REGULATION OF AQUaporin-1 ION CHANNEL FUNCTION BY INTRACELLULAR SIGNALING PATHWAYS

by

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DEDICATION

This dissertation is dedicated to my son Bryce Alan Birdsell born 11/29/04.
# TABLE OF CONTENTS

LIST OF FIGURES .................................................................................................................. 8

LIST OF TABLES ..................................................................................................................... 11

ABSTRACT ................................................................................................................................ 12

CHAPTER 1. Background and Literature Review ........................................................................ 14

## 1.1 MAJOR INTRINSIC PROTEIN (MIP) FAMILY .................................................................. 14

1. Structure and Function of MIPs ......................................................................................... 16
2. Ion Channels ........................................................................................................................ 32
   a. Ion Channels of Aqp0 ....................................................................................................... 32
   b. Ion Channels of Aqp1 ....................................................................................................... 35
   c. Ion Channels of Aqp6 ....................................................................................................... 41

## 1.2 POTENTIAL ROLES OF AQP1 IN THE PROCESS OF FLUID TRANSPORT ................. 43

1. Angiogenesis and Endothelial cell (EC) permeability ......................................................... 44
2. Cerebral Spinal Fluid Production and Renal Function ......................................................... 49

## 1.3 GENERAL HYPOTHESIS AND AIMS ......................................................................... 51

Chapter 2. AQUAPORIN-1 ION CHANNEL FUNCTION IS REGULATED BY INTRACELLULAR SIGNALING ACTIVATED BY THE INSULIN SIGNALING PATHWAY .................................................................................................................. 70

## 2.1 INTRODUCTION .......................................................................................................... 70

## 2.2 MATERIALS AND METHODS ...................................................................................... 73

1. Molecular techniques .......................................................................................................... 73
2. Oocyte preparation and injection ......................................................................................... 75
3. Electrophysiological recordings ........................................................................................... 76
4. Osmotically-induced swelling assay .................................................................................... 77
5. Cellular fractionation ............................................................................................................ 78
6. Immunoprecipitation and Western blotting ....................................................................... 78
7. Immunofluorescence of intact oocytes ............................................................................... 80

## 2.3 RESULTS ................................................................................................................... 81

## 2.4 DISCUSSION ............................................................................................................... 96
# TABLE OF CONTENTS (continued)

CHAPTER 3. DIVERSE GATING MODES OF cGMP-ACTIVATED CURRENT IN AQP1-EXPRESSING OOCYTES ........................................................................................................... 122

3.1 INTRODUCTION ........................................................................................................ 122
3.2 MATERIALS AND METHODS .................................................................................. 125
   (1) Molecular techniques ......................................................................................... 125
   (2) Oocyte preparation .......................................................................................... 125
   (3) Electrophysiological recordings ....................................................................... 125
3.3 RESULTS .................................................................................................................. 127
3.4 DISCUSSION .......................................................................................................... 138

SUMMARY AND CONCLUSIONS ............................................................................ 172

REFERENCES ......................................................................................................... 186
LIST OF FIGURES

Figure 1.1 Predicted topology of Aquaporin1 subunit as a six Transmembrane (TM) spanning protein with intracellular amino and carboxyl Termini………………………………………………………………….54
Figure 2.2 The function of Aqp-1 as a cGMP-regulated ion channel is enhanced by insulin-activated tyrosine kinases in the Xenopus oocyte expression system……………………………………………………55
Figure 1.3 Side view of one subunit of Aquaporin 1…………………………56
Figure 1.4 Alignment of the Loop B domains of AQPs show conserved patterns across two segments shown in blue and red…57
Figure 1.5 Alignment of a segment of Loop E in AQPs shows a conserved pattern shown in blue………………………………………………….59
Figure 1.6 Schematic depiction of the selectivity filter of one subunit of Aquaporin and disruption of the single file column of water molecules……………………………………………………………… 61
Figure 1.7 Extracellular view of Aquaporin-1 homotetramer……………62
Figure 1.8 Intracellular view of crystal structure of one subunit of AQP1 from bovine (left) and human (right) ………………………………………63
Figure 1.9 Differences in reported endogenous whole cell current of HEK cells in a conventional patch clamp mode………………………………...64
Figure 1.10 Diagram illustrating the proposed interactions of junctional Proteins…………………………………………………………………65
Figure 1.11 Diagram proposing how cGMP activation of Aqp1 ion channels in EC may contribute to the increase of endothelial permeability…..66
Figure 1.12 Schematic diagram of the Choroid Plexus epithelia……………68
Figure 1.13 Schematic diagram of solute transport pathways across a proximal tubule cell. …………………………………………………………..69

Figure 2.1 SNP-induced Aqp1 current response is highly variable across experiments…………………………………………………………102
Figure 2.2 The function of Aqp-1 as a cGMP-regulated ion channel is enhanced by insulin-activated tyrosine kinases in the Xenopus oocyte expression system………………………………………………………105
Figure 2.3 No effect of season on insulin potentiation of SNP-induced Aqp1 ion current…………………………………………………………...107
Figure 2.4 Insulin preincubation does not enhance water permeability of Aqp1-expressing oocytes……………………………………………….108
Figure 2.5 Putative regulatory domains of Aqp1 along the carboxyl tail………109
Figure 2.6 Aqp1 is not phosphorylated at a tyrosine residue………………….111
LIST OF FIGURES (continued)

Figure 2.7 The lack of Aqp1 PDZ binding domain or mutation of putative tyrosine phosphorylation site does not interfere with water channel function when expressed in *Xenopus* oocytes……………………112

Figure 2.8 Carboxyl tail truncation mutants express at the plasma membrane and functions as water channels……………………………………114

Figure 2.9 Aqp1 PDZ binding domain does not mediate the potentiating effects of insulin-preincubation………………………………………………116

Figure 2.10 SNP activates ionic current in Aqp1 lacking the putative cGMP-binding domain when expressed in oocytes…………………………118

Figure 2.11 Aqp1 is phosphorylated at a serine residue………………………120

Figure 3.1 The rapid gating mode of SNP-activated Aqp1 current displays properties of rapid deactivation after SNP removal and a linear current-voltage relationship………………………………………………156

Figure 3.2 The slow gating mode of SNP-activated Aqp1 current displays properties of delayed deactivation after SNP removal and outward rectification. ……………………………………………………………158

Figure 3.3 Rapid and slow SNP gated currents have reversal potentials that are significantly different. ……………………160

Figure 3.4 Outward rectification of current positively correlates with the percent rapidly deactivating current in the absence of SNP. …………161

Figure 3.5 The PDZ-binding domain does not mediate the rapid gating properties observed in SNP-activated Aqp1 current. ……………162

Figure 3.6 The PDZ-binding domain does not mediate the slow gating properties observed in SNP-activated Aqp1 current. ……………164

Figure 3.7 The putative cGMP-binding domain does not mediate the rapid gating observed in SNP-activated Aqp1 current ……………………166

Figure 3.8 The activation rates of SNP-stimulated Aqp1 current display kinetic properties reflecting either a plateau or a linear/exponential trend by 500 seconds after SNP stimulus……………………………………………168

Figure 3.9 Rapidly gated currents have plateau activation profile and slowly gated currents have a non-plateau activation profile………………169

Figure 3.10 Summary histogram of the effect of insulin treatment on SNP-induced current properties displayed in Aqp1-expressing oocytes…………………………………………………………….171
LIST OF FIGURES (continued)

Figure 4.1  Alignment of domains in the amino and carboxyl termini of Aqp1 channel across the vertebrae taxa................................................182

Figure 4.2  The insulin-activated signaling pathway. Insulin peptide binding to insulin receptor leads to activation by autophosphorylation of the insulin receptor.................................................................184
LIST OF TABLES

Table 2.1 No pattern of seasonal influence affecting Aqp1 channel response to SNP…………………………………………………………………..104

Table 4.1 Diverse mechanisms that regulate AQP expression and facilitation of solute permeation…………………………………………………………185
ABSTRACT

My dissertation work has focused on identifying regulatory mechanisms that govern Cyclic Guanosine Monophosphate (cGMP)-activation of Aquaporin-1 (Aqp1) ion channels. Aquaporins serve as pores for water thus allowing enhanced water permeability in biological membranes (Preston, et al., 1992). A subset of Aquaporin proteins behave as ion channels regulated by intracellular signaling pathways (Anthony, et al., 2000; Ehring, et al., 1990; Yasui, et al., 1999a). cGMP is necessary for Aqp1 ion channel activation, but only a small subpopulation of Aqp1 proteins function as cGMP-activated ion channels. This observation indicates the involvement of additional regulatory mechanisms in the gating Aqp1 ion channels.

