The Combinatorial Nature of Osmosensing in Fishes

Organisms exposed to altered salinity must be able to perceive osmolality change because metabolism has evolved to function optimally at specific intracellular ionic strength and composition. Such osmosensing comprises a complex physiological process involving many elements at organismal and cellular levels of organization. Input from numerous osmosensors is integrated to encode magnitude, direction, and ionic basis of osmolality change. This combinatorial nature of osmosensing is discussed with emphasis on fishes.

For fishes and other aquatic animals, environmental osmolality is equal to the salinity (the concentration of inorganic ions) of their aquatic habitat. Estuaries, the intertidal zone, and desert lakes all represent habitats in which salinity (and accordingly osmolality) fluctuates greatly over different temporal scales (daily, tidally, and seasonally). An increase in water salinity causes hyperosmotic stress because it leads to passive water loss from fish. Thus fish become dehydrated not unlike terrestrial animals that become dehydrated due to evaporative and excretory water loss. Tissue dehydration also occurs in ectothermic polar animals as a result of freezing at subzero environmental temperatures (effectively leading to aqueous solvent removal due to ice formation). These animals have evolved mechanisms of freeze avoidance and freeze tolerance to cope with adverse effects of severe dehydration (24). Conversely to hyperosmotic stress, hypo-osmotic stress occurs when environmental salinity decreases and fish gain water passively and, thus, become overly hydrated.

Because animal cell membranes are semipermeable (i.e., having high water but low ion permeability), a change in hydration state of fish is reflected in an altered cellular hydration state. A deviation of cellular hydration state from the optimal set point interferes with macromolecular and cellular structure and function. The reason for this deleterious effect of osmotic stress is that cell metabolism and biochemical properties of macromolecules have been selected for functioning optimally at a specific and highly stable intracellular ionic milieu (195, 196). This internal milieu presumably reflects the conditions under which cells, the smallest units of life, have evolved.

Fishes are able to perceive and compensate for changes in environmental salinity of their aquatic habitat. From a mechanistic perspective, they utilize 1) osmosensors for perceiving osmotic stress, 2) osmosensory signaling mechanisms for transducing information about osmotic stress, and 3) osmoregulatory effector mechanisms for alleviating osmotic stress (57). Osmosensing is the physiological process of perceiving a change in environmental osmolality. In this review, the term osmosensor refers to molecules that 1) directly sense changes in osmolality and 2) generate an intracellular signal that contributes to the alleviation of osmotic stress. In contrast, the term osmoreceptor is used for cells that are unique to osmoregulating animals (fishes and other vertebrates) and are capable of producing a specific para-, neuro-, or endocrine signal that contributes to the maintenance of body water and fluid homeostasis. The concept of a combinatorial nature of osmosensing that is developed in this review refers to the multitude of sensory elements involved in the perception of environmental osmolality, their different effective operating ranges, and the convergence of specific combinations of signals emanating from those osmosensors. Such combinatorial osmosensing allows for finely tuned, qualitatively and quantitatively appropriate responses to changes in environmental osmolality.

What osmosensors do fishes utilize and how do they work? This question is not easy to answer. The problem is not the lack of knowledge of potential osmosensors, but, on the contrary, there are too many proteins (and other macromolecules) that fulfill the criteria for an osmosensor. As outlined in what follows, essentially every protein is affected in its structure/function by osmotic stress (169), and a large number of intracellular signaling pathways are involved in controlling osmoregulatory effector mechanisms (47, 48, 57, 101, 102). What sets them apart is their relative sensitivity toward osmotic stress and, hence, their effective operating range (see below). Therefore, I had previously proposed that animal cells integrate input from multiple osmosensors with different sensitivity ranges to accurately quantify environmental osmolality and osmotic stress (97). Here, I will review the available evidence supporting such a combinatorial nature.
of osmosensing and what we know about some of the key elements that are involved in the osmosensing network. Although the emphasis will be on fishes, the discussion presented here has broader applicability to other organisms as well, and some supporting evidence is borrowed from organisms other than fish if necessary to illustrate the general concept.

**Evolutionary Driving Forces for Combinatorial Osmosensing**

Bacteria use simple two-component systems consisting of histidine kinases and a transcriptional response regulator for osmosensing (133). Orthologous histidine kinases are also used as osmosensors by some unicellular eukaryotes such as slime molds, yeast, and some plants (143, 158, 182). However, the genomes of fishes and other animals do not contain homologous proteins. During the evolution of metazoans, alternative mechanisms of osmosensing have been selected, which include a much larger number of proteins than osmosensory bacterial two-component systems. As organismal complexity and the number of differentiated cell types with specialized functions within a single organism increases, so does the complexity of combinatorial logic underlying intracellular signaling networks, including those that are involved in osmosensing. There are several strong evolutionary selection pressures that drive this phenomenon. First, the number of protein-coding genes in simple vs. highly complex organisms does not increase proportionally to the number of differentiated cell types and states nor to the number of specialized physiological functions. Thus the available genomic space for supporting increased cellular and tissue specialization and differentiation is rather limited (89). Second, cells and cellular organelles such as nuclei have an optimal size and volume, and their capacity for accommodating a finite number of macromolecules (chiefly proteins and nucleic acids) and micromolecules (metabolites, electrolytes, etc.) is limited (63). Third, the risk of system failure increases with the number of elements (e.g., proteins) constituting the system if each protein has only one specialized function and each function is only mediated by one specific protein. These selection pressures have resulted in the re-utilization of available proteins for novel functions and increased redundancy and robustness of signaling networks and other biological processes (64). Much innovation with regard to physiological mechanisms appears to have evolved via novel combinations and compartmentation of protein/macromolecular interactions rather than invention of novel genes/proteins. Large multi-protein complexes are common in higher organisms, and how individual proteins function often depends on the temporal-spatial dynamics of their interactions with other macromolecules and their compartmentation, which, in turn, is governed by environmental and developmental cues (32, 130, 156, 167, 183). This combinatorial nature of macromolecular dynamics in cells has profound physiological implications, for instance for the regulation of transcriptional networks, neural circuits, and developmental pattern formation (70, 72, 163).

