50 μM) for 15 to 30 min at 23°C did not affect permeability. No effect on permeability was found with cAMP-agonists used previously in oocytes [(S)p-cAMPS or chlorophenylthio-cAMP/forskolin/isobutylmethylxanthine mixture (*2*)], with a cell-permeable cAMP-antagonist [(R)p-cAMPS], or with a protein kinase C agonist (phorbol myristate acetate). In these experiments we used three separate batches of oocytes, and measurements were made on six to nine oocytes for each protocol in each batch.

Because oocyte expression studies can be subject to variability between laboratories, measurements were also done on human erythrocytes and inside-out erythrocyte vesicles (IOVs). All solutions contained 0.8 mM Ca. Stopped-flow light scattering measurement of erythrocyte shrinkage in response to a 50 mM NaCl gradient gave a P_f value of 0.018 cm/s at 23°C. With less than a 5% standard error, erythrocyte permeability was not affected by incubation (15 to 60 min) with the cAMP agonists and antagonists listed above. Further, permeability in IOVs was not affected by in vitro phosphorylation with MgATP (2 mM) and the catalytic subunit of protein kinase A (100 U, 23°C, 60 min). Finally, erythrocyte cation (KCl) leak was measured from the kinetics of shrinkage after replacing extracellular NaCl by isosmolar sucrose. Minimal leak (<1% per minute) was found with or without forskolin. Although our data do not provide an explanation for the findings of Yool *et al.*, we are concerned that oocyte viability and membrane permeabilities might be perturbed by high expression of foreign membrane proteins and modulation of membrane trafficking by cAMP.

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REFERENCES

1. A. J. Yool *et al.*, *Science* **273**, 1216 (1996).
2. H. Hasegawa *et al.*, *ibid.* **258**, 1277 (1992).

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We have not been able to detect cation conductance in AQP0 or AQP1 when it is expressed in *Xenopus* oocytes, but neither had we tested it in the presence of forskolin or cAMP. Therefore, we felt the need to repeat the work of Yool *et al.* (*1*). We isolated *Xenopus* oocytes and injected each with 10 ng cRNA that encodes human AQP1. After 2 days of incubation at 18°C in modified Barth's solution [0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 1 mM Hepes (pH = 7.5), and 0.82 mM $MgSO_4$], we tested six oocytes with a standard swelling assay. All six revealed a high osmotic water permeability, P_f (mean value 274 ± 43 μm/s) as opposed to that of water-injected oocytes serving as controls (10 ± 3 μm/s). Forskolin (10 μM) and 3-isobutyl-1-methylxanthine (IBMX, Sigma, St. Louis, MO) (1 mM) did not influence the permeability.

The same day, 10 AQP1-expressing oocytes were analyzed with the two-electrode voltage clamp technique, with isosmotic NaCl saline, under conditions described by Yool *et al.* (*1*). The mean membrane potential was −30.8 ± 5.7 mV. The basal unstimulated conductance, obtained from application of voltage ramps, ranging from −100 mV to +50 mV in 2.0 s, was identical for oocytes that express AQP1 and for water-injected oocytes serving as controls (1.75 ± 0.38 μS and 1.88 ± 0.50 μS, respectively; n=10). Incubation with forskolin and IBMX did not induce an increase in conductance (1.90 ± 0.47 μS). Our conclusion is that cAMP does not activate a cation conductance in AQP1.

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REFERENCES

1. A. J. Yool *et al.*, *Science* **273**, 1216 (1996).

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Yool *et al.* (*1*) describe increases in osmotic permeability and conductance of oocytes that express AQP1 after exposure to forskolin or cAMP. We sought to reproduce the increases in permeability and injected *Xenopus laevis* oocytes with 1.25 ng of cRNA that encodes AQP1. Oocytes were then incubated for 4 days at 18°C in ND96 medium (*1*).

