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Proteomics and AQP2 Regulation

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Abstract

The advent of modern quantitative protein mass spectrometry techniques around the turn of the 21st century has contributed to a revolution in biology referred to as ‘systems biology”. These methods allow identification and quantification of thousands of proteins in a biological specimen, as well as detection and quantification of post-translational protein modifications including phosphorylation. Here, we discuss these methodologies and show how they can be applied to understand the effects of the peptide hormone vasopressin to regulate the molecular water channel aquaporin-2 (AQP2). The emerging picture provides a detailed framework for understanding the molecular mechanisms involved in water balance disorders.

Keywords

collecting duct; kidney; protein kinase A; AQP2; protein mass spectrometry; next-generation DNA sequencing

Index words:

LC-MS/MS; proteomics; phosphoproteomics; aquaporin-2; vasopressin

I. Background and Introduction

The peptide hormone arginine vasopressin (AVP) regulates renal water excretion principally through the control of the osmotic water permeability of collecting duct cells. When circulating AVP levels increase, the osmotic water permeability increases, allowing water movement across the collecting duct epithelium from the tubule lumen to the blood. AVP does this by binding to the V2 vasopressin receptor (Gene symbol: *Avpr2*), a G-protein coupled receptor (GPCR) that signals chiefly through $G_{\alpha}s$ and activation of adenylyl cyclase 6 (*Adcy6*) to produce cyclic AMP (cAMP). V2 receptor activation also mobilizes

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intracellular calcium (Star *et al.*, 1988), which is seen as calcium pulses that increase in frequency with vasopressin (Yip, 2002; Pisitkun *et al.*, 2008). However, vasopressin does not do this by increasing inositol 1,4,5-trisphosphate (IP_3) levels or activating protein kinase C (PKC) in collecting duct cells (Chou *et al.*, 1998) as is commonly seen with GPCR coupling to the G-protein alpha subunit $G_{q/11}$. Instead, the calcium mobilization appears to be a result of PKA-mediated phosphorylation of IP_3 receptors that lowers the threshold for calcium-stimulated calcium release in the endoplasmic reticulum (Section VI below). Although other cAMP effectors are present in collecting duct cells, experiments involving CRISPR deletion of both protein kinase A (PKA) catalytic genes (*Prkaca* and *Prkacb*) in mouse mpkCCD cells have revealed that vasopressin effects in collecting duct cells are almost completely dependent on PKA activation (Isobe *et al.*, 2017; Datta *et al.*, 2020), consistent with the conventional view in the renal vasopressin field (Jung & Kwon, 2016; Ranieri *et al.*, 2019; Salhadar *et al.*, 2020). Given the essential role of PKA in V2 receptor-mediated signaling in renal collecting duct principal cells, we focus here on cellular effects of PKA activation in the regulation of osmotic water permeability. The downstream molecular target of vasopressin signaling is the water channel protein, aquaporin-2 (AQP2).

II. The AQP2 Water Channel

Vasopressin controls collecting duct water permeability chiefly through regulation of the molecular water channel aquaporin-2 (AQP2, gene symbol: *Aqp2*) (Nielsen *et al.*, 2002). In collecting duct cells, AQP2 is responsible for water movement across the apical plasma membrane, whilst two other water channels, aquaporin-3 (AQP3) and aquaporin-4 (AQP4), mediate water movement across the basolateral plasma membrane (Nielsen *et al.*, 2002). Generally, water permeation across the apical plasma membrane is rate-limiting for transepithelial water transport, allowing control of AQP2 to regulate transepithelial water permeability.

Studies in rodents have revealed two main modes of vasopressin-mediated regulation of AQP2: *short-term regulation* occurring over a time period of a few minutes and *long-term regulation* occurring over a time period of hours to days. The short-term regulation of AQP2 is the result of membrane trafficking of AQP2-containing vesicles to and from the apical plasma membrane (Nielsen *et al.*, 1995). The net translocation of AQP2 to the apical plasma membrane occurs as a result of two actions, namely increased exocytic insertion into the apical plasma membrane and decreased endocytic internalization, removing AQP2 from the apical plasma membrane (Nielsen & Knepper, 1993; Brown, 2003). The ability of vasopressin to affect AQP2 trafficking is believed to be mediated in part by changes in AQP2 phosphorylation at four sites in the cytosolic COOH-terminal tail (Nishimoto *et al.*, 1999; Hoffert *et al.*, 2006). Vasopressin massively increases AQP2 phosphorylation at Ser269, which is thought to be responsible for inhibition of AQP2 endocytosis (Hoffert *et al.*, 2008; Moeller *et al.*, 2010), increasing osmotic water permeability. Exocytosis of AQP2-containing vesicles requires phosphorylation at a different site, namely Ser256 (Fushimi *et al.*, 1997; Katsura *et al.*, 1997; van Balkom *et al.*, 2002). Phosphorylation of AQP2 at Ser256 has been shown to be necessary for phosphorylation at Ser269 and therefore it appears to be important for regulation of both endocytosis and exocytosis of AQP2 (Hoffert *et al.*, 2008). In contrast, vasopressin *decreases* phosphorylation at Ser261 by decreasing

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the activities of ERK1/2 and p38 kinase (Nedvetsky *et al.*, 2010; Rinschen *et al.*, 2010). Decreased phosphorylation of AQP2 at Ser261 appears to increase the stability of the AQP2 protein (Nedvetsky *et al.*, 2010) but does not appear to affect AQP2 trafficking (Lu *et al.*, 2008).