A combinatorial nature of osmosensing is suggested by the ubiquitous effects of osmotic stress on many (signaling and other) proteins. Moreover, fishes and other osmoregulators combine osmotic stress signals that are generated in multiple tissues to coordinate and integrate mechanisms for maintaining osmotic homeostasis of their extracellular body fluid (ECF). In addition, differential sensitivity of osmoreactive proteins allows for fine-grained physiological adjustments to be mounted to exactly match environmental osmolarity changes in quantity and quality. I will discuss some specific examples of fish osmosensors that have different sensitivity ranges after briefly reviewing the general principles of osmosensing.

**Principles of Cellular Osmosensing**

**The Threat of Osmotic Stress and Its Effects on Animal Cells**

The solute composition of cells has been subject to stringent selection during evolution, and responses to any form of osmotic stress (dessication, salinity fluctuations, freezing) are evolutionarily highly conserved (169, 196). Osmotic stress (i.e., water stress) is caused by changes in intracellular water activity (solvent capacity), which can be due to I) changes in water (solvent) content or 2) changes in electrolyte (charged solute) content. Both of these scenarios alter the rates of biochemical reactions because the activities of macromolecules are exquisitely sensitive to water activity and depend on the dynamics of the hydrogen bonding network of water (51, 63).

Cells of fishes and other animals are enclosed by semi-permeable membranes but lack cell walls and thus are unable to maintain a turgor pressure or osmotic difference between extracellular and intracellular fluid. However, intracellular solute composition is very different from extracellular solute composition, with high intracellular K+ being a key variable for physiological homeostasis (169, 196). Cells rigorously defend the intracellular inorganic ion composition and overall electrolyte concentration during osmotic stress to maintain proper metabolism and function. From an evolutionary perspective, mechanisms for maintaining an intracellular milieu that is compatible with cell
metabolism during osmotic stress have been subject to strong selection pressure. Homeostasis of the intracellular milieu is absolutely essential for 1) adequate macromolecular structure and function (stability) and 2) adequate macromolecular “regulatory responsiveness” (flexibility) (169). In particular, proteins are highly responsive to changes in water activity by changing their structure, interactions, posttranslational states, and/or compartmentation, all of which are properties that are critical for intracellular signal transduction.

Acute osmotic stress brought about by impermeable solutes (i.e., hyper-/hypotonicity) causes osmosis and changes cell volume because of the semipermeable nature of the plasma membrane. Hypertonicity and hypotonicity do not perturb only cell volume but also intracellular inorganic ion concentration. In the case of hypertonicity, cells passively lose water due to osmosis, and the intracellular osmolality equilibrates with extracellular osmolality. It is important to remember here that animal cells are not able to maintain a constant intracellular osmolality setpoint. Any change in extracellular osmolality is accompanied by a shift in intracellular osmolality to a new setpoint (hyperosmolality prevails) rather than reestablishment of osmotic homeostasis. This circumstance may have favored the evolution of mechanisms for maintaining osmotic homeostasis in extracellular body fluids (ECF) of osmoregulators. In these highly evolved animals, including bony fishes and all mammals, the ECF effectively dampens environmental osmolality fluctuations. Hypertonicity not only resets the intracellular osmolality but also causes cell shrinkage (volume stress) and crowding of macromolecules and micromolecules (metabolites and inorganic ions) (FIGURE 1, scenario A). Unlike alterations in the osmotic setpoint, cells cannot tolerate permanent changes of cell volume or intracellular inorganic ion strength. Therefore, a two-phase adaptive response is mounted consisting of 1) cell volume regulation and 2) replacement of excessive inorganic ions by compatible organic osmolytes (FIGURE 1). Regulatory volume increase (RVI) is achieved by active uptake of inorganic ions, which are then replaced by either compatible or counteracting osmolytes. Compatible osmolytes are small uncharged or zwitter-ionic organic metabolites that, unlike inorganic ions, have no net charge and do not alter macromolecular structure

**FIGURE 1. Effects of hyperosmotic stress on animal cells**

A: acute, severe hyperosmotic stress caused by membrane-impermeable solutes leads to hypertonicity, cell shrinkage (volume stress), and macromolecular crowding. Hypertonic cell volume stress is relieved by regulatory volume increase resulting from active transport of inorganic ions. Subsequently, inorganic ion stress is relieved by accumulation of compatible or counteracting osmolytes. B: less severe, gradual osmotic stress caused by increased extracellular inorganic ion concentration can be isovolumetric, i.e., have no noticeable effect on cell volume. In this scenario, inorganic ion stress still needs to be relieved by compatible or counteracting osmolyte accumulation. C: less severe, gradual osmotic stress caused by increased extracellular compatible or counteracting osmolytes is isionic when these osmolytes permeate cell membranes with similar efficiency as water. In this scenario, no volume or inorganic ion stress is imposed, but overall molecular density is still increased and water activity (solvent capacity) is decreased. The dissimilar consequences of different types of osmotic stress provide a way for sensory perception of its nature. For hypo-osmotic stress, similar scenarios are applicable, except that the events proceed in the opposite direction. Yellow hexagons are macromolecules; red pentagons are inorganic ions; green dots are water molecules; blue rectangles are compatible/counteracting osmolytes.
and function over a very wide concentration range (196). Counteracting osmolytes are pairs of organic osmolytes whose effect on macromolecular structure and function cancels each other out over a wide range of concentrations and at a given ratio. An example for a pair of counteracting osmolytes is the combination of urea and trimethyl-amine oxide (TMAO), whose strong individual effects on macromolecules cancel each other out at a ratio of ~2:1 (196).