Work from this dissertation provides the first insight into the potential mechanism that dictates Aqp1 ion channel availability to respond to the cGMP signal. I show here that insulin-activated tyrosine kinases positively regulate cGMP-mediated activation of Aqp1 ion channels when expressed in Xenopus oocytes. In addition, I show that Aqp1 currents have rapid and slow gating modes with distinct current properties which may reflect distinct gating mechanisms. Treatment of Aqp1-expressing oocytes with insulin enhanced the Aqp1 ion channel function but did not influence the relative frequency of the two
gating modes. The Carboxyl (C)-terminus of Aqp1 encodes a PSD-95/DLG/ZO-1 (PDZ) ligand binding domain and a number of putative regulatory domains. The potential regulatory domains were systematically truncated to test the contribution of each of these regions for ion channel availability and current gating. Through this approach I have demonstrated that the enhancement of Aqp1 ion channel by insulin treatment was not mediated by regions of the distal C terminus. I also showed that the regulatory regions of the putative cGMP-binding domain are necessary for the slow but not the rapid gating mode. In summary, results from this dissertation support a hypothesis that Aqp1 is targeted convergently by intracellular signaling pathways which dictate the ion channel status of Aqp1. Work from this dissertation provides evidence that Aqp1 is phosphorylated by an endogenous serine/threonine kinase native to the oocyte. Tyrosine kinases and other signals may serve as ‘master switches’ governing Aqp1’s ability to behave as an ion channel.
CHAPTER 1. Background and Literature Review

1.1 MAJOR INTRINSIC PROTEIN (MIP) FAMILY

The existence of a molecular element specialized in transporting water through plasma membranes was postulated based on experimental evidence of rapid, mercury-sensitive water transport that could not be accounted for by simple diffusion across the lipid bilayer (Macey, 1984). The serendipitous discovery of a red blood cell protein led to the characterization of a water channel latter named Aquaporin-1 (Aqp1) (Preston, et al., 1992). Expression of Aquaporin 1 proteins in *Xenopus* oocytes facilitated rapid osmotically-driven water permeability that was blocked by mercurial compounds (Preston, et al., 1992). This discovery initiated the study of water transport through channel proteins.

Aqp1 is classified as a member of the major intrinsic protein (MIP) family based on strong sequence homology with other known MIP channels (Preston and Agre, 1991). MIP channels are an ancient family of channels that are expressed across phyla (Agre, et al., 2002). MIP channels are specialized as solute transporters (primarily for water and glycerol as well as ions). The bovine lens protein, Aqp0, was the first MIP channel cloned (Gorin, et al., 1984). Aqp0 is thought to serve as a part of a junctional protein complex of the lens (Girsch and Peracchia, 1985; Nikaido and Rosenberg, 1985); functional studies have revealed ion and water conducting capacities. Subsequent cloning of other
proteins including the glycerol facilitator GlpF (from *E. coli*) and Nodulin26 (from soybean root nodules) and others, prompted classification as the MIP family (Baker and Saier, 1990). The MIP family includes more than 200 members (Agre, et al., 2002).

To date, there are eleven mammalian aquaporins (0-10) identified (Agre, et al., 2002; Morinaga, et al., 2002). Aquaporin channels are broadly classified based on permeability to water (Aquaporins) and to small chain hydrocarbons (Aquaglyceroporins; Park and Saier, 1996). This classification system may require revision based on the discovery that some Aquaporins also function as ion channels. Aqp0 (Ehring, et al., 1990) functions as an ion channel when reconstituted in lipid bilayer system. Aqp6 was shown to function as a Cl⁻ channel regulated by pH and Hg²⁺ when expressed in *Xenopus* oocytes (Hazama, et al., 2002; Yasui, et al., 1999a). Aqp1 functions as a cGMP-activated ion channel when expressed in *Xenopus* oocytes (Anthony, et al., 2000). Other members of the MIP family function as regulated ion channels. Big Brain (BIB) functions as a tyrosine kinase regulated cation channel when expressed in *Xenopus* oocytes (Yanochko and Yool, 2002) and Nodulin26 (Weaver, et al., 1994) when reconstituted in lipid bilayer. The distinct properties of ion selectivity and regulation of these MIP channels would be predicted to correlate with divergent sequence elements in an otherwise common structural organization. This prediction launched my interest in the structural basis of
solute selectivity and regulation of permeability in Aquaporins, using Aqp1 as a target for investigation.

Aqp1 is a multifunctional channel with the capacity for regulated permeation of both ions and water. There is much work to be done towards understanding the structural basis of its ion pore pathway, mechanisms of regulation, and the how this multifunctional feature contributes to physiological processes.

(1) Structure and Function of MIPs

Crystal studies have confirmed the general structural organization of Aqp1 that was predicted by amino acid sequence analyses and functional studies. The 28 KDa Aqp1 protein transverses the plasma membrane a total of six times, forming 3 extracellular loops and two intracellular loops, and intracellular facing NH₂ and COOH termini (Figure 1.1). Aqp1 Loops A, C, and E are extracellular and Loops B and D are intracellular (Preston and Agre, 1991). This basic topology was confirmed by cleavage analysis with a chymotrypsin cleavage site inserted at various positions along Aqp1 protein (Preston, et al., 1994), and by antibodies generated against peptides from Loop C, E, and the C-terminus (Stamer, et al., 1996). There is considerable homology between the amino and carboxyl halves of all MIP proteins, a pattern thought to have originated from the duplication of an ancient gene (Pao, et al., 1991). Within each MIP protein subunit there are internal repeats of an Asn-Pro-Ala (NPA) motif within Loops B
and E (Heymann, et al., 1998; Heymann and Engel, 2000; Reizer, et al., 1993). In addition to the NPA motif, the amino and carboxyl half also possess conserved glutamate (E) residues in transmembrane (TM) helices 1 and 4, and conserved glycine (G) residues within in TM helices 3 and 6 (Heymann, et al., 1998; Reizer, et al., 1993).

The highly conserved nature of the NPA motif the surrounding residues in Loops B and E suggests that this region serves a prominent function among MIP channels (Reizer, et al., 1993). The reduction or ablation of water permeability of Aqp1 channels with mutations in the NPA motifs provided evidence that these residues are integral to forming the aqueous pathway (Jung, et al., 1994). A “hourglass” structural model (Figure 1.2) was proposed in which the intracellular Loop B connects with the extracellular Loop E within the transmembrane domain to form the aqueous pathway (Jung, et al., 1994). Atomic imaging confirmed the “hourglass model” (Figure 1.3). Projection maps of Aqp1 derived from 3-D electron cryocrystallography at 7 Å resolution showed an intramolecular 2-fold symmetry in the hydrophobic core of the bilayer (Cheng, et al., 1997; Walz, et al., 1997). The pseudo 2-fold symmetry of the amino and carboxyl halves of the subunit reflects the invagination of the small helices of Loops B and E into the membrane (Murata, et al., 2000; Ren, et al., 2000b; Ren, et al., 2001). Interaction of the NPA motifs at the center of the monomer is stabilized by proline residues (Murata, et al., 2000) to provide bidirectional water flow (Cheng, et al., 1997). Loops B and E form the pore-lining domains within each subunit, as
demonstrated by high resolution structural images of GlpF or Aqp1 that showed glycerol or water molecules in transit (Fu, et al., 2000; Sui, et al., 2001).