In addition to the short-term action of vasopressin to regulate AQP2 trafficking, vasopressin exerts a long-term action over hours to days associated with an increase in the total amount of the AQP2 protein in collecting duct cells, amplifying the short-term response (DiGiovanni *et al.*, 1994; Kishore *et al.*, 1996). Direct measurements in isolated perfused collecting ducts established that the vasopressin-stimulated increase in AQP2 protein abundance are paralleled by an increase in the osmotic water permeability of the collecting ducts (DiGiovanni *et al.*, 1994).

III. The Systems Biology Concept

We focus the remainder of this review on the question, "*What are the molecular mechanisms by which vasopressin, acting through the V2 receptor and PKA activation, alters AQP2 trafficking and increases AQP2 protein abundance in renal collecting duct cells?*'. The question posed is complex and is perhaps best addressed by discovery approaches that comprise the field of *systems biology*.

Systems biology can be defined as "*The study of a biological process in which all of the relevant components are studied together in parallel to discover mechanism* (Knepper, 2015)". Systems biology is often contrasted with reductionist scientific approaches that resolve problems into their elements, which are then studied independently. Much of the foundation for our understanding of biological mechanisms comes from reductionist studies. However, with the success of the various genome projects at the beginning of this century, providing nearly comprehensive mapping of genes and their nucleotide base sequences, systems level ("-Omics") have become feasible, facilitating large-scale discovery of genes and proteins involved in biological processes. Systems biology methods can sometimes identify so-called '*emergent properties'* of complex systems that are not recognizable through reductionist studies of individual proteins. An example is the role of partial epithelial-to-mesenchymal transition as a mechanism for loss of AQP2 expression with sustained vasopressin secretion in the syndrome of inappropriate antidiuresis (SIAD) discovered via transcriptomics (Lee *et al.*, 2018).

Among the most important of the so-called '-Omic' methodologies used in systems biology investigations is 'proteomics', employing protein mass spectrometry (LC-MS/MS). In the following, we describe common methods in quantitative proteomics and illustrate the use of these methods in the study of vasopressin-mediated regulation of AQP2 in the renal collecting duct.

IV. Quantitative Proteomics: Proteome-Wide Response to Vasopressin

Protein mass spectrometry has undergone rapid improvements in the past decade allowing tens of thousands of proteins to be identified and quantified in individual samples (Rinschen *et al.*, 2018). Most often, bottom-up strategies are employed, whereby proteins are digested

with proteases, usually trypsin, to generate short peptides that can be individually sequenced and quantified. There are various strategies for quantification, one of which is "SILAC" (Stable Isotope Labeling by Amino acids in Cell culture) (Figure 1A) (Ong *et al.*, 2002). With this method, cells are grown alternatively in medium containing the amino acids lysine and arginine with C-12 and N-14 (light amino acids) or with lysine and arginine with C-12 replaced by C-13 and N-14 replaced by N-15 (heavy amino acids). This allows two differently treated samples (e.g., vasopressin vs vehicle) to be mixed and read out in a single LC-MS/MS run distinguishing tryptic peptides from the two samples based on their difference in mass. SILAC was employed for proteome-wide quantification of the long-term response to the V2 receptor-selective agonist dDAVP in vasopressin-responsive mouse mpkCCD cells (Figure 1B) (Khositseth *et al.*, 2011). These results confirmed the prior observations by immunoblotting (DiGiovanni *et al.*, 1994; Hasler *et al.*, 2002) that vasopressin V2 receptor occupation is associated with a large increase in the abundance of the AQP2 protein in collecting duct cells (>20-fold in these studies). The advance realized from proteomic profiling compared with the prior reductionist studies is recognition of the high degree of selectivity of the response. Only a few proteins among many thousands of proteins quantified showed significant increases in abundance in response to dDAVP and none of these responses rivaled AQP2 in magnitude.

V. Dynamic SILAC: Vasopressin Effects on Protein Half Lives and Translation Rates

The large increase in AQP2 protein abundance in response to vasopressin could theoretically be due to an increase in AQP2 protein half-life or to an increase in AQP2 protein production by translation. Both responses were measured using a modification of the SILAC method called *dynamic SILAC* (Figure 2A,B) (Sandoval *et al.*, 2013). With this method, the light amino acids lysine and arginine are replaced in the cell culture medium at time zero with heavy versions of lysine and arginine (or vice versa) and the light-to-heavy ratio is measured at different times to measure protein turnover. If steady state conditions are maintained throughout the experiment for variables other than the masses of arginines and lysines, both protein half-life and translation rate can be ascertained in thousands of proteins simultaneously. Notably, unlike conventional measurements of protein half-life that requires pharmacological blockade of translation (typically with cycloheximide), dynamic SILAC does not require this intervention. In mpkCCD cells, half-life was measured for 4403 proteins, providing a substantial resource that is useful to understanding regulatory processes in epithelia (https://esbl.nhlbi.nih.gov/Databases/ProteinHalfLives/). Of special note are transcription factor proteins. Some groups of transcription factors have exceptionally low half-lives (less than10 hours versus the global median half-life of 31 hours), including Hox proteins, AP-1 components, nuclear receptors, and some subclasses of zinc finger proteins. The low half-lives of these transcription factors are necessary for rapid transcriptional regulation (*vide infra*). Only a handful of proteins underwent significant changes in response to dDAVP including AQP2, which showed an increase in half-life from 9.8±1.3 to14.2±1.1 hours with dDAVP exposure (Sandoval *et al.*, 2013). Thus, vasopressin increases AQP2 protein stability in collecting duct cells, a finding consistent with observations from reductionist studies (Nedvetsky *et al.*, 2010; Moeller *et al.*, 2016; Centrone *et al.*, 2017).