Less acute and more moderate hyperosmotic stress can be isovolumetric (non-hypertonic), not causing a change in cell volume or macromolecular crowding (FIGURE 1, scenario B). Isovolumetric regulation in various cell types depends on the rate and magnitude of osmolality change (25, 116, 151, 155, 170). Because in fishes and other osmoregulators the ECF greatly dampens environmental salinity fluctuations (with regard to both acuteness and magnitude), isovolumetric regulation may be more common for most fish tissues in vivo than hypertonicity. Possible exceptions include epithelial cells that are directly exposed to external salinity, e.g., gill epithelial cells and epithelial cells lining the GI tract. During isovolumetric regulation, the signals generated by volume changes and macromolecular crowding are absent, and only intracellular inorganic ion strength has to be restored to the homeostatic setpoint.

A third possibility for responding to osmotic stress is by isoionic regulation, which could occur in circumstances of mild and gradual osmotic stress when intracellular compatible or counteracting osmolyte concentrations can be adjusted very rapidly to offset water movement by osmosis (FIGURE 1, scenario C). For instance, urea and glycerol are organic osmolytes that cross plasma membranes with similar ease as water. These organic osmolytes and water have long been assumed to freely permeate cell membranes, but it is questionable whether this assumption holds true under all circumstances. If it did, then the presence of aquaporins and urea transporters in cell membranes would seem counterintuitive. In any case, isoionic regulation is similar to isovolumetric regulation except that compatible or counteracting osmolytes are utilized right away to close the osmotic gap instead of first relying on inorganic electrolytes. Isoionic regulation requires compatible osmolytes to be rapidly adjustable, e.g., during hypo-osmotic stress via rapid osmolyte release or during hyperosmotic stress via rapid uptake (e.g., uptake of urea from elasmobranch ECF or mammalian renal medullary interstitium, or glycerol uptake from teleost ECF). Even though no volume stress, macromolecular crowding, or inorganic ion stress occurs during isoionic regulation, water activity (solvent capacity) and overall molecular density (micromolecules + macromolecules) are still altered in this scenario.

From this brief survey of osmotic effects on animal cells, we can see that direct consequences of osmotic stress differ depending on the direction (hyper- vs. hypo-osmolality), acuteness, magnitude, and the ionic nature of the osmolality change. In addition, the state of the cell and its microenvironment (e.g., how readily compatible osmolytes can be recruited to close the osmotic gap) modulates osmotic stress effects. Differences in these parameters are utilized for the generation of intracellular signals, the specific combination of which encodes the quantity and quality of osmotic stress to produce appropriate physiological responses.

Molecular Osmosensors

Molecular osmosensors are directly altered by at least one of the consequences of osmotic stress outlined above. In response to such alteration, they produce a signal that contributes to the regulation of osmoregulatory effector mechanisms. In fishes (and other highly complex animals), signals that emanate from numerous osmosensors appear to be logically integrated in a combinatorial fashion that allows highly specific outcomes despite the lack of exclusively specific osmosensors (95). Osmosensors include enzymes whose activity is stringently correlated with intracellular electrolyte concentration, proteins that are regulated by ion concentration or osmotically altered membrane properties, proteins associated with cytoskeletal organization, ion channels, and proteins that monitor the degree of macromolecular (protein and DNA) damage (FIGURE 2).

Osmolality changes directly affect the reaction rates of enzymes by altering their catalytic efficiency ($k_{cat}$) and Michaelis-Menten constant ($K_m$) (169). The osmolality threshold at which such effects manifest themselves is enzyme specific. Theoretically, any protein kinase, protein phosphatase, or other intracellular signaling protein could contribute to osmosensing via direct osmotic effects on their catalytic rates and enzymic efficiency. It is, therefore, not surprising that many signaling proteins such as numerous protein kinases and phosphatases have been implicated in intracellular osmosensing. Since most intracellular signaling may be mediated via the dynamic assembly and disassembly of multimeric protein complexes, the ubiquitous osmotic effects outlined above are of fundamental importance (see The Role of Protein Phosphorylation for Osmosensing below). They could explain why other sensory modalities, e.g., conspecific recognition, are altered when fishes are faced with environmental salinity change (75). Nonetheless, convergence of a multitude of intracellular signaling pathways...
into unique combinations could produce stressor-specific output even if each pathway is also involved in numerous other processes.

The formation of multi-protein complexes depends on electrostatic interactions that are highly sensitive to water activity and intracellular inorganic ion strength (169). For instance, the self-assembly of major cytoskeletal proteins (actin, tubulin) is sensitive to intracellular inorganic ion concentration (150, 177). Cytoskeleton strain has been proposed to be one of the main inputs into combinatorial osmosensing, but, based on the evidence outlined above, it is conceivable that cytoskeletal effects of osmotic stress also manifest themselves in the absence of changes in cell volume or shape (95, 102). The actin-based cytoskeleton has been shown to participate in the salinity-dependent regulation of Na\(^+/K^+\)/2Cl\(^-\) (NKCC) cotransporters (114). Furthermore, changes in ion transport across intestinal epithelium of euryhaline eels require intact F-actin and microtubules (113), and the open/closed state of apical crypts of gill chloride cells also depends on the actin cytoskeleton (26). In addition, osmotic stress-induced actin remodeling invokes changes in the morphology of tight junctions. For instance, in primary cultures of trout gill cells, tight-junction adjustments to osmotic stress require claudin 28b and the supporting F-actin ring (162).

Acute and severe osmotic stress triggers changes in cell volume and membrane organization. Changes in membrane organization, including lipid packing density and membrane tension, are important parameters that regulate the activity of membrane-associated proteins. An example is phospholipase A2 (PLA\(_2\)), which is activated by membrane lipid rearrangement (109). PLA\(_2\) catalyzes the hydrolysis of membrane glycerophospholipids, resulting in liberation of arachidonic acid (AA), an important signaling molecule in cells (82, 142). One function of AA is to activate AA-regulated Ca\(^{2+}\) selective (ARC) channels in the plasma membrane to increase intracellular

**FIGURE 2.** Molecular osmosensors that contribute to the perception of osmotic stress in fish cells

A few intracellular messenger compounds and a few effector mechanisms are depicted, but no detail of the osmotic stress signaling network, which is still insufficiently understood, is shown. Please refer to the text for a discussion of the role of each of the depicted elements in fish osmosensing. SHR, steroid hormone receptor; ARC, arachidonic acid-regulated Ca\(^{2+}\) selective channels; ANXA11, annexin A11; AA, arachidonic acid; PLA2, phospholipase A2; CaSR, calcium sensing receptor; AC, adenylate cyclase; AQP, aquaporins; TRPV4, transient receptor potential vanilloid 4 cation channel; CR, cytokine receptor.
calcium concentration (FIGURE 2), which is a common response of cells exposed to acute and severe osmotic stress (168).