Despite the overall homology between the amino and carboxyl halves of Aqp1, the primary amino acid sequences of Loops B and E (which might be thought to share a common evolutionary origin) have diverged substantially. Residues in Loop B are more hydrophobic than the corresponding residues in loop E. Site-directed mutagenesis studies showed that mutations in the NPA motif of Loop B are more tolerated (indicated by preserved water channel function) than mutations in corresponding residues of Loop E (Jung, et al., 1994). The difference in the effects of mutation also suggests that the contributions to the water pore pathway of the NPA motif of Loops B and E are not identical. These observations suggest that the homologous structures have essential but not identical functions. MIP channel proteins have greater sequence homology in TM regions 1-3 than in TM regions 4-6. These structural patterns suggest that TM1-3 (amino half of MIP channels) are important for general features (channel structure, tetrameric assembly, or membrane insertion) (Reizer, et al., 1993) and that TM4-6 (carboxyl half of MIP channels) are important for specialized functions (e.g. solute selectivity or regulation) (Reizer, et al., 1993).

The AQP Loop B shows two highly conserved domains (Figure 1.4) (Yool, 2003). Site-directed mutagenesis of a conserved residue (A73M) in SGAH domain (highlighted in blue) (Loop B) of Aqp1 resulted in disruption of tetramer formation detected by velocity sedimentation (Mathai and Agre, 1999). This
result suggests that Loop B is important for tetramer assembly. The mutation in corresponding residue C189M (Loop E) did not disrupt the tetramer assembly (Jung, et al., 1994). However, Loop E also contributes to assembly. The conserved serine of the NPARS domain (Figure 1.5) (found in Loop E of many AQPs) is required for tetramer formation in the insect AQP (AQPcic) (Lagree, et al., 1998). Therefore, perhaps not surprisingly, tetrameric assembly involves both amino and carboxyl halves of MIP channels.

Domains in the amino half of MIP channels are important for solute-selectivity. Mutation of Lys-72 (Figure 1.4 shown as underlined) of Aqp6 to Glu resulted in increased permeability of cations over anions (Yasui, et al., 1999a). In addition, substitution of Asn-60 of Aqp6 to Gly (TM2; region not shown in Fig 1.4) enhanced water permeability and abolished anion permeability (Liu, et al., 2005). These findings clearly demonstrate that the structures of the amino half of MIP are important for solute selectivity. Images derived from X-ray crystallography studies showed that Loop B contributes to the selectivity filter for glycerol or water (Fu, et al., 2000; Sui, et al., 2001) and indirectly supports structural domains of the water or glycerol pore pathway. Due to the direct and indirect contributions of Loop B, domains of the amino half of MIP channel are involved in both the general and specialized features of channel function.

Regions of the carboxyl half of MIP channels appear to be important for specialized function of MIP channels (i.e. solute selectivity and regulation). Water-selective AQPs have a conserved serine in the NPARS domain (Figure
1.5 Loop E) while aquaglyceroporins (selective for both water and glycerol) have a conserved Asp. The serine is suggested to be important for tetramer assembly (Lagree, et al., 1998), required for water channel function (Mathai and Agre, 1999). Molecular images of Aqp1 derived from X-ray crystallography suggests that Loop E contains residues important for solute-selectivity. H182 (in Loop E) of Aqp1 promotes high water throughput and is thought to hinder glycerol transport (Sui, et al., 2001). TM6 influences solute selectivity between water and glycerol. A mutational study showed that switching tyrosine and tryptophan residues in the TM6 region of AQPcic (an aquaporin from the insect Cicadella viridis) to corresponding amino acids of GlpF (P,L) resulted in the permeability of glycerol instead of water (Lagree, et al., 1999). The striking feature of this finding is that despite the evolutionary distance between AQPcic and GlpF channels, corresponding positions of the COOH half of these distantly related channels appear to confer solute selectivity in a precisely similar manner. This finding suggests that the structural organization MIP channels are highly conserved and subtle residue differences at corresponding positions across MIP channels confer the specialized properties of specific channels.

A number of substitution studies suggested that domains of the carboxyl half of MIP channels, particularly TM5 and Loop E are important for determining rate of solute permeation. Aqp0 has a 13-fold lower water permeability than Aqp2 (Yang and Verkman, 1997). Chimeric constructs of Aqp0 incorporating the Loop E of Aqp2 showed a water permeability that was approximately 1.5 fold greater
than that of Aqp0 wild-type (Kuwahara, et al., 1999). Incorporation of both the
distal TM5 and Loop E regions of Aqp2 into Aqp0 gave rise to permeation rates
similar to that of Aqp2 wild-type (Kuwahara, et al., 1999). Similarly, the
substitution of the TM5 domain of other high throughput channels (Aqp1 or Aqp4)
to Aqp0 increased the water permeability of the Aqp0 chimera by 2.6 and 3.3
folds (Kuwahara, et al., 1999). These studies demonstrate that the TM5
sequence is critical for determining the rate of water permeation. Specifically, the
presence of a valine in the second half of TM5 (conserved among AQP5s with
high permeability) was found to confer maximal water permeability (Kuwahara, et
al., 1999). A proline residue in the TM5 is conserved in a number of AQP5s with
high throughput except AQP4. Substitution of proline in place of valine at
position 160 in the first half of TM5 of Aqp0 resulted in an increase of water
permeability of 50 ± 20% (Mulders, et al., 1995), demonstrating that subtle
changes of residues of TM5 can increase or decrease water permeability
dramatically.

Solute selective MIP channels depends on a combination of a narrow
diameter at the selectivity filter and structural residues that differentially favor
interaction with solute molecules to permit transport (Figure 1.6). GlpF and Aqp1
show interesting differences in the pore-lining region with respect to structure
diameter and chemical properties. The narrowest region of the pore (constriction
site) is composed of aromatic side chains of Phe and His, and positively charged
Arg abbreviated “P” (de Groot and Grubmuller, 2001; Fu, et al., 2000; Sui, et al.,
2001). Strict selectivity for water by Aqp1 is in part mediated by the 2.8 Å
diameter at the constriction site (which is too narrow for glycerol) as well as the
polar nature of residues that line the pore structure (Sui, et al., 2001). In
contrast, the constriction site of the GlpF glycerol pore is wider (3.8 Å) in
diameter and is comparatively hydrophobic (Sui, et al., 2001). Water conduction
through GlpF is thought to be limited by energetic constraints imposed by the
more hydrophobic environment at the constriction site (Fu, et al., 2000). Glycerol
selectivity over water is determined by two perpendicular aromatic rings W48
(TM 2) and F200 (loop E) that form a hydrophobic “corner” which may interact
with the carbon backbone of glycerol (Fu, et al., 2000). In Aqp1 channels, the
hydrophobic corners in the pore are smaller and more polar than in GlpF
because Trp48 is typically replaced by Phe or His; and Phe 200 is typically
replaced by Ala, Thr, or Cys (Fu, et al., 2000). The selectivity filter of GlpF is
strongly amphipathic (Fu, et al., 2000) while the selectivity filter of Aqp1 is
generally hydrophobic with dispersed regions of water-binding residues that give
rise to hydrophilic nodes (Sui, et al., 2001). These hydrophilic nodes are dense
enough to reduce the energy barrier for highly efficient water transport in Aqp1
but sparse enough to maintain strict water selectivity (Sui, et al., 2001). Studies
from molecular imaging have identified two interacting sites in the solute pore
pathway. Comparing molecular images of GlpF and Aqp1 has permitted the
formulation of a hypothesis for the structural basis of solute-selectivity.
The conservation of the NPA motif as a signature sequence across all MIP channels suggests that this motif is not involved in conferring solute selectivity, even though mutation of these residues results in compromised water permeability in AQP1 (Jung, et al., 1994).