We preincubated oocytes for 20 min with forskolin and then measured permeability in comparison with untreated (unincubated) oocytes. Permeability was determined (*2*) by challenging the cells with ND96 hypotonic saline diluted from 198 to 100 mOsm. The permeability increase of treated as compared with untreated oocytes was significant (p = 0.0004): P_f (mean + SEM) = 559 ± 16 μm (n = 7) and 420 ± 25 μm (n = 5), respectively.

We performed the experiment again with a second batch of oocytes. This time the permeability differences were not significant: P_f (mean + SEM) = 330 ± 25 μm (n = 8) and 309 ± 19 μm (n = 5), treated to untreated oocytes, respectively.

In the same two batches, water-injected oocytes (n = 5), otherwise similarly treated, serving as a control, did not show significant increases. Our data suggest that, while forskolin can increase the permeability of AQP1, variability among oocyte batches could make detection of such an effect problematic.

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REFERENCES

1. A. J. Yool *et al.*, *Science* **273**, 1216 (1996).
2. J. Fischbarg *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 3244 (1990).

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We have carried out experiments similar to those of Yool *et al.* (*1*) with AQP1 cloned from ocular ciliary epithelium as part of our work on aquaporins. We microinjected 2 ng of bovine *AQP1* cRNA into *Xenopus* oocytes to study the forskolin stimulation of water permeability of AQP1. Oocytes were incubated in 200 mOsm modified Barth's buffer at 18°C for 72 hours, after which they were transferred to 70 mOsm Barth's buffer diluted with distilled water, and the time course of osmotic volume increase was monitored at 20°C. Osmotic water permeability (P_f) was calculated as previously described (*2*). The forskolin effect was examined by incubating oocytes in Barth's buffer containing 10 μM concentrations of the reagent for 15 min before P_f was measured. We performed these experiments using three different oocyte batches. The values of P_f at 20°C (mean ± SE) were 42 ± 5 μm/s (n = 8), 38 ± 5 μm/s (n = 7), and 39 ± 4 μm/s (n = 8) for untreated AQP1 injected oocytes; 69 ± μm/s (n = 10), 66 ± 5 μm/s (n = 9), and 41 ± 5 μm/s (n = 10) for forskolin-stimulated oocytes; and 4 ± 1 μm/s (n = 7), 5 ± 2 μm/s (n = 8), and 6 ± 1 μm/s (n = 8) for water injected oocytes. Our data suggest that stimulation of AQP1 activity for forskolin (seen in two out of three experiments) may vary with the oocyte batch.

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REFERENCES AND NOTES

1. A. J. Yool *et al.*, *Science* **273**, 1216 (1996).
2. G. M. Preston *et al.*, *ibid.* **256**, 385 (1992).
3. Supported by the National Eye Institute, grant EY10423.

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Response: As detailed in our report (*1*), increases in cationic conductance in *Xenopus* oocytes injected with *AQP1* cRNA were obtained not only with forskolin but with cyclic 8-bromo-adenosine 3′,5′-monophosphate and by direct injection of the catalytic subunit of protein kinase A (PKA). More recently, we have coexpressed AQP1 with an adenylyl cyclase–stimulatory receptor and have activated cationic conductance by stimulation of the receptor with its endogenous ligand (*2*). As noted by Agre *et al.*, we have recorded forskolin-activated currents in eggs that were sent to us after being injected in his laboratory. This does not mean that all AQP1-injected oocytes show forskolin-activated currents. In our hands, approximately 15% of the oocytes injected with AQP1 do not show currents. However, these same oocytes usually fail to swell and burst after being placed in water, which suggests problems with oocyte viability, or the expression of AQP1, or both. We have not found this forskolin-activated cationic conductance in any oocytes that were injected with water (as controls).