The Role of Inorganic Ions for Osmosensing

Multiple mechanisms for sensing concentrations of inorganic ions and intracellular ionic strength have evolved (95, 102). Because life originated in the primordial ocean, evolution has favored mechanisms for monitoring concentrations of Na\(^+\), K\(^+\), Ca\(^2+\), and Mg\(^2+\), which constitute the most prevalent cations in seawater and cells, having strong effects on macromolecular function (131, 169). Transient receptor potential (TRP) cation channels have been shown to sense Na\(^+\), Ca\(^2+\), and osmolality in fish and other animals (8, 17, 19, 66, 110, 127, 154, 194, 199). Several isoforms of TRP channels, including osmosensory TRP vanilloid 4 (TRPV4), are expressed in many zebrafish tissues (122, 134). TRPV4 is also expressed and salinity-regulated in gill epithelial cells of euryhaline sea bass (8). The osmosensory function of TRPV4 is very highly evolutionarily conserved as evidenced by functional complementation of nematode OSM-9 mutants (which lack a functional TRPV type channel) with mammalian TRPV4 (111). TRPV channels are highly sensitive to changes in osmolality and inorganic ions, and, like ARC channels (see above), their activation modulates intracellular Ca\(^2+\) (110).

Calcium signaling pathways and intracellular calcium concentration play a central role for controlling osmosensory signal transduction pathways in fishes (FIGURE 2). For instance, hyposmotic stimulation of prolactin secretion by rostral pars distalis cells isolated from tilapia pituitary gland (hypophysis) requires changes in intracellular calcium levels (166). In addition, the osmoregulatory hormone cortisol inhibits prolactin secretion via reduction of free intracellular calcium (78), whereas angiotensin II, another osmoregulatory hormone, increases free intracellular calcium in fish tissues (161). A key role of intracellular Ca\(^{2+}\) for fish osmosensory signal transduction independently emerged when modeling a signaling network based on 20 immediate early genes that are rapidly induced during salinity stress in tilapia gill (55).

Extracellular Ca\(^{2+}\) concentration also plays a critical role for osmosensing by directly modulating the activity of a transmembrane glycoprotein, the calcium sensing receptor (CaSR) (FIGURE 2). First cloned in the Hebert Lab in 1994, this protein is important for Ca\(^{2+}\) homeostasis and osmoregulation in fishes and other vertebrates (1, 13, 74, 117). In a plasma-hyperosmotic environment, the effective Ca\(^{2+}\) concentration for CaSR regulation corresponds to the range found in the external milieu, and this protein has been proposed to be a salinity sensor in fish (145). CaSR is expressed in osmoregulatory tissues of diverse fishes, including elasmobranchs and teleosts (53, 54, 62, 83, 84, 118). In fish gills, CaSR has been localized to mitochondria-rich cells (chloride cells) (117, 145). In fish pituitary gland, CaSR has been localized to the rostral pars distalis and the pars intermedia (118). Pituitary CaSR mRNA abundance decreases rapidly (by 50% within hours) in tilapia exposed to hyperosmotic stress, indicating that it is subject to feedback regulation during osmotic stress (184). Such feedback regulation of CaSR mRNA abundance was not observed in flounder gill and corpuscles of Stannius (11, 71). However, CaSR undergoes a shift in molecular mass when flounder is exposed to osmotic stress, suggesting possible posttranslational modification, which is consistent with a proximal osmosensory role of this protein (71). CaSR has been shown to activate downstream signaling pathways in euryhaline tilapia in response to salinity change, including phospholipase C-dependent and mitogen-activated protein kinase (MAPK)-dependent signaling pathways (119). Because CaSR is a trimeric G-protein-coupled receptor that acts through modulation of adenyl cyclase activity, its activation also affects intracellular cAMP levels (31) (FIGURE 2). A role of adenyl cyclase in osmosensing of fishes is supported by electrophysiological data on isolated opercular membranes of euryhaline killifish. Using these preparations, it has been demonstrated that forskolin (an adenyl cyclase agonist) modulates transepithelial chloride secretion (128). In addition, forskolin also markedly alters osmoregulatory hormone/cytokine secretion from tilapia pituitary gland (50).

The Control of Fish Spermatocyte Motility by Inorganic Ions

In fish species with external fertilization, spermatocyte motility is controlled primarily by osmolality and the intra- and extracellular concentration of cations (2, 3, 23, 175). The molecular regulation of motility/flagellar activity of fish spermatocytes promises to be a valuable simple model for deciphering the logic of osmosensory signal transduction networks in highly complex animals because the combination of sensors, signals, and effectors involved in this system appears more tractable under physiological conditions than for other models. In marine teleosts, spermatocyte motility increases rapidly (within seconds) after release into a plasma-hyperosmotic environment (i.e., seawater). In FW teleosts, spermatocyte motility increases with comparable rapidity when released into a plasma-hypoosmotic environment (FW) (175, 192). This difference in osmotic effects on fish spermatocyte motility is clearly a result of adaptation to living in marine vs. limnic environments since the selection pressure on spermatocyte motility is very strong.
Such evolutionary (genetic) adaptation illustrates that the “interpretation” of osmotic stress can be adjusted to generate a specific set of intracellular signals for activation of appropriate effector mechanisms. Of particular interest in this regard are euryhaline fishes that are capable of reproducing in both marine and FW environments, e.g., tilapia and sticklebacks (41, 138). These strongly euryhaline fish species employ physiological acclimation mechanisms for changing the “interpretation” of osmotic stress signals by spermatocytes. In such species, osmosensory signal transduction is apparently reset to switch the trigger for initiation of spermatocyte motility from hypo- to hyperosmolality after acclimation of fish to a plasma-hypersomatic environment (and vice versa). Not much is known about the mechanisms associated with such resetting, which seems to include differential phosphorylation of antioxidant proteins (138).