A central question is how do Aquaporins permit efficient rapid water transport but yet exclude protons? Protons are known to transfer across lipid bilayers through the single file columns of water molecules that are aligned in a uniform orientation in Gramicidin A channels for example (Pomes and Yu, 2003). In contrast, Aqp1 excludes protons. Murata et al postulated that blocking of protons was achieved by re-orientation of water molecules in the pore of Aqp1 through hydrogen bond formation with N residues (in the NPA motif) (Figure 1.6). Simulation studies show that NPA is involved in disrupting the single file alignment of water or glycerol molecules (Tajkhorshid, et al., 2002). The NPA region of Loop B and E is proposed to be the proton filter because the interaction of water with the Asn (N) of this region contributes to the reorientation of water molecules which in turn abrogates proton transport (Tajkhorshid, et al., 2002).

Before structural data were available, several lines of functional evidence supported the idea that the conserved loops B and E directly contribute to the pore-lining region of AQP channels. The involvement of Loop E in the aqueous pathway was suggested when a cysteine at position 189 (Loop E) was identified as the residue mediating the blocking effect of Hg^{2+} on Aqp1 water permeability (Preston, et al., 1993). Conservative substitution of C189 with serine abrogated
the Hg$^{2+}$-sensitivity (Preston, et al., 1993). To ask whether the residue at the corresponding position in the Loop B of Aqp1 also was involved in forming the aqueous pathway, the effects of A73 substitution with cysteine were tested (Jung, et al., 1994). A73C/C189S mutants showed mercury-sensitivity that suggested involvement in the formation of the water pore similar to C189, but the degree of Hg$^{2+}$-sensitivity was less (Jung, et al., 1994). The authors proposed that the cysteine substituted in position 73 was less accessible to Hg$^{2+}$ because structures of intracellular loop B are less exposed to the extracellular side. The blocking effect of Hg$^{2+}$ when interacting with cysteine sites that were endogenous at position 189 or engineered at 73, indicates that these corresponding residues are located near a critical narrowing structure of the water pore pathway. An additional study confirming the involvement of Loop E resulted from the discovery of tetramethylammonium (TEA) acting as a partial inhibitor of Aqp1 water permeability. Tyrosine at position 186 (Loop E) was identified as the residue mediating the blocking effect of TEA (Brooks, et al., 2000).

Scanning mutagenesis was used to assess the contribution of Loop B residues to the channel aperture. Hydrophobic residues at position 70, 73, 75, 81, and 84 were substituted with phenylalanine (F) (Jung, et al., 1994). Oocytes expressing A73F channels had no water permeability ($P_t$) and L75F channels had a reduced $P_t$ (Jung, et al., 1994), consistent with the direct involvement of loop B, but the alternative possibility of reduced expression of mutant channels at the plasma membrane was not addressed. To test if
residues of corresponding positions between the two halves of Aqp1 have similar functional contributions, the same substitutions were introduced at corresponding residues in loops B and E at positions 73 and 189, 76 and 192, 78 and 194 (Jung, et al., 1994). Two significant patterns were observed. First, the size of the residue was critical to channel function or plasma membrane expression (Jung, et al., 1994). Second, substitutions in Loop B were better tolerated (as indicated by $P_l$) than those in corresponding positions in Loop E (Jung, et al., 1994). The difference in tolerance for structural alterations is consistent with the idea that the carboxyl half of MIP structure mediates subtype-specific channel functions.

Although imaging data conclusively showed that permeation of water or glycerol is mediated through the individual subunit, the functional unit of AQPs is the homotetramer (Figure 1.7). The exact purpose of tetrameric assembly for water or glycerol permeation is not known. The most robust evidence for the dependence of water permeability on tetrameric assembly was provided from a study that showed an Aqp1 mutant, A73M, imposed a dominant negative effect on wild-type water permeability (Mathai and Agre, 1999). Tandem dimers were constructed of Aqp1-Aqp1, Aqp1-C189M, and Aqp1-A73M (Mathai and Agre, 1999). Testing of water permeability of the dimers in Xenopus oocytes and yeast expression systems showed that the Aqp1-Aqp1 dimer functioned equivalently to wild-type, Aqp1-C189M had functionality reduced by half, and Aqp1-A73M lacked function all together. In addition, velocity sedimentation studies from yeast
extracts showed that Aqp1-Aqp1 and Aqp1-C189M dimers occupied fractions that corresponded to tetramers, while Aqp1-A73M dimers were in fractions that corresponded to dimers and monomers (Mathai and Agre, 1999). The result of matching water permeabilities with velocity sedimentation is consistent with the idea that tetrameric assembly is necessary but not sufficient for water permeation through individual subunits. The equivalent water permeability of yeast systems expressing Aqp1-Aqp1 dimer proteins or native wild-type channels indicated that the fusion proteins displayed correct folding and targeting. Results from this study suggest that proper function of the individual subunit is dependent on the tetrameric assembly, therefore the individual subunit is not truly independent.

Structural studies suggest that the pore pathway of the individual subunit is incompatible with ion transport due to a lack of structures that would aid in stripping the shield of hydration from ions (Murata, et al., 2000). Yet, a subset of AQP proteins are permeable to ions (Ehring, et al., 1990; Yasui, et al., 1999; Yool, et al., 1996). Aqp1 is a cGMP-gated cation channel (Anthony, et al., 2000) shown to be permeable to K⁺, Na⁺, Cs⁺, and to a lesser degree TEA⁺ (Yool, et al., 1996). Aqp6 is an acidic pH gated anion channel permeable to Cl⁻ and NO₃⁻ (Ikeda, et al., 2002; Yasui, et al., 1999a). While it has been conclusively determined that the pore pathway for water or glycerol exists in the individual subunit, the ionic pore pathway is still unknown. Crystal studies of the GlpF channel showed two Mg²⁺ ions located in the central cavity of the 4-fold axis of symmetry coordinated with W42 and E43 of TM2 (Fu, et al., 2000). The central
cavity of the tetrameric subunits or pseudosubunits serves as the ion pore in ion channels that include K⁺ channels (Doyle, et al., 1998). GlpF channels and MIP channels resolved by crystallography show similarities to K⁺ channels in that individual subunits form homotetramer, the extracellular side of the central cavity is more narrow, and the motif of loop and helix projecting into the membrane (Doyle, et al., 1998). The difference is that for K⁺ channels the helix proceeds the loop (Doyle, et al., 1998). The resemblance of the design of MIP and K⁺ channels and the common function of ion permeation could be an example of convergent evolution. In line of this idea, the hypothesis of the central cavity serving as the ionic pore pathway is especially attractive.