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However, the demonstrated 45 percent increase in AQP2 half-life falls far short as a potential explanation for the 20-fold increase in AQP2 protein abundance shown in Figure 1B. Indeed, the AQP2 translation rate measured in the same study was increased by more than 10-fold (Figure 2C). Thus, the increase in AQP2 protein abundance in response to vasopressin is largely dependent on an increase in AQP2 translation. The study also demonstrated changes in translation rates for several other proteins with potential roles in vasopressin action, including multiple small GTP-binding proteins and proteins associated with glutathione metabolism (Figure 2C).

The vasopressin-induced increase in AQP2 protein synthesis (increased AQP2 translation) in response to vasopressin could be due either to increased AQP2 mRNA abundance or to selective regulation of AQP2 translation. To determine the effects of vasopressin (dDAVP) on levels of all expressed mRNAs, RNA-seq was performed in cultured mouse mpkCCD cells after 24-hour exposure to dDAVP (Sandoval *et al.*, 2016). In parallel with changes in AQP2 protein abundance, there was a highly selective increase in AQP2 mRNA. Of the 8393 transcripts quantified (https://esbl.nhlbi.nih.gov/Databases/Vasopressin/), only a very small fraction showed significant changes in abundance, including AQP2 which was increased more than 20-fold. Consequently, the increase in AQP2 translation is principally due to an increase in AQP2 mRNA levels. The increase in AQP2 mRNA in response to vasopressin was anticipated from prior reductionist results (Ecelbarger *et al.*, 1997; Matsumura *et al.*, 1997; Hasler *et al.*, 2002), although the marked selectivity of the response for AQP2 was a surprise. The data also pointed to two protein kinases whose mRNAs are increased by vasopressin, namely salt-inducible kinase 1 (Sik1) and PCTAIRE kinase 3 (Cdk18). These kinases could accordingly be involved in the overall vasopressin response. Other protein kinases downstream from PKA activation are considered below (Section VI).

The increase in AQP2 mRNA seen in collecting duct cells in response to vasopressin could be a result either of an increase in *Aqp2* gene transcription or an increase in AQP2 mRNA stability. To address the former possibility, we performed ChIP-seq (chromatin-immunoprecipitation followed by DNA-sequencing) using antibodies to the A subunit of the RNA polymerase II complex (Pol2ra). If vasopressin increases *Aqp2* gene transcription, RNA-polymerase II occupancy over the Aqp2 gene body should be increased. Indeed, there was a 7-fold increase in RNA-polymerase II occupancy in dDAVP-treated cells versus vehicle controls, supporting the view that vasopressin signaling results in a marked increase in *Aqp2* gene transcription (Sandoval *et al.*, 2013).

VI. Quantitative Phosphoproteomics: Identification of Signaling Mechanisms Activated by the Vasopressin V2 Receptor

Cell signaling is in large part governed by phosphorylation events. Thus, identification of the processes that connect vasopressin binding to its receptor in collecting duct cells to effects on regulation of AQP2 requires global identification and quantification of protein phosphorylation. This task involves quantitative mass spectrometry-based phosphoproteomics typically carried out using SILAC for quantification in cultured collecting duct cells (Figure 1A) or tandem mass tagging (TMT) in native kidney collecting

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ducts (Figure 3A). The latter technique allows up to 16 samples to be multiplexed in a single mass spectrometry (LC-MS/MS) run. Unlike SILAC, TMT adds mass-tagging labels after samples are collected and digested with trypsin. After enrichment of phosphopeptides by affinity chromatography, fragmentation of individual phosphopeptides in the mass spectrometer liberates a series of TMT mass tag peaks that give a readout of the relative amount of the phosphopeptide in each sample (Figure 3A, **bottom**).

Building on breathtaking progress in mass spectrometer performance, it has now become possible to carry out very deep identification and quantification of vasopressin-mediated phosphorylation changes in native rat inner medullary collecting ducts (IMCDs) using TMT (Deshpande *et al.*, 2019) and in mouse cultured mpkCCD cells using SILAC (Datta *et al.*, 2020). In both studies, vasopressin exposure resulted in phosphorylation changes in only a minority of phosphorylation sites (<5 percent), indicative of highly selective regulation. There were 51 phosphorylation sites that were common to the two studies in 45 phosphoproteins (Tables 1 and 2) and the corresponding phosphoproteins are candidates to play roles in regulation of AQP2 trafficking or transcription. Vasopressin-mediated phosphorylation at all of these sites, with the exception of Ser256 of AQP2, were prevented when PKA-Cα and PKA-Cβ were deleted using CRISPR-Cas9, supporting the conclusion that PKA-mediated phosphorylation is central to vasopressin signaling (Datta *et al.*, 2020; Datta *et al.*, 2021).