In addition to osmolality, fish spermatocyte motility strongly depends on extracellular Ca\(^{2+}\) (106, 140). The effect of extracellular Ca\(^{2+}\) on spermatocyte motility varies somewhat between different species of fish and is pH dependent (4, 139, 148). Uncoupling extracellular Ca\(^{2+}\) from osmolality has a strong effect on spermatocyte motility in tilapia, independent of which salinity the animals had been acclimated to (112). These phenomena suggest that osmosensory input via CaSR may be critical for resetting the osmotic stress signaling network during salinity acclimation of those euryhaline fish that can reproduce in marine habitat as well as in FW. Interestingly, osmotic stimulation of spermatocyte motility and flagellar beating is mediated via intracellular Ca\(^{2+}\) signaling in both marine and FW fish (3, 12). In euryhaline tilapia, intracellular Ca\(^{2+}\) of spermatocytes increases during both hypotonic and hypertonic stress, but the Ca\(^{2+}\) source for this increase is different. During hypotonic stress, intracellular Ca\(^{2+}\) store depletion occurs, whereas during hypertonic stress extracellular Ca\(^{2+}\) is taken up (140, 141). In common carp, however, extracellular Ca\(^{2+}\) uptake may also play a role during hypotonicity (91). In puffe rfish, it has been demonstrated that an increase in Ca\(^{2+}\)/calmodulin interaction is required for triggering spermatocyte motility (92). These data strongly reinforce the notion that Ca\(^{2+}\) is of central importance for osmosensing (see *The Role of Inorganic Ions for Osmosensing* above).

In addition to Ca\(^{2+}\), fish spermatocyte motility also depends on extra- and intracellular K\(^{+}\) (and to a lesser extent Na\(^{+}\) and possibly Cl\(^{-}\)) in FW and marine fishes (2, 3, 33, 136, 137, 174, 175). Thus inorganic ion concentrations represent critical signals for osmosensory signal transduction in fish spermatocytes. These signals mediate differential phosphorylation of flagellar proteins during osmotic activation of spermatocytes in marine (140, 200, 202) and FW teleosts (141). In marine species, hypertonicity-induced spermatocyte shrinkage is intensified by aquaporins (201) and results in the activation of adenyl cyclase and cAMP signaling (200) even though cAMP is not necessary for initiation of spermatocyte motility in all species (159). Alternative pathways (possibly with redundant functionality to compensate for lack of cAMP signaling under certain conditions) may be involved in some species.

### The Role of Protein Phosphorylation for Osmosensing

Reversible phosphorylation/dephosphorylation of proteins represents a major mechanism of intracellular signal transduction, which dynamically alters the composition and localization of protein complexes and controls cell function and physiological responsiveness via adjustment of effector protein abundance and activity (18, 171, 185). Any search/screen for protein kinases and phosphatases involved in osmosensing is bound to be provocative, not because none can be identified but, on the contrary, because too many of them are affected by one form of osmotic stress or another (see general consideration of osmotic effects on proteins discussed above). To illustrate the physiological significance of reversible, dynamic protein phosphorylation for osmosensory signal transduction in fishes, I will now highlight some selected examples of kinases, phosphatases, and protein phosphorylation events (and the corresponding osmoregulatory effectors) involved in this process.

A possible link between cytoskeletal proteins that may sense osmotic stress (see discussion above) and osmoregulatory effectors is focal adhesion kinase (FAK), which is an enzyme that responds to altered cytoskeleton dynamics by promoting stabilization of the cytoskeleton (5, 49, 120, 121, 188). In gill and opercular epithelia of euryhaline killfish exposed to hypotonicity, FAK is dephosphorylated in an integrin-dependent manner (125). Such FAK dephosphorylation has been shown to alter the activity of the Na\(^{+}/K\(^{+}\)/2Cl\(^{-}\) (NKCC) cotransporter and the cystic fibrosis transmembrane conductance regulator (CFTR) Cl\(^{-}\) channel in these tissues in response to salinity change (124, 126). In primary cultures of Japanese eel gill cells, hypertonic induction of the Na\(^{+}/Cl\(^{-}\)/taurine co-transporter, which represents an important effector of osmotic stress signaling pathways, depends on myosin light chain kinase (MLCK) (21). In this tissue, MLCK-dependent phosphorylation of a major cytoskeletal building block that associates with tight junctions (MLC) is accompanied by redistribution of F-actin to the cell periphery (21).
Another link between osmotic effects on cytoskeletal proteins and phosphorylation-based signal transduction is apparent for mitogen-activated protein kinase (MAPK) cascades. All three major MAPKs, in particular the stress-activated protein kinases (SAPKs) p38 and jun-N-terminal kinase (JNK), are activated by small GTPases of the Rho family that are linked to the cytoskeleton (187). Even though MAPKs are highly evolutionarily conserved and utilized for osmosensory signal transduction in all eukaryotic cells (98, 101), the regulation of MAPKs by activators and inhibitors and MAPK substrates are far less conserved between diverse groups of animals and other eukaryotes (57). MAPK cascades seem to have evolved as a major cell signaling node capable of integrating diverse combinations of inputs to produce relevant signaling output. We have shown that the activity of p38, JNK, and extracellular signal-regulated (ERK) MAPK cascades is rapidly altered in gills of euryhaline killifish after exposure to salinity stress in vivo (100). Osmotic regulation of p38 and JNK phosphorylation was also observed in killifish isolated opercular epithelium. In such in vitro preparations, chloride secretion is inhibited by a pharmacological p38 blocker (107). In addition, regulatory volume decrease of isolated hepatocytes from turbot (Scophthalmus maximus) depends on p38 MAPK (132). Furthermore, an upstream regulator of MAPK cascades, mitogen-activated protein kinase kinase kinase 7 interacting protein 2 (TAK 1 binding protein 2 = TAB 2) is induced as an immediate early gene during hyperosmotic stress in tilapia gill epithelium (46). TAB2 induction is very rapid (within 2 h at the mRNA level) and transient, which is consistent with a role in osmotic stress signaling. In euryhaline medaka, activation of the JNK pathway is required for osmotic induction of the osmotic stress transcription factor 1b (OSTF1) (160). OSTF1 has been discovered after a suppression subtractive hybridization screen for salinity-induced genes in Mozambique tilapia gill epithelium (58). This protein is a homolog of mammalian transforming growth factor β (TGF-β)-stimulated clone 22 3b (TSC22–3b) (56, 59). A role for OSTF1 in osmotic stress signaling also has been demonstrated for other euryhaline fishes, including black porgy (20), Japanese eel (22, 179), Nile tilapia (12), and medaka (180). A role of OSTF1 in osmosensory signal transduction is not limited to fishes but is also evident in mammalian cells, where we identified TSC22D2 as an OSTF1 ortholog that is activated and alternatively spliced in response to hypertonicity with similar kinetics as in fish gill cells (59). Overexpression of mammalian TSC22D2 increases osmotolerance of murine inner medullary collecting duct (mIMCD3) cells, suggesting that its function during osmotic stress is evolutionarily conserved in vertebrates (59).