After discussions with Agre *et al.*, a simple explanation for our different results has not emerged; an unresolved issue is the criteria used to select oocytes for recordings. For example, Agre *et al.* record from oocytes with resting membrane potentials that are below (more negative than) −35 mV; we use a cutoff of −20 mV. Our data show that for AQP1-injected oocytes in which forskolin-activated currents were obtained, only 30% had resting membrane potentials below −35 mV (the remainder being in the range of −20 to −35 mV). By contrast, for AQP1-injected oocytes in which currents were not obtained, and for water-injected cells (controls), nearly 80% had resting membrane potentials below −35 mV. This suggests that in oocytes that express AQP1, there is a slight depolarization of the resting membrane potential, which is consistent with the higher initial conductances in AQP1-injected oocytes as compared with water-injected cells (3 to 9 versus 1 to 4 μA/V, respectively) and a reversal potential near −20 mV (*1*). Therefore, the selection of oocytes with resting membrane potentials below −35 mV may favor oocytes that do not express AQP1.

That aquaporin2 (AQP2), the vasopressin-sensitive water channel, does not show a forskolin-activated cationic conductance (see the comment of Sasaki *et al.*) bears further evidence that the individual members of this family of proteins can differ. For example, among the aquaporins, which all show relatively high permeability to water, only aquaporin3 is permeable to glycerol (*3*). The major intrinsic protein of lens (MIP), a founding member of this family (*4*), has extensive homology with the aquaporins but is significantly less permeable to water (*5*). Furthermore, in reconstituted lipid bilayers, MIP shows a voltage-dependent anion conductance (*6*); a property shared with nodulin-26, another member of the MIP family found in the nitrogen-fixing root nodules of plants (*7*). Nodulin-26, however, is not permeable to water nor is the glycerol facilitator (glpF), a MIP family member found in *Escherichia coli* (*8*). Amino acid similarities have been identified between members of the MIP family of proteins and the transmembrane domains of voltage-dependent K^+ channels (*9*), which raises the possibility that these two families of proteins are related and may underlie a structural basis for the formation of a channel.

Intra- and interlaboratory variability exists with respect to the effects of forskolin on the osmotic water permeability of AQP1. Conflicting results have also been described for AQP2, which may reflect the state of the art. Thus, it was initially found (*10*) that forskolin could directly increase the permeability of AQP2 in *Xenopus* oocytes that express these channels, but studies with rat papillary endosomes attribute this increase solely to the translocation of AQP2 to the plasma membrane and not to a direct effect on the channel (*11*). On the other hand, the results obtained for α-TIP (*12*), an aquaporin found in seeds, have not been disputed. In these studies the P_f values of oocytes expressing α-TIP increased 80 to 100% after stimulation of cAMP formation by forskolin. It was also found that the increase in water permeability involved the phosphorylation of α-TIP by PKA at three potential sites, two of which are similar to a site present in the carboxyl terminus of AQP1. Some might regard the aquaporins as purely constitutive channels, but our data for AQP1 and those for AQP2 and α-TIP say otherwise. More work will be needed to settle this issue, to determine whether these effects occur in vivo, and to see whether they contribute to the physiology of water and ion transport.

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REFERENCES

1. A. J. Yool *et al.*, *Science* **273**, 1216 (1996).
2. A. J. Yool and J. W. Regan, unpublished results.
3. K. Ishibashi *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 6269 (1994).
4. A. B. Chepelinsky, in *Handbook of Membrane Channels* (Academic Press, New York, 1994), pp. 413–432.
5. S. M. Mulders *et al.*, *J. Biol. Chem.* **270**, 9010 (1995).
6. G. R. Ehring *et al.*, *J. Gen. Physiol.* **96**, 631 (1990).
7. C. D. Weaver *et al.*, *J. Biol. Chem.* **269**, 17858 (1994).
8. C. Maurel *et al.*, *EMBO J.* **12**, 2241 (1993).
9. J. Fischbarg, personal communication.
10. M. Kuwahara *et al.*, *J. Biol. Chem.* **270**, 10384 (1995).
11. M. B. Lande *et al.*, *ibid.* **271**, 5552 (1996).
12. C. Maurel *et al.*, *EMBO J.* **14**, 3028 (1995).

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