Among the 33 phosphorylation sites that were increased in abundance by vasopressin (Table 1), 23 had basic (positively charged) amino acids, i.e. arginine (R) and lysine (K), in positions −2 and −3 relative to the phosphorylated amino acid, an (R/K-(R/K)-X-(S/T) motif, consistent with an increase in phosphorylation at these sites by PKA (Figure 3B). Among the 18 phosphorylation sites that were decreased (Table 2), 15 had a proline in position +1 relative to the phosphorylated amino acid (Figure 3B), consistent with downregulation of MAP kinases and/or cyclin-dependent kinases in response to vasopressin or with upregulation of a proline-directed protein phosphatase such as phosphatase 2B (Deshpande *et al.*, 2019). Previous studies using immunoblotting to detect active site phosphorylation in the MAP kinases ERK1 and ERK2 supported the conclusion that vasopressin inhibits these kinases in collecting duct cells (Pisitkun *et al.*, 2008).

Among the 45 vasopressin-regulated phosphoproteins in Tables 1 and 2, 18 are involved in various aspects of intracellular signaling including 5 protein kinases (Camkk2, Cdk18, Erbb3, Mink1 and Src), 2 protein kinase A regulators (Akap12 and Prkar2a), a cyclic nucleotide phosphodiesterase (Pde7a), 5 regulators of small GTP binding proteins (Ralgapa2, Agfg1, Als2, Arfgef1, and Myo9b), a phospholipid kinase (Pi4kb) and 4 proteins involved in calcium signaling (Stim1, Itpr1, Itpr2, and Itpr3). PKA-mediated phosphorylation of Itpr1 has been proposed to account for vasopressin-stimulated calcium mobilization in collecting duct cells (Isobe *et al.*, 2017). Six regulated phosphoproteins are involved in membrane trafficking (Bin1 [Amphiphysin II], Bloc1s5 [Dysbindin], Itsn1 [Intersectin-1], Sec22b, Stxbp5 [Tomosyn-1] and Wdr7). Four are transcriptional regulators and coregulators (Ctnnb1 [Beta-catenin], Hdac4, Lrrfip1, and Tsc22d4) that are candidates for roles in vasopressin-mediated regulation of *Aqp2* gene transcription.

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CRISPR-phosphoproteomics.

To develop causal models of vasopressin signaling, it is necessary to map the regulated phosphorylation sites to the protein kinases that phosphorylate them. This can be achieved in part through deletion of individual candidate protein kinases in cultured cell models or in mice using genome editing techniques, followed by phosphoproteomics analysis to determine what phosphorylation sites are perturbed. At this writing, this goal has been accomplished for three protein kinases in cultured mpkCCD cells, viz. the two PKA catalytic units (PKA-cα and PKA-cβ) (Isobe *et al.*, 2017; Datta *et al.*, 2020; Raghuram *et al.*, 2020), as well as myosin light chain kinase (Mylk) (Isobe *et al.*, 2020).

Protein kinase A is coded by two paralogous genes (*Prkaca* and *Prkacb*) that produce proteins with nearly identical catalytic regions, viz. PKA-cα and PKA-cβ. These genes have been deleted in vasopressin-responsive mpkCCD cells using CRISPR-Cas9, both singly and as a double knockout (dKO). In the PKA dKO cells (Isobe *et al.*, 2017), there were 273 phosphorylation sites that showed decreases in abundance, most of which were in a –(R/K)-(R/K)-X-p(S/T) motif characteristic of previously identified PKA sites (for a full listing, see https://esbl.nhlbi.nih.gov/Databases/PKA-Targets/). These data greatly expanded the number of known PKA phosphorylation sites. Given the virtually identical nature of the catalytic regions of PKA-cα and PKA-cβ, it could be anticipated that the two single knockout cell lines would identify the same list of PKA-dependent phosphorylation sites. Instead, it was found that PKA-cα and PKA-cβ deletions resulted in nearly completely independent phosphorylation target lists (Raghuram *et al.*, 2020) with PKA-cα favoring sites in proteins associated with cell membranes and membrane vesicles and PKA-cβ favoring sites in cytoskeletal proteins and cell junction proteins. Thus, PKA-Cα and PKA-Cβ serve substantially different regulatory functions in renal collecting duct cells. The differences in phosphorylation targets are believed to be due to differences in subcellular localization of the two kinases, perhaps mediated by A-kinase anchor proteins or binding to PDZ-domain proteins.

The PKA dKO cells have also been employed to ask whether there is PKA-independent vasopressin signaling in mpkCCD cells (Datta *et al.*, 2020). For this, phosphoproteomics was carried out in PKA dKO cells treated with dDAVP versus vehicle. The results showed that V2R-mediated vasopressin signaling is predominantly, but not entirely, PKA-dependent. The sites increased by dDAVP in PKA dKO cells were chiefly those previously characterized as being phosphorylated by SNF1-family kinases, e.g., salt-inducible kinase 2, MAP/microtubule affinity-regulating kinase 3, or AMP-activated kinase. Interestingly, in the PKA dKO, dDAVP still increased phosphorylation of AQP2 at Ser256, a site widely believed to be phosphorylated by PKA, suggesting that some other basophilic kinase could be responsible for Ser256 phosphorylation. Bayesian integration of multiple NGS and protein mass spectrometry data sets has identified Ca^{2+}/calmodulin-dependent protein kinase type II-delta (Camk2d) as an alternative protein kinase for this site (Bradford *et al.*, 2014; Yang *et al.*, 2015).