The Role of Macromolecular Crowding and Damage for Osmosensing

Macromolecular crowding (or dilution) and macromolecular damage are direct physical consequences of salinity stress that contribute to the combinatorial signaling logic of osmosensing. These effects are mostly encountered during severe/acute osmotic stress (FIGURE 1). Changes in macromolecular density are intimately linked to cell volume changes. Experiments with primary fish cells isolated from a wide variety of tissues have shown an abundance of molecular responses to osmotically induced changes in cell volume, and the corresponding literature is too extensive to be reviewed here (6, 16, 40, 73, 88, 107, 108, 178, 193). Some of these studies imply that membrane stretch is a trigger for osmosensory signal transduction. However, it seems unlikely that changes in cell volume lead to overall changes in membrane tension such as stretch because most cells (in particular epithelial cells) are characterized by basolateral and apical plasma membrane infoldings (at least in vivo). It is more likely that cell volume changes are sensed via changes in macromolecular density in general or in certain localized areas (such as specialized membrane lipid rafts). Isolated fish cells respond to anisotonic osmotic stress by activating a multitude of diverse signaling pathways and adaptive effector mechanisms for counteracting osmotic stress-induced volume changes (6, 16, 88, 108, 178).

Macromolecular damage resulting from perturbed macromolecular density and intracellular ionic strength represents an important signal for perception of osmotic stress (35–37, 93, 94, 96, 103, 105). Thus mechanisms for protein, DNA, and RNA stabilization are activated during osmotic stress in fish. HuR is an mRNA stabilizing protein that is induced during hyperosmotic stress in tilapia gill epithelium (55). It is possible that HuR relays information about mRNA stability during hyperosmolality to the osmosensory signal transduction network. Another protein that is rapidly upregulated in tilapia gill epithelium during hyperosmotic stress is a ubiquitin E3 ligase (a Grail/Goliath homolog) (55). Tilapia Grail was localized to the tips of secondary lamellae in fish gill epithelium and shown to increase osmotic stress tolerance of HEK293 cells when overexpressed in this human cell line (60). A similar ubiquitin E3 ligase (an Rbx1 homolog) is also induced by hyperosmotic salinity stress in salmon (153). Ubiquitin E3 ligases may sense protein damage by quantifying the amount of substrates and/or integrating signals transduced via diverse proteins that are being tagged with
ubiquitin (123). In most cases, such protein substrates are terminally damaged and destined for proteolytic degradation and removal, but specific signaling proteins also transduce information via reversible mono-ubiquitination (15, 85, 189). Some targets of ubiquitin E3 ligases are osmoregulatory effector proteins. For instance, in mammalian kidneys, the interaction of Nedd4 E3 ubiquitin ligase with epithelial Na⁺ channels (ENaC) is controlled by osmolality, vasopressin and 14-3-3 proteins (81). Whether these osmoregulatory effectors are also targets of the osmotic stress-induced ubiquitin E3 ligases in fish remains to be determined. Ubiquitin-mediated protein degradation prevents nonspecific aggregation of terminally damaged proteins, i.e., proteins with exposed hydrophobic residues that cannot be repaired/correctly refolded. Molecular chaperones (including heat shock proteins) are utilized and induced during many forms of environmental stress to either direct such proteins toward proteolytic degradation or, alternatively, to refold them into their native state to prevent nonspecific protein aggregation (52, 77, 152, 160). Many examples (too many to be mentioned here) for molecular chaperone activation in fish challenged by osmotic stress have been documented (28–30, 99, 197). The latter function of molecular chaperones resembles that of compatible osmolytes in the context of osmotic stress illustrating that macromolecules and micro-molecules act in concert to maintain optimal protein conformation during osmotic stress. Notably, molecular chaperones themselves are subject to modulation by osmolytes (172). Because unfolding and degradation of specific signaling proteins during osmotic stress will alter intracellular signal transduction, it is conceivable that cells monitor how much overall protein damage is accumulated during osmotic stress via the activity of molecular chaperones. A mechanism for such monitoring and induction of appropriate responses is exemplified by transcriptional regulation via heat shock factors (e.g., HSF1) and the regulation of these proteins via sequestration by molecular chaperones (157, 186). HSF sequestration is in competition with binding of other substrates (i.e., unfolded proteins) to molecular chaperones. As a result of such competition, the transcriptional activity of HSF represents a readout of the amount of protein damage in cells. Similar regulatory circuits operate to sense and quantify cellular DNA damage (115, 129). Macromolecular damage quantitation may not be stressor-specific (unless certain types of damage are associated with only one specific form of stress), but it contains information about the severity of stress. Combined with signals from other osmosensors, macromolecular damage sensors contribute to encode an overall integrated signal that allows for a qualitatively and quantitatively appropriate and specific response at the cellular level.