Structural analyses of Aqp1 have shown that the central cavity of the tetramer is formed by TM2 and TM5 of each monomer and is shaped irregularly where the extracellular side is more narrow than on the intracellular side (Ren, et al., 2001). Glutamine residues (Q47) of TM2 domains of each subunit contribute to the narrowest constriction (~ 3 Å diameter) of the 4-fold axis domains (Ren, et al., 2001). At this diameter, the central cavity would have to undergo conformational change to accommodate the passage of TEA⁺ ion. Atomic models of Aqp1 show that the TM2 and TM5 fit within rigid grooves (Murata, et al., 2000), thus the investigators proposed that Aqp1 proteins have very little potential for conformational change. Comparing recent crystal studies with functional studies may replace the old idea that MIP channels are highly rigid with very little potential for conformational change. Recent crystal studies of
Aqp0 at 2.2 Å resolution show that the aqueous pore pathway narrows to a diameter of 1.5 Å (Harries, et al., 2004). The size of this diameter would restrict the permeation of water, unless conformational changes occur to increase the diameter to at least 2.8 Å. The idea of Aqp0 changing conformation to accommodate water passage is supported by functional studies. In addition to the fact Aqp0 channels permeate water molecules, this function appears to be gated by acidic pH and high cytosolic Ca\(^{2+}\) (Nemeth-Cahalan and Hall, 2000; Nemeth-Cahalan, et al., 2004). These observations suggest several things. First, that AQPs can undergo conformational change affecting the diameter and the chemical composition of the aqueous pore pathway. Second, that conformational changes could be gated by intracellular signaling pathways. It is reasonable to speculate that conformational changes in the individual subunits can lead to changes in the central cavity of the 4-fold axis of symmetry. These changes could result in a diameter size or chemical composition of the central cavity that would accommodate the passage of ions. When deglycosolated Aqp1 was crystallized in a dioleoyl phosphatidylcholine (DOPC) bilayer, the tetramer packing had flexibility depending on the lipid and Mg\(^{2+}\) concentration (Ren, et al., 2000a). Results of this study suggest that the Aqp1 tetramer packing is not rigid, but rather flexible with various polymorphic arrangements that depend on the lipid characteristic. This supports the idea AQP has conformational flexibility not conveyed in crystal structures.
Current structural and simulation data have yet to provide conclusive evidence identifying the location of the ionic pore pathway. Molecular dynamic (MD) simulation study showed no permeation of water or ions in the central 4-fold axis cavity (de Groot and Grubmuller, 2001) and the environment of the central cavity is hydrophobic thus concluded by the authors to exclude water (Murata, et al., 2000). The results of crystal and simulation studies have limited interpretation. Both the MD simulation and structural studies model Aqp1 proteins lacking portions of the distal C-terminus. The C-terminus truncated form of Aqp1 proteins lack the known PDZ-ligand binding domain as well as a number of diverse putative regulatory domains. In addition, structural and simulation analyses characterize the function of Aqp1 in a non-regulated state. Functional studies show that MIP channels are regulated by a number of intracellular signaling molecules. The ion channel function of Aqp1 is regulated by cGMP, therefore implying that the pathway compatible with ion permeation would not be observable in models derived from crystal structures. Since ion flux is a gated event, structural studies would not be able to demonstrate this event.

Structural studies fail to capture the potential ranges of protein conformations due to molecular imaging at a single point in time. While viewing images of Aqp1 of human and bovine origin, Yool and Tajkhorshid made a novel observation that loop D was shown to exists in two possible conformations (Figure 1.8), leading to the exciting suggestion that this loop has flexibility. The significance of the potential flexible Loop D is the identification of a potential
steric or electrostatic barrier to ion permeation. The basis of loop D flexibility could be from G166 on Aqp1, a residue conserved in Aqp1 across all species. Glycine is unique in that it can adopt a large range of main-chain dihedral angles and therefore be flexible at specific points of protein structures (Jiang, 2002). Glycine at the intracellular domain is conserved throughout the diverse family of K⁺ channels and is thought to serve as a gating hinge (Jiang, 2002). Aqp1 ion current has been shown to be enhanced upon stimulation by depolarization steps (Anthony, et al., 2000). It was hypothesized that this Loop D containing negative charges due to the arginines, was the molecular domain thought to carry this sensitivity (Boassa, 2001).

Residues that determine water or glycerol-selectivity appear to influence the stability of tetrameric assembly. This supports the idea that the pore pathway within the individual subunit affects tetramer structure. Crystal studies have confirmed the tetrameric structure of both AQPs and GlpF channels predicted by sedimentation studies. But, functional studies demonstrated differences in tetrameric stability between glycerol facilitating channels and water-selective channels. Results from sucrose sedimentation studies suggest that aquaglyceroporins function as monomers which differ than the tetrameric water-selective AQPs (Lagree, et al., 1998). The conversion of the insect water-selective channel AQPcic into a glycerol facilitating channel was achieved by substituting two amino acids that corresponded with GlpF channels (Lagree, et al., 1999). AQPcic wild-type had velocity sedimentation coefficients indicative of
tetramers but AQPCic mutant (now a glycerol-selective channel) had coefficients indicative of monomers (Lagree, et al., 1999). These results suggest that changing structures of the pore pathway of the individual subunit alters tetrameric stability. Extraction of GlpF with SDS results in monomers, but extraction with urea (4M) results in molecular weights that are consistent with monomers, dimers, trimers, tetramers (Manley, et al., 2000). This result suggests that the oligomermization state of GlpF channels, but not water-selective AQP channels, is subject to influence by environmental factors. In addition, this result suggests that GlpF channels can exist in multiple oligomermization states (monomers, dimers, trimers, tetramers) whereas water-selective channel are strictly tetramers. The oligomermization state of E. coli glycerol facilitator GlpF, but not water-selective channel AqpZ, was sensitive to the composition of sucrose gradient. Increasing the ionic strength of the sucrose gradient favors tetrameric oligomermization states of GlpF channels (Borgnia and Agre, 2001). This result suggests that oligomermization of GlpF channels are weaker than AQPs, but nevertheless can form tetramers. Taken together, these observations support the idea that structural arrangements of the pore of the individual subunit affect tetrameric assembly. Based on this, it is reasonable to speculate that conformational changes of the individual subunit can transfer changes in the central cavity of the 4-fold axis of symmetry. Perhaps gated conformational change provides the pore environment that could facilitate ion permeation.
(2) Ion Channels

While all known mammalian aquaporins (Aqp0-10) are permeable to water, a subset of aquaporins (Aqp0, Aqp1, Aqp6) are also permeable to ions. Solute permeabilities of Aquaporins are regulated or modulated by a number of classic mechanisms including post-translational modification (phosphorylation/dephosphorylation), protein-protein interaction, ligand binding, translocation, and voltage. While the pore pathways for water and glycerol have been proven to be within the monomer, the pathway for ion permeation remains undetermined. Currently, functional data combined with structural data suggest that the center of the tetramer at the 4-fold axis may serve as the ionic pore pathway (Figure 1.7). Channels belonging to MIP family are highly conserved with similar structural organization. Structural studies of the *E. coli* GlpF show two Mg\(^{2+}\) binding sites at the 4-fold axis of the tetramer, suggesting that this region could potentially be a pore pathway for ions.

a. Ion Channel of Aqp0:

The MIP channel from lens fiber (Aqp0) was the first MIP channel to be cloned and was found to comprise more than 60% of the membrane proteins found in the lens fiber. For some time the function of Aqp0 was not obvious, but the dysfunction or the lack of Aqp0 resulted in cataracts (Berry, et al., 2000; Shiels and Bassnett, 1996; Shiels and Griffin, 1993). Reconstitution of Aqp0 channels in lipid bilayers showed conductance of large non-specific ion currents
(Na⁺ and Cl⁻) (Ehring, et al., 1990; Shen, et al., 1991; Zampighi, et al., 1985) and increased permeability to neutral solutes (water and glycerol) (Nikaido and Rosenberg, 1985; Peracchia and Girsch, 1985). The facts that the interior of the lens is slightly acidic (pH 6.5) (Mathias, et al., 1991) and that disturbance of Ca²⁺ concentrations is associated with cataracts (Paterson, et al., 1997) have led a number of investigators to test the role of pH and Ca²⁺ in the function of Aqp0.