Similar experiments using CRISPR-Cas9, observed the effect of deletion of myosin light chain kinase (Mylk) on the phosphoproteome of cultured mouse mpkCCD cells (Isobe *et al.*, 2020). Immunocytochemistry and electron microscopy demonstrated a defect in

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the processing of AQP2-containing early endosomes to become late endosomes. The phosphoproteomics experiments revealed that more than 100 target proteins showed changes in phosphorylation in response to Mylk deletion, in addition to the canonical target site in myosin regulatory light chain. There was significant overlap between the derived Mylk signaling network and a previously determined PKA signaling network. The presence of multiple proteins in the actomyosin category prompted experiments showing that Mylk deletion inhibits the normal effect of vasopressin to depolymerize F-actin, providing a potential connection to AQP2 trafficking. It was concluded that Mylk is part of a multicomponent signaling pathway in both the cytoplasm and nucleus that includes much more than simple regulation of conventional myosins.

VII. Effects of Kinase Inhibitors on Phosphoproteome: H89

Protein kinases have been one of the most prioritized drug targets and at this writing 55 protein kinase inhibitors have been approved by the US FDA (Bournez *et al.*, 2020; Roskoski, 2020) and many more are under evaluation. These protein kinase inhibitors are used for a variety of neoplastic and non-neoplastic diseases. Since protein kinases are critical mediators of intracellular signaling in all cells, off target effects of kinase inhibitor drugs are likely, including possible untoward effects on transport in the kidney. Quantitative phosphoproteomics approaches are being applied to determine what phosphorylation sites are affected by individual kinase inhibitors (Pan *et al.*, 2009; Oppermann *et al.*, 2013; Daub, 2015). Another useful technique is kinase inhibitor profiling, consisting of in vitro assays to profile inhibitor specificity versus an array of recombinant protein kinases (Davies *et al.*, 2000; Bain *et al.*, 2007; Karaman *et al.*, 2008). These studies found that most of the kinase inhibitors tested had specificities that were much broader than presumed, sometimes inhibiting off-target kinases much more potently than their anticipated targets. These studies have been extended to 243 commonly used kinase inhibitors, many of which are in clinical use, and the data have been made available as a web resource at http://www.kinase-screen.mrc.ac.uk/kinase-inhibitors. In the context of vasopressin signaling in the renal collecting duct, data for the presumed PKA-specific inhibitor H-89 (*N-[2-p-bromocinnamylamino-ethyl]-5-isoquinolinesulphonamide*) indicates that H-89 has a broader specificity than just PKA (Davies *et al.*, 2000). The PKA dKO mpkCCD cells were employed to test whether this is true in living cells (Limbutara *et al.*, 2019). Indeed, it was found that H-89 at concentrations normally employed in physiological studies reduced phosphorylation at numerous sites in the PKA dKO cells, leading to the conclusion that H-89 inhibits basophilic protein kinases other than PKA. Thus, the observation that H-89 affects a particular cellular process should not be regarded as sufficient evidence for PKA involvement in the process. The full phosphoproteomic dataset showing changes in phosphorylation in PKA-intact and PKA dKO cells in response to H-89 is provided at https://esbl.nhlbi.nih.gov/H89/volcano/.

VIII. AQP2 Interactome

Quantitative mass spectrometry can also be used to identify proteins that interact with AQP2, the so-called “AQP2 interactome”. Three types of studies have been performed for this purpose. First, Barile et al (Barile *et al.*, 2005) used an AQP2 antibody to immuno-

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isolate intracellular AQP2 vesicles in native rat IMCD cells. Second, Chou et al (Chou *et al.*, 2018) used an AQP2 antibody to pull-down AQP2 from native rat IMCD cells, followed by mass spectrometry to identify proteins directly bound to AQP2. Both of the first two studies identified multiple Rab family small GTP binding proteins as AQP2 interactors. Third, Zwang et al (Zwang *et al.*, 2009) identified binding partners for phosphorylated versus nonphosphorylated forms of the AQP2 COOH-terminus via a targeted comparative proteomic approach.

The Barile et al study (Barile *et al.*, 2005) identified Rab GTPases 4, 5, 18, and 21 (associated with early endosomes); Rab7 (late endosomes); and Rab11 and Rab25 (recycling endosomes) in AQP2 vesicles indicating that a substantial fraction of intracellular AQP2 is present in endosomal compartments. In addition, several endosome-associated SNARE proteins were identified including syntaxin-7, syntaxin-12, syntaxin-13, Vti1a, vesicle-associated membrane protein 2, and vesicle-associated membrane protein 3. Also, AQP2 intracellular vesicles contained several markers of the trans-Golgi network, components of the exocyst complex, and several motor proteins including myosin 1C, non-muscle myosins IIA and IIB, myosin VI, and myosin IXB.