### Osmosensing by Multiple Tissues

In multicellular animals, the combinatorial nature of osmosensing is compounded at higher levels of organization. In particular osmoregulators have multiple osmosensory tissues with specialized osmoreceptor cells that monitor ECF osmolality. Some osmosensory tissues are also target tissues for systemic osmoregulation (e.g., epithelia that are directly exposed to the external environment) (FIGURE 3). Although osmotic stress has consequences for all cell types, their sensitivity toward osmotic stress and capacity for generating systemic signals in response to osmotic stimulation differs (e.g., specialized osmoreceptor cells are highly sensitive, whereas other cell types are less sensitive). The combination of signals generated in multiple tissues by osmoreceptor cells and other osmosensory cell types (e.g., certain epithelial cells) and their relative strengths provides a means for integrating and coordinating osmotic stress responses at the whole organism level.

#### Cerebral, Hypophyseal, and Systemic Osmoreceptor Cells

Most fishes are osmoregulators that maintain osmotic homeostasis of their ECF constant even when environmental salinity/osmolality is altered. Evolution of this physiological mechanism/trait in ancestral bony fishes was an important prerequisite for tetrapods to become independent of aquatic habitats and conquer land. During exposure of euryhaline fish to severe osmotic stress (e.g., transfer from FW to SW or vice versa), the internal osmolality setpoint of ~300 mosmol/kg can deviate temporarily by as much as ±100 mosmol/kg (34, 86, 104, 184). Smaller environmental salinity changes lead to proportionally smaller changes in ECF osmolality and to shorter periods of deviation of ECF osmolality from the optimal setpoint. Because the constancy of the “milieu interieur” is pivotal and tightly regulated in all vertebrates, any deviations from the homeostatic optimum trigger compensatory responses that are aimed at restoring osmotic homeostasis of ECF. Special osmoreceptor cells have evolved in fishes to monitor body fluid and electrolyte homeostasis and provide sensitive regulatory feedback upon deviation from the optimal setpoint. Their function illustrates the importance of sensory feedback as a general principle for maintaining homeostasis of physiological systems (147). These specialized osmoreceptor cells are superior over other cell types in converting osmotic signals into specific physiological outcomes. In the brain, such osmoreceptor
cells are neurons whose action potentials, firing rate, and neurotransmitter secretion are tightly controlled by ECF osmolality (10). In mammals, these neurons are localized to distinct circumventricular brain areas including the organum vasculosum laminae terminalis (OVLT), subfornical organ, area postrema, and posterior pituitary (9). In fishes, the OVLT is present and well developed but has not been studied with regard to its role for osmosensing (65). However, the area postrema and subfornical organ are known to participate in the control of osmoregulatory actions of atrial natriuretic peptide and renin-angiotensin hormone systems in euryhaline eels (146, 176, 181). Thus it appears that osmoreceptor cells in the brain are conserved in all vertebrate classes, consistent with the conserved physiological capacity for maintaining a constant ECF osmolality.

The posterior pituitary (i.e., neurohypophysis derived from neural ectoderm) representing an extension of the brain containing a collection of hypothalamic neuronal projections harbors mammalian osmoreceptor cells (9). Of interest, however, in fishes, the most prominent hypophyseal osmoreceptor cells have been identified in the adenohypophysis, specifically in the pro-adenohypophysis (rostral pars distalis), which is derived from oral ectoderm (7, 43, 69, 149). Of all the brain structures mentioned above, the role of the hypophyseal rostral pars distalis for osmosensing in fishes is best understood. This discrete tissue consists to a large degree of prolactin-producing cells, which can be isolated and cultured in vitro (42). Experiments on such isolated cells provided proof that they are functioning as osmoreceptors. Prolactin cells are capable of sensing subtle extracellular osmolality changes in the range of plasma osmolality variation experienced in teleosts exposed to salinity stress (69). These cells swell in response to hypo-osmotic stress-induced decrease of ECF osmolality. Prolactin cell swelling, in turn, triggers release of prolactin, which is an important hormone/cytokine for coordinating acclimatory responses to plasma-hypo-osmotic environments (191). The swelling-induced prolactin release is mediated by stretch-activated ion channels, aquaporin 3, and the second messengers Ca²⁺ and cAMP (164–166, 190).

Endothelial ECF volume-/pressure-sensing neurons are located in the vasculature of gill arches in several teleost and elasmobranch species (14). These cells correspond to the mammalian baroreceptor cells that are located in the carotid and aortic arches, which are evolutionarily derived from fish gill arches (132). In mammals, these baroreceptors contribute (at least in the short term) to body fluid osmotic and volume homeostasis by signaling to osmoregulatory effector tissues that control blood pressure via adjustment of water intake and urinary excretion (27, 68, 90). Coordination of neurotransmitter signals from cerebral, hypophyseal, and systemic osmoreceptor neurons contributes to synchronizing the whole organism physiological response to salinity stress in fishes. Another important element for coordination of whole organism responses are osmoregulatory hormones/cytokines.