The water channel function of bovine Aqp0 is gated by acidic pH and binding with Ca²⁺/Calmodulin (CaM) (Nemeth-Cahalan and Hall, 2000; Nemeth-Cahalan, et al., 2004). Residue H40 of loop A has been identified the pH sensor based on mutagenesis studies (Nemeth-Cahalan and Hall, 2000). Ca²⁺ and Calmodulin (CaM) presence reduced water permeability of liposomes reconstituted with Aqp0 (Peracchia and Girsch, 1985). Cleaving the carboxyl tail of Aqp0 carboxyl tail by trypsin ablated the effects of Ca²⁺ and calmodulin (Peracchia and Girsch, 1985), thus identifying the carboxyl tail as mediating the Ca²⁺-sensitivity. A study of the amino acid sequence in the Aqp0 carboxyl tail showed amphiphilic regions characteristic of CaM-binding domain (Peracchia and Girsch, 1989). Purified polypeptide of Aqp0 C-tail containing the putative CaM-binding domain interacted with CaM (Peracchia and Girsch, 1989), thus providing evidence that CaM directly interacts with Aqp0 C-terminus. Gating effect of Ca²⁺ on Aqp0 channels expressed in oocytes was observed when intracellular (not extracellular) Ca²⁺ levels were elevated (Nemeth-Cahalan and Hall, 2000), thus indicating that the Aqp0 structures mediating the Ca²⁺ gating
effect are facing the cytoplasm. Ca\textsuperscript{2+} is coupled to Aqp0 gating in the presence of CaM (Nemeth-Cahalan, et al., 2004). The addition of a CaM mutant unable to bind to Ca\textsuperscript{2+} prevented Ca\textsuperscript{2+} gating of Aqp0 (Nemeth-Cahalan, et al., 2004), thus demonstrating that CaM binding directly on Aqp0 alone is not sufficient but the gating requires Ca\textsuperscript{2+} bound CaM.

Modulated ionic conductance was observed in lipid bilayers when Aqp0 was reconstituted. Bovine Aqp0 reconstituted in the lipid bilayers showed voltage-dependent ion channel function (Ehring, et al., 1992; Shen, et al., 1991; Zampighi, et al., 1985). Single channel analysis revealed two conductance states of 380 pS and 160 pS and membrane depolarization shifted the Aqp0 channel to a lower conductance state (Ehring, et al., 1990). Voltage-dependence of Aqp0 ion channel function is regulated by phosphorylation (Ehring, et al., 1992). PKA phosphorylation of Aqp0 increases voltage-dependent closure of Aqp0 channels by increasing the rate and lifetime of channel closure (Ehring, et al., 1992). Chicken Aqp0 reconstituted in the bilayer also showed voltage-dependent ion channel function with reported unitary conductances of 60 and 290 pS (Modesto, et al., 1996). Ca\textsuperscript{2+} reduced the channel mean open time (Modesto, et al., 1996).

Ionic conductance of Aqp0 appears to be highly regulated when expressed in cell systems with intact signaling pathways. When expressed in oocytes, Aqp0 showed no overt ion channel function (Kushmerick, et al., 1995; Mulders, et al., 1995). The expression of frog Aqp0 in oocytes altered the
reversal potential by 10 mV, indicating a change in ion selectivity although the authors concluded that Aqp0 did not function as an ion channel because the overall current amplitude did not change (Kushmerick, et al., 1995). A change in reversal potential indicates a change in ion selectivity. This change in reversal potential suggests that the expression of Aqp0 channels either altered the ion selectivity of endogenous channels or serve as an ion channel in the oocyte system. Rat Aqp0, heterologously expressed in mouse erythroid leukaemia cells or baculovirus-infected cells, showed acidic-pH-dependent ion channel activity (Drake, et al., 2002). Antibodies targeting the NPA of the amino half of rat Aqp0 reduced ion conduction, therefore suggesting that this region is involved in the ion channel function (Drake, et al., 2002).

b. Ion Channel function of Aqp1:

Aqp1 forms a non-specific cation-selective channel activated in the presence of cGMP when expressed in oocytes (Anthony, et al., 2000; Boassa and Yool, 2003; Yool, et al., 1996) or reconstituted in the bilayer (Saparov, et al., 2001). Controversy persists as to the actual existence of Aqp1 ion channel function in real physiological systems. When GFP-Aqp1 fusion proteins (missing the first 2-3 residues from the amino terminus) were expressed in HEK293 cells and currents were recorded by whole-cell patch clamp mode, cGMP stimulation of GFP-Aqp1 expressing HEK cells did not increase macroscopic current from the ~20 pA background current (Tsunoda, et al., 2004). This GFP-Aqp1 fusion
protein displayed similar levels of water permeability to wild-type channels (Tsunoda, et al., 2004). Based on these results the investigators concluded that Aqp1 proteins function exclusively as water channels in physiology. Several publications have characterized the endogenous channels of HEK293 cells by the whole-cell patch clamp method. These studies showed voltage-sensitive endogenous currents with amplitudes of ~500 pA (Jiang, et al., 2002; Zhu, et al., 1998). Results from my own pilot studies confirmed the findings of these publications (Figure 1.9). In addition, I found current amplitudes of ~20 pA in cell patches with excellent seals but no break in the membrane directly under the patch (Figure 1.9). Once the membrane directly under the patch was broken, intracellular electrical access became evident by the increase of macroscopic current from ~20 pA to ~500 pA with voltage-sensitive characteristics consistent with reports from previous publications. When comparing this finding with the ~20 pA background current reported by Tsunoda and colleagues the status of intracellular electrical access is naturally questioned. A positive control to provide evidence of cell patches with intracellular electrical access would be to test cGMP-induced current activation in HEK cells expressing CNG channels. This type of evidence was not provided by Tsunoda and colleagues. Because the status of intracellular electrical access remains uncertain and true wild-type Aqp1 channels were not recorded from in the study by Tsunoda et al, the conclusion that Aqp1 is not an ion channel when expressed in HEK cells remains to be demonstrated.
Several lines of evidence show Aqp1 conduction of cations involves cGMP. Patch clamp experiments showed cGMP-induced ion channel activity with large conductance (~150 pS) and long open times in Aqp1-expressing oocytes but not in controls (Anthony, et al., 2000). cGMP binding to the membrane of Aqp1-expressing SF9 cells suggests that cGMP directly binds to Aqp1 (Anthony, et al., 2000). The carboxyl tail of Aqp1 contains amino acid residues conserved in other cGMP-binding proteins (CNG channels and PDE) shown to be important for cGMP binding (Boassa and Yool, 2002). But mutagenesis studies substituting two conserved residues reduced but did not abolish cGMP activation of Aqp1 ion current (Boassa and Yool, 2003). The tolerance of mutations in these conserved residues suggests that these residues are not cGMP interacting moieties, but rather they influence the efficacy of cGMP activation. In addition, complete mutation of the putative cGMP-binding (Boassa and Yool, unpubl) or partial truncation (see chapter 2) did not ablate the cGMP ionic response. cGMP activation was not ablated by inhibiting the cAMP/PKA signaling pathway (Anthony, et al., 2000), indicating that the effects of cGMP is not through signaling cross-talk with PKA pathway. The mechanism that regulates cGMP activation of Aqp1 ion channel is currently unknown and is the focus of this dissertation work.