The Chou et al study (Chou *et al.*, 2018) compared the AQP2 interactome with the interactome of another vasopressin-regulated protein, UT-A1. Among the proteins unique to the AQP2 interactome were several Rab proteins (Rab1a, Rab2a, Rab5b, Rab5c, Rab7a, Rab11a, Rab11b, Rab14, Rab17) involved in membrane trafficking. Other AQP2 interacting proteins potentially involved in AQP2 trafficking included clathrin heavy chain (Cltc), Vacuolar protein sorting-associated protein 35 (Vps35), ALG-2-interacting protein 1 (Pdcd6ip), and LPS-responsive beige-like anchor (Lrba). A recent study (Hara *et al.*, 2022) confirmed that Lrba colocalizes with AQP2 in vivo and showed that *Lrba* knockout mice display a polyuric phenotype with severely impaired AQP2 phosphorylation. The authors implicated Lrba as an AKAP protein that organizes AQP2 interaction with PKA regulatory subunits.

The Zwang et al study (Zwang *et al.*, 2009) used mass spectrometry to identify binding partners to phosphorylated versus nonphosphorylated forms of the AQP2 COOH-terminus. Cytosol from inner medullary collecting ducts isolated from rat kidneys was incubated with “bait” peptides, representing the COOH-terminal AQP2 tail in its nonphosphorylated and phosphorylated forms. The captured proteins were identified by LC-MS/MS analysis. The studies confirmed previously identified interactions between AQP2 and HSP70, HSP70–1 and –2 (Lu *et al.*, 2007), as well as annexin II (Noda *et al.*, 2005; Tamma *et al.*, 2008). These proteins were found to bind less to Ser-256-phosphorylated AQP2 than to the nonphosphorylated form. In contrast, another heat shock protein, HSP70–5 (BiP/grp78), bound to phosphorylated AQP2 more avidly than to nonphosphorylated AQP2. These results suggest that phosphorylation of AQP2 at Ser-256 may regulate AQP2 trafficking in part by mediating differential binding of HSP70 family proteins to the COOH-terminal tail.

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IX. Data Sharing

Systems level data, if made available, can be used by the members of the research community to guide further experimental studies. While data sharing is common, e.g., *ProteomeXchange* for proteomic data, it mostly focusses on unprocessed data that are not immediately useful to users without a high level of computational expertise. In general, there is no standard mechanism for sharing curated data. However, for kidney-specific data, we have developed the *Kidney -Omics* website (https://esbl.nhlbi.nih.gov/Databases/KSBP2/) that allows users to browse, search or download kidney-relevant curated data sets. Much of the data described in this review is accessible there.

X. Conclusion

In this review, we have described application of proteomic methods focusing on discovery of mechanisms whereby vasopressin regulates the molecular water channel AQP2. Proteomics and other systems level approaches have generated an 'ocean' of new information and new hypotheses. Going forward, it will be important to prioritize the observations described here to develop a deeper analysis of AQP2 regulation by vasopressin using classical reductionist tools.

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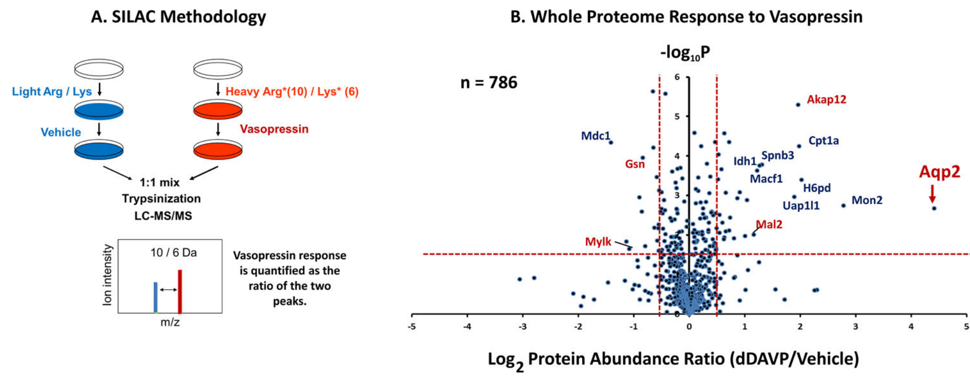

Figure 1. SILAC quantification of protein abundance changes in response to vasopressin analog dDAVP.

(A) Cultured cells are grown and equilibrated in medium containing either arginine and lysine with the normal abundance C and N isotopes, i.e. C-12 and N-14 (‘light medium’) or with arginine and lysine containing C-13 and N-15 (‘heavy medium’). Samples are trypsinized, mixed 1:1 and subjected to LC-MS/MS-based quantification. Individual tryptic peptides are ‘seen’ twice by the mass spectrometer at different molecular masses. The relative height of the light and heavy peaks reports the relative abundance of the parent protein in the original samples. (B) ‘Volcano plot’ reports the proteome-wide response to dDAVP versus vehicle, showing n=786 quantified proteins. Vertical axis is the negative of the base 10 logarithm of calculated P values (from t-statistic) for multiple replicates. Horizontal axis the base 2 logarithm of the abundance ratio dDAVP/Vehicle. Note the marked increase in abundance of AQP2 protein, which exceeds that of all other proteins.