**Osmosensing by Osmoregulatory Epithelia**

Epithelial cells directly facing the external milieu such as gill epithelial cells and cells lining the gastrointestinal tract including the esophagus are...
prime candidates for sensing changes in environmental salinity and evoking rapid anticipatory responses even before disruption of physiological homeostasis. In fishes, neuroepithelial cells are embedded in the gill epithelium (39), and some of these cells are in direct apical contact with the external environment (198). Neuroepithelial cells are known to produce and respond to cytokines (144, 173), and they were suggested to have local, paracrine functions in euryhaline fishes (67, 135). Osmoregulatory cytokines that are synthesized locally in fish gills include endothelins and urotensins (46, 79). Nevertheless, the paradigm for osmosensing in vertebrates emphasizes brain and systemic osmoreceptor neurons discussed in the preceding paragraph. These neurons convert an osmotic stimulus into an electrical signal that can be transduced along axons and then converted into a neurotransmitter response at efferent nerve endings (9). However, (at least some) osmotic stress induced re-differentiation and re-programming of mitochondria-rich cells in tilapia gill and yolk sac epithelia, and primary cultures occur independent of systemic (neuroendocrine) signals (56, 76, 87). Experiments on isolated gill cells of euryhaline fish have shown that they are capable of responding directly to osmotic stress by activating genes involved in cytokine production, including c-fos and osmotic stress transcription factor 1 (OSTF1) (56, 99). Moreover, cytokines such as interleukin 8 (IL-8) and tumor necrosis factor α (TNF-α) are part of the osmotic stress signaling network in tilapia gill epithelium (55). In addition, proteome analysis of euryhaline leopard sharks suggested that TNF-α is an important element in the osmotic stress response network of gill and rectal gland epithelium (38). Therefore, cytokines that act as para-, auto-, and/or endocrine messengers represent an additional avenue for transducing osmotic signals within and between cells and tissues (95).

The term “cytokine” is used as a common denominator of a diverse array of soluble proteins and peptides that regulate cell and tissue functions at low concentrations (80). Important cytokines in fishes include known osmoregulatory growth factors and peptide hormones (e.g., growth hormone, insulin-like growth factor 1, atrial natriuretic peptide, and prolactin). TNF-α and ILs are cytokines that can act in autocrine and paracrine fashions and, therefore, reinforce and coordinate epithelial responses to salinity change (FIGURE 4).

Another group of cytokines that are involved in fish osmosensing are bone morphogenetic proteins (BMPs). A BMP receptor (BMPR2) increases rapidly during hyperosmotic stress in tilapia gill

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**FIGURE 4. Integration of auto-, para-, and endocrine cytokine and hormone signals with osmosensing in gill epithelial cells during osmotic stress**

Osmotic stress responses of different cell types are unequal because they have different molecular phenotypes (e.g., they express distinct quantities of cytokine receptors and osmosensors). Distinct responses of different cell types underlie extensive remodeling of gill epithelium in euryhaline teleosts exposed to a reversal of the osmotic gradient between extracellular fluid and the environment. For clarity, general impacts of cytokines and hormones are only indicated for a single mitochondria-rich cell. The same principle applies to the other cells, but the corresponding relationships are not depicted. Shown are pavement cells (green), mitochondria-rich (chloride) cells (orange), neuroepithelial cells (blue), and undifferentiated progenitor cells (white).
epithelium (55). In zebrafish gills and yolk sac epithelium, BMPs control the balance between Delta-Notch signaling and, correspondingly, the activity of the transcription factors glial cell missing 2 (Gcm2) and Foxi3a (44, 45). When Notch function is impaired by mutation, Foxi3a expression is increased, and differentiation of precursor cells is shifted toward generation of H^+ATPase-rich mitochondria-rich cells (45). Conversely, overexpression of Notch drives precursor cell differentiation toward the keratinocyte cell fate (45). Collectively, these findings indicate that BMP cytokines contribute to remodeling of gill epithelium via mitochondria-rich cell fate determination by Delta-Notch and Foxi3a signaling pathways, even though the source of BMP (auto-, para-, or endocrine) is currently unknown (FIGURE 4).

Responses of osmoregulatory target tissues to systemic or local extracellular signals are likely “primed” by the internal state of cells in those tissues. For instance, cytokine responsiveness is determined by the presence of corresponding receptors in target cells and thus can be modulated via regulation of receptor abundance (see BMPR2 above). Another mechanism for such modulation is the expression of decoy cytokine receptors. An example is the osmotic induction of a short splice variant of tilapia prolactin receptor 2 that lacks most of its extracellular prolactin binding domain (61) (FIGURE 5). Differential responsiveness of cells to extracellular signals such as cytokines alters the integration of these signals into intracellular osmosensory signaling networks and contributes to the generation of cell type-specific outcomes that result from osmotic stress.

Conclusions

Changes in environmental osmolality have profound effects on macromolecules, cells, and organisms that need to be compensated for to maintain adequate physiological functionality at all levels of organization. In fishes and other vertebrates, perception of altered environmental osmolality (osmosensing) is a highly complex physiological process that involves many elements at multiple levels of organization. To coordinate osmoregulatory responses of multiple tissues and cells and to mount appropriate effector mechanisms that match the magnitude, ionic nature, and other aspects of osmotic stress, many signals (none of which may be stressor specific) are integrated in a combinatorial manner. In theory, such combinatorial osmosensing allows for fine-grained transduction of information about the specific nature of osmotic stress, which enables activation of appropriate effector mechanisms. In praxis, we do not (yet) know the code that governs how multifaceted osmosensory input is integrated in signal transduction networks to yield specific qualitatively and quantitatively accurate outcomes. To decipher this code, we need to know and understand in detail the behavior of

A: in plasma hypo-osmotic environments, functional (long) forms of both PRLRs are expressed. These receptors homo- or heterodimerize and effectively bind PRL, which is present in high concentration in plasma. PRL binding activates PRLR dimers and induces the Jak2/Stat5 pathway, among other pathways. B: when fish are transferred from a plasma hypo- to a plasma hyperosmotic environment, then alternative splicing leads to a rapid increase in the short form of PRLR2, which lacks a large part of the extracellular domain. The short form sequesters functional (long form) PRLRs via heterodimer formation, which prevents these dimers from binding PRL. Thus gill epithelial cells are rapidly rendered less responsive to circulating PRL to suppress this cytokine’s stimulating effect on active NaCl absorption and to allow gill epithelial cells to rapidly switch to active NaCl secretion.
the key elements that comprise the osmosensory signaling network, not just in any given cell but also at the whole organism level. Based on our current knowledge, it appears that calcium is the most critical intracellular messenger for transduction information about osmotic stress in fish. But there are many additional elements, which have been briefly outlined in this essay, that contribute to osmosensory signaling. Defining the role and relative importance for osmosensing of each of these elements in various physiological contexts and cell types represents a formidable challenge.

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