I suggest that Aqp1 is regulated by multiple signaling pathways that control ion and water permeabilities. Despite presumably saturating levels of cGMP, only a small subset of Aqp1 proteins serve as ion channels when
expressed in oocytes (Yool and Weinstein, 2002) and when reconstituted in lipid bilayers (Saparov, et al., 2001). This suggests that the functional availability of Aqp1 proteins to serve as cGMP-regulated ion channels could be dictated by additional separate signaling pathways. Aqp1 ion channels are activated by forskolin treatment (Yool, et al., 1996) and direct injection of the PKA catalytic subunit in Aqp1-expressing oocytes increases current amplitudes, an effect not observed in controls (Yool, et al., 1996). These data suggest that the effect of forskolin is through the activation of PKA. Consensus sites for PKA (Yool, et al., 1996) and PKG (Kennelly and Krebs, 1991) phosphorylation have been identified in the Aqp1 carboxyl tail. The PKA catalytic subunit does phosphorylate Aqp1 from mouse kidney (Han and Patil, 2000) and purified fusion protein of Aqp1 carboxyl tail (Yool, et al., 1996). Although the connection between PKA and cGMP-activation is not known, perhaps PKA phosphorylation transforms Aqp1 into a functional cGMP-regulated ion channel. Forskolin treatment does enhance water permeability (Han and Patil, 2000; Yool, et al., 1996), thus suggesting one possible effect of forskolin is mediated through altering the conformation of Aqp1 protein. Altering channel conformation can be achieved either through direct PKA phosphorylation, phosphorylation by other PKA-activated kinases, or interaction with a PKA-regulated accessory protein. Taken together, additional intracellular signaling pathways regulate the ion and water channel functions of Aqp1, including the PKA signaling pathway.
Insulin treatment of Aqp1-expressing oocytes potentiates cGMP-activated ion current (please see chapter 2), suggesting that insulin activated signaling pathways may be involved in dictating functional availability of Aqp1 proteins. Regulatory effects of insulin on ion channel function are commonly mediated through channel phosphorylation by insulin-activated serine/threonine or tyrosine kinases. A putative tyrosine phosphorylation site (Songyang and Cantley, 1995) has been identified on Aqp1 carboxyl tail, but it is not known if phosphorylation of this residue mediates the potentiation effect of insulin treatment (Birdsell and Yool, unpubl). The homologously related Drosophila MIP channel, Big Brain (BIB), is a tyrosine kinase regulated ion channel (Yanochko and Yool, 2002). BIB is phosphorylated at tyrosine residues on the carboxyl terminus and the ion channel function is negatively regulated by insulin. Lavendustin A (a tyrosine kinase inhibitor) treatment potentiates channel activity when BIB is expressed in oocytes (Yanochko and Yool, 2002). The potentiating effect of Lavendustin A is removed by the deletion of part of the carboxyl terminus that contains tyrosine phosphorylation sites (Yanochko and Yool, 2002). This shows that the ion channel function of a MIP protein is regulated by insulin-activated intracellular signaling pathways.

AQP5s may participate in coordinated signaling processes mediated by macromolecular protein complexes. A growing body of evidence show that protein-protein interaction regulates major cell events such as intracellular signaling, ion channel gating, trafficking, targeted proteolysis, cytoskeletal...
organization, and gene expression. Aqp1 contains a PDZ ligand binding domain on the carboxyl tail and shown to colocalize with receptor tyrosine kinase EphB2 presumably through the interaction with a PDZ-containing linker protein PICK-1 (Cowan, et al., 2000). PICK-1 aggregates a number of transmembrane proteins into macromolecular signaling complexes (Cowan, et al., 2000). When EphB2 is not properly colocalized in signaling complexes due to disrupt binding to PDZ ligand binding domain, the result is abnormal development of the inner ear in mouse (Cowan, et al., 2000). This result provides another example of how an individual protein function is modified by the composition of the signaling complex. What is not known is the consequence of Aqp1 ion or water channel function when associated with PICK1 or other PDZ containing proteins. Aqp1 interacts with intracellular proteins through protein-protein interactions (Abu-Hamdah, et al., 2004; Cowan, et al., 2000). Interestingly, recent studies show evidence of gated Aqp1 water permeability through protein-protein interaction. Immunoisolation of Aqp1 from zymogen granules (ZG) (membrane bound secretory vesicles in exocrine pancreas) resulted in the coprecipitation of associated proteins that include PLA2, G_{a3}, potassium channel IRK-8, and ClC-2 chloride channel (Abu-Hamdah, et al., 2004). Aqp1 water channel function is gated by GTP in ZG (Cho, et al., 2002). Blocking of potassium current or inhibiting PLA2 proteins significantly inhibited the GTP-induced ZG swelling (Abu-Hamdah, et al., 2004). These results suggest that the function of Aqp1
proteins are regulated by signaling pathways coupled through protein-protein interactions.

While most epithelia and endothelia constitutively express Aqp1 at the plasma membrane, Aqp1 expression in cholangiocytes (bile epithelia) is regulated by translocation. Aqp1 channels are translocated from intracellular vesicles to the plasma membrane. Secretin hormone increases ductal bile secretion by causing the insertion of Aqp1 into the apical side of the plasma membrane through a microtubule-dependent mechanism (Marinelli, et al., 1999). The intermediate pathways of secretin induced translocation of Aqp1 are unknown.

c. Ion channel function of Aqp6:

Aqp6 forms an acidic-pH gated anion channel when expressed in HEK293 cells and *Xenopus* oocytes (Ikeda, et al., 2002; Yasui, et al., 1999a). The ion channel function was confirmed subsequently in excised patches from Aqp6-expressing oocytes (Hazama, et al., 2002). Unstimulated Aqp6 has low water permeability similar to Aqp0 and no detectable ionic current; low pH or 100 µM of Hg$^{2+}$ reversibly induces a rapid rise in water permeability, and activates an ionic conductance that is nearly voltage-independent (Yasui, et al., 1999a). The effects of Hg$^{2+}$ or low pH were unique to Aqp6 channels, and not seen for Aqp0, 1, or 2. Ion selectivity was found to depend on the method of activation; Hg$^{2+}$ induced a current with equal permeability to both cations and anions (Hazama, et
al., 2002; Yasui, et al., 1999a), whereas acidic-pH induced selective anion permeation (Ikeda, et al., 2002; Yasui, et al., 1999a). In mammalian cells, Aqp6 expression is restricted to the intracellular vesicles within acid-secreting α intercalated cells of the renal epithelia and colocalizes with H+-ATPases (Yasui, et al., 1999b). The proposed physiological function of Aqp6 is to serve as anion channel in acidified intracellular vesicles to maintain electroneutrality (Yasui, et al., 1999b).

Mutagenesis studies have provided insight to structures of Aqp6 involved in the ion selectivity and gating. Residues K72 (Yasui, et al., 1999a) and Thr63 (Ikeda, et al., 2002) are important for anion selectivity. Asn60 appears is critical for anion permeability; a point mutation of Asn60 to glycine converted Aqp6 into a constitutive water channel that was no longer permeable to anions (Liu, et al., 2005). The position corresponding to Asn60 is a glycine in all other aquaporins. Mutating this asparagine to glycine in Aqp6 was found to endow rapid water permeability and abolish anion conductance (Liu, et al., 2005). Gating of Aqp6 by Hg^{2+} is mediated by residues C155 and C190 (Yasui, et al., 1999a), but the residues involved in sensing pH remain unknown. Single channel studies of Aqp6 expressed in oocytes determined the Hill coefficient for Hg^{2+} binding to be nearly 1, suggesting the pore pathway could be through the individual monomer (Hazama, et al., 2002). The idea that water and ions share the same pathway is supported circumstantially by the fact that both fluxes are gated by the same activator (acidic-pH and Hg^{2+}). But other observations argue against a shared
pore pathway. For example, Aqp6 channels activated by Hg$^{2+}$ are non-selective whereas acidic-pH activated Aqp6 channels are anion-selective. Hazama et al reported Aqp6 had a single channel conductance of ~49 pS. If there were four ion pathways per tetramer of Aqp6, then I would predict that patch clamp data should show multiple levels of unitary channel events of 49 pS. Multiple levels of conduction were not reported. It will be interesting to resolve the structure of Aqp6 at high resolution in different pH conditions to determine if changes in the diameter of the aqueous pore pathway can accommodate ion permeation.

1.2 POTENTIAL ROLES OF AQP1 IN THE PROCESS OF FLUID TRANSPORT.

A subset of tissues highly involved in fluid absorption or secretion such as choroid plexus of the brain, proximal tubules of the kidney, and endothelia lining the vascular system abundantly express the water channel, Aqp1, reflecting the importance of Aqp1 in the process of fluid balance in these tissues. Process of fluid secretion and absorption in these tissues are intimately linked to the activation of the nitric oxide cascade involving soluble guanylyl cyclase (sGC) and the production of cGMP, and to receptor guanylate cyclases regulating the cGMP-signaling pathway. Due to the capacity of Aqp1 to function as both a cGMP-regulated ion channel and constitutive water channel, the direction or magnitude of Aqp1-mediated water flux could be influenced by the inward cation flux provided by activated Aqp1 ion channels. This section reviews possible roles for Aqp1 ion channels.
The dual function of Aqp1 opens the possibility that the direction of Aqp1-mediated water flow is influenced by the direction of Aqp1-mediated ion flux. Parallel ion fluxes of ions and water could promote water flux in the absence of an overall net osmotic gradient, described by a phenomenon known as “solute drag”. For example, Na⁺ coupled cotransporters enable water uptake during glucose transport due to localized increases in osmotic pressure (Loo, et al., 1996; Meinild, et al., 1998; Zeuthen, 1994; Zeuthen, et al., 1997; Zeuthen, et al., 2002). The antibiotic, Gramicidin A, forms ion channels that couple water molecules with cation transport (Levitt, et al., 1978; Rosenberg and Finkelstein, 1978). Since Aqp1 is both an osmotic water channel and cGMP gated ion channel, it is conceivable that, local ion accumulation in the channel vestibule could influence the direction of water flux. While this idea remains to be proven, examples from the literature are consistent with the proposed role of cGMP-induced Aqp1 channel activity in governing fluid transport and cell volume.