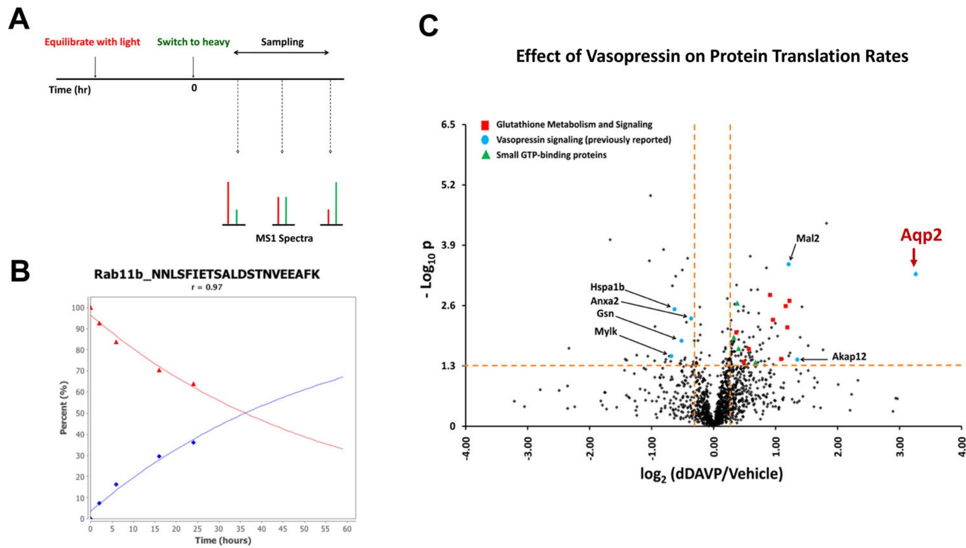

Figure 2. Dynamic SILAC-based global determination of protein half-lives and translation rates.
(A) Cultured cells are grown and equilibrated in medium containing arginine and lysine with the normal abundance C and N isotopes, i.e., C-12 and N-14 (‘light medium’). At time zero, cells are switched to ‘heavy medium’ with arginine and lysine containing C-13 and N-15. Thus, individual tryptic peptides are ‘seen’ twice by the mass spectrometer at different molecular masses indicating peptide synthesized before and after the medium switch. Sampling at different times allows assessment of the rate of protein turnover. (B) An example of data from the method quantifying a tryptic peptide corresponding to the small GTP-binding protein Rab11b (amino acid sequence: NNLSFIETSALDSTNVEEAFK). Data are fit to single-exponential curves with high precision. Both degradation rate (red) and production rate (blue) can be calculated from the data, which are equal if steady state conditions are maintained. Typically, several tryptic peptides derived from a single protein can be quantified, giving a very high degree of overall precision. (C) Volcano plot showing whole proteome quantification of effect of vasopressin analog dDAVP on translation rates of individual proteins. Labeled proteins have been identified in prior studies as having roles in vasopressin signaling or AQP2 regulation. Note that dDAVP exposure results in a multi-fold increase in AQP2 translation far exceeding changes in all other proteins.

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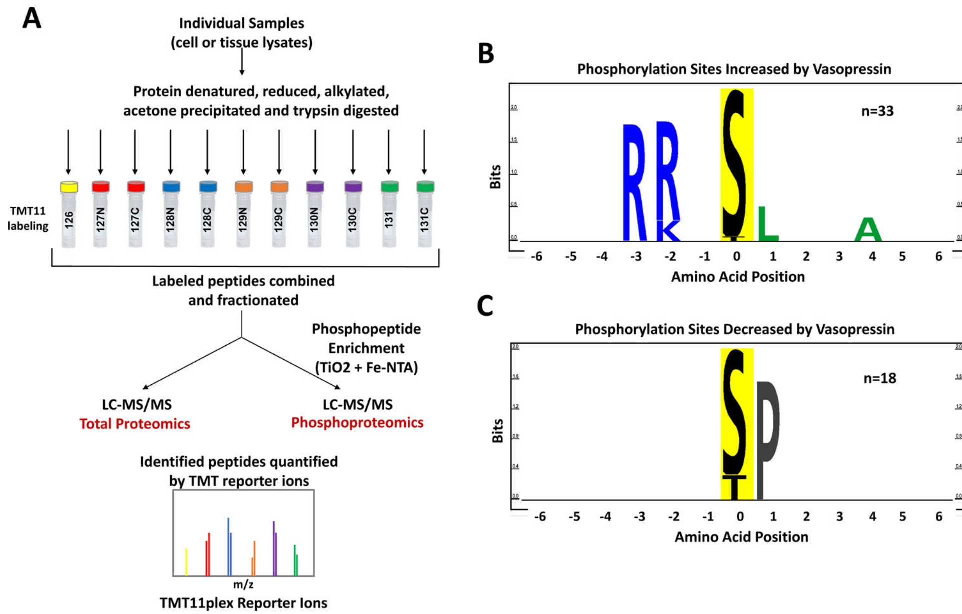

Figure 3. Phosphoproteomics.

(A) TMT for quantification in protein mass spectrometry. (B) Sequence logo based on the 33 phosphorylation sites that are increased by vasopressin in both mpkCCD cells and native IMCD cells (Table 1). (C) Sequence logo based on the 18 phosphorylation sites that are decreased by vasopressin in both mpkCCD cells and native IMCD cells (Table 2).

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Table 1.

Phosphorylation sites increased in abundance by vasopressin in both mouse mpkCCD cells and native rat IMCD cells

Gene Symbol	Site	Annotation	Centralized Sequence*
Aqp2	**S256**	**AQP2**	**RR**QŜVEL
Arhgef2	**S885**	**rho guanine nucleotide exchange factor 2**	**RR**RŜLPA
Bin1	**S304**	**myc box-dependent-interacting protein 1**	PDGŜPAA
Bloc1s5	**S25**	**biogenesis of lysosome-related organelles complex 1 subunit 5**	**KR**DŜLGT
Borcs6	**S173**	**BLOC-1-related complex subunit 6**	DSRŜLDG
Camkk2	**S510**	**calcium/calmodulin-dependent protein kinase kinase 2**	RSLŜAPG
Camkk2	**S494**	**calcium/calmodulin-dependent protein kinase kinase 2**	**RK**RŜFGN
Cdk18	**S66**	**cyclin-dependent kinase 18**	**RR**FŜMED
Clip1	**S347**	**CAP-Gly domain-containing linker protein 1**	**RK**IŜGTT
Ctnnb1	**S552**	**catenin beta-1**	**RR**TŜMGG
Ctnnb1	**T551**	**catenin beta-1**	QRRT̂SMG
Erbb3	**S980**	**receptor tyrosine-protein kinase erbB-3**	**KR**AŜGPG
Igsf5	**S334**	**immunoglobulin superfamily member 5**	QRSŜLPQ
Itpr1	**S1589**	**inositol 1,4,5-trisphosphate receptor type 1**	**RR**DŜVLA
Itpr1	**S1756**	**inositol 1,4,5-trisphosphate receptor type 1**	**RR**EŜLTS
Itpr2	**S1709**	**inositol 1,4,5-trisphosphate receptor type 2**	GDHŜVGV
Itpr3	**S1832**	**inositol 1,4,5-trisphosphate receptor type 3**	RVSŜFSM
Itsn1	**S315**	**intersectin-1**	RSGŜGMS
Lrrfip1	**S88**	**leucine-rich repeat flightless-interacting protein 1**	**RR**GŜGDT
Luzp1	**S261**	**leucine zipper protein 1**	**RK**GŜLDY
Myo9b	**S1649**	**unconventional myosin-IXb**	**RR**KŜELG
Nsfl1c	**S176**	**NSFL1 cofactor p47**	**RR**HŜGQD
Pde7a	**S28**	**high affinity cAMP-specific 3’,5’-cyclic phosphodiesterase 7A**	**RR**GŜHPY
Pi4kb	**S511**	**phosphatidylinositol 4-kinase beta**	**RR**LŜEQL
Prrc2a	**S455**	**protein PRRC2A**	**RK**QŜSSE
Rmdn3	**S46**	**regulator of microtubule dynamics protein 3**	RSQŜLPN
Sec22b	**S137**	**vesicle-trafficking protein SEC22b**	NLGŜINT
Specc1l	**S385**	**cytospin-A**	**RK**GŜSGN
Specc1l	**S833**	**cytospin-A**	**RR**SŜTSS
Src	**S17**	**neuronal proto-oncogene tyrosine-protein kinase Src**	**RR**RŜLEP
Stxbp5	**S760**	**syntaxin-binding protein 5**	**RK**LŜLPT
Veph1	**S430**	**ventricular zone-expressed PH domain-containing protein 1**	**RR**YŜLDH
Zfp36l1	**S54**	**mRNA decay activator protein ZFP36L1**	**RR**HŜVTL

*Seven amino acid sequence surrounding the phosphorylated site (^). Basic amino acids in both position −3 and −2 underlined and bold.

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Table 2.

Phosphorylation sites decreased in abundance by vasopressin in both mouse mpkCCD cells and native rat IMCD cells

Gene Symbol	Site	Annotation	Centralized Sequence
Agfg1	**S181**	**arf-GAP domain and FG repeat-containing protein 1**	PTQŜ**P**VV
Akap12	**S469**	**A-kinase anchor protein 12**	TDLŜ**P**EE
Als2	**S486**	**alsin**	SQVŜ**P**RL
Aqp2	**S261**	**AQP2**	ELHŜ**P**QS
Arfgef1	**S1566**	**brefeldin A-inhibited guanine nucleotide-exchange protein 1**	SQKŜVDI
Exoc7	**S250**	**exocyst complex component 7**	VPYŜ**P**AI
Hdac4	**S245**	**histone deacetylase 4**	KTAŜEPN
Mink1	**S782**	**misshapen-like kinase 1**	LDSŜ**P**VL
Pdlim5	**S111**	**PDZ and LIM domain protein 5**	PITŜ**P**AV
Prkar2a	**S97**	**Protein kinase A, type II-alpha regulatory subunit**	RRVŜVCA
Prrc2a	**T783**	**protein PRRC2A**	ERGT̂**P**PV
Ralgapa2	**S820**	**ral GTPase-activating protein subunit alpha-2**	RSSŜ**P**AE
Sept9	**T125**	**septin-9**	GHKT̂**P**EP
Slc9a3r1	**S277**	**Na(+)/H(+) exchange regulatory cofactor NHE-RF1**	ASEŜ**P**RP
Stim1	**S575**	**stromal interaction molecule 1**	LPDŜ**P**AL
Tanc1	**S1658**	**protein TANC1**	SSGŜ**P**SS
Tsc22d4	**T223**	**TSC22 domain family protein 4**	AAGT̂**P**PL
Wdr7	**S935**	**WD repeat-containing protein 7**	ARDŜ**P**PA

[*] Seven amino acid sequence surrounding the phosphorylated site (^). Proline in position +1 underlined and bold.