(1) Angiogenesis and Endothelial cell (EC) permeability

Increased vascular permeability is a prerequisite for the development of new blood vessels from pre-existing vessels (angiogenesis) (Bates and Harper, 2002; Bates, et al., 2002; Kutryk and Stewart, 2003; Michel and Curry, 1999; Murohara and Asahara, 2002) and is limited by regulatory barrier cells of the endothelia that line the blood vessels. Vascular permeability is markedly increased in diseases such as heart disease, peripheral vascular disease, stroke,
cancer, and type 2 diabetes, where pathological angiogenesis ensues (Bates and Harper, 2002). Vascular paracellular EC permeability is normally limited by adhesive interaction of tight junctions between neighboring cells, creating a highly resistant barrier (Alexander and Elrod, 2002). The formation of tight junctions involves numerous proteins such as occludin, claudin family members, junctional adhesion molecules, and linker proteins such as the ZO-family members (Figure. 1.10).

Angiogenic mediators such as vascular endothelial growth factor (Senger, et al., 1990), Platelet Activating Factor (PAF) (Noel, et al., 1996; Ramirez, et al., 1996; Ramirez, et al., 1995), Bradykinin (BK) (Mayhan, 1992), histamine (Buckley and Ryan, 1969), estrogen (Cho, et al., 1998), and Substance P (Baluk, et al., 1997) cause rapid increase of EC permeability to water and solutes. Vessel wall shear-stress, a known physiological stimulator of angiogenesis, also enhances EC permeability (Ando, et al., 1993; Noria, et al., 1999). What is striking is that all of these EC permeability-promoting factors converge on the nitric oxide (NO)-cGMP signaling pathway (Michel and Curry, 1999) which is clearly involved in promoting endothelial permeability in vivo (Cho, et al., 1999; He, et al., 1997; He, et al., 1998; He, et al., 2000; Noel, et al., 1996; Wu, et al., 1996). Studies in vivo have shown cGMP is both necessary (Cho, et al., 1999; DeFouw and DeFouw, 2001; Meyer and Huxley, 1992; Wu, et al., 1996) and sufficient for increasing vascular EC permeability (He, et al., 1998; Michel and Curry, 1999; Sarker and Fraser, 2002). Conflicting results were reported for
studies in vitro performed on large vessels or nonexchange microvessels (Draijer, et al., 1995; Westendorp, et al., 1994; Yuan, 2002). These studies showed that cGMP elevation decreased vascular permeability. EC monolayer cultures are problematic because the normal expression of native EC proteins is altered (Yuan, 2002). As a consequence, experimental results from in vitro studies require careful interpretation as well as independent confirmation. Inhibiting cGMP production alone is sufficient to block the enhanced permeability effects of angiogenic factors (Michel and Curry, 1999; Noel, et al., 1996), thus demonstrating that enhanced EC permeability is mediated through the cGMP signaling pathway.

Angiogenic mediators are thought to increase vascular permeability by disrupting adhesive junctional proteins (Baldwin and Thurston, 2001; Dejana, 1997; Haselton, et al., 1989; van Nieuw Amerongen, et al., 2000). Initially, it was proposed that intercellular gaps form when adjacent EC contract, disrupting tight junction interactions, thus creating intercellular spaces (Majno, et al., 1969). Numerous studies contradict this hypothesis. It is well established that cGMP induces EC relaxation, not contraction (Davis, et al., 2002; Giardina, et al., 2002; Rivero-Vilches, et al., 2003). In addition, PAF- and BK-mediated permeability was not profoundly reduced by blocking actin-myosin contractile machinery (Adamson, et al., 2003) nor was cGMP-mediated permeability affected by intracellular Ca^{2+} levels which dictates EC contractions (He, et al., 1998). These results suggest that increased permeability can occur independent of EC
contraction and that cGMP regulation of other molecular targets must lead to the increased EC permeability.

The disruption of junctional integrity appears to be in part due to cytoskeletal rearrangement. Ultrastructural studies showed that vessel wall shear-stress, a physiologic mediator of cGMP production in EC, causes cell swelling (Masuda, et al., 2003; Noria, et al., 1999) and appears to disrupt adhesive interactions of junctional proteins (Noria, et al., 1999). Ischemia is a potent stimulator for vascular permeability and angiogenesis (Scholz, et al., 2003). Interestingly, ischemic tissues from the lung and muscle were shown to have swollen ECs (Kim, et al., 2001; Thomson, et al., 1996). One study found that non-specific prolonged depolarization induced with various depolarizing agents, including gramicidin D, formed intercellular gaps in monolayers of bovine corneal EC (Chifflet, et al., 2003). The intercellular gap formations were associated with actin and microtubule reorganization (Chifflet, et al., 2003). Although the authors attributed the gap formations solely to the effects of depolarization, the mechanism of how depolarization led to cytoskeletal reorganization was not addressed. I suggest that the effect of depolarization on cytoskeletal reorganization is partly mediated by the influx of ions through gramicidin D followed by the obligatory influx of water through Aqp1 proteins. Cell swelling transfers mechanical tension to the cytoskeleton and leads to the remodeling of cytoskeleton by the polymerization and depolymerization of actin (Jakab, et al., 2002; Sun and Levitan, 2003). In vascular EC, Na⁺ influx through
activated Aqp1 ion channels could increase cell volume altering cell shape that could lead to disruption of tight junction integrity leading to increased paracellular permeability of fluids (Figure 1.11). Aqp1-mediated EC swelling could account for the cGMP-coupled rapid and reversible intercellular gap formation.

The molecular targets of cGMP that lead to increase EC permeability are not well defined. The currently known candidates for cGMP signaling include cAMP-specific phosphodiesterases (PDEs) that modulate cAMP levels (He, et al., 2000) and cGMP-dependent protein kinases (PKG) (Hofmann, et al., 2000 136). It is not clear exactly what role PKG might serve in EC permeability. PKG activation was shown to reduce cadherin expression (Park, et al., 1999) and shown to increase permeability by decreasing cAMP levels (Defouw and Defouw, 2000). Studies have shown that elevated cAMP reduces EC permeability (DeFouw and DeFouw, 1999; Draijer, et al., 1995; He, et al., 1998; He, et al., 2000; Morel, et al., 1990). I suggest that Aqp1 channels are a logical candidate for cGMP-mediated enhanced permeability and Aqp1 channel functions work in concert with other cGMP-mediated processes leading to increased vascular permeability (Figure 1.11).

(2) Cerebral Spinal Fluid Production and Renal Function

The cGMP-elevating protein hormone atrial natriuretic peptide (ANP), modulates fluid transport in a number of epithelia in which Aqp1 is expressed. For example, cerebral spinal fluid (CSF) secretion is decreased with elevated
cGMP in choroid plexus cells. Aqp1 is expressed exclusively on the apical side of the choroid plexus and is thought to enable water pathway for this side. Figure 1.12 depicts a model in the choroid plexus epithelia (review; Boassa and Yool, in press) in which ANP-elevated cGMP decreases the production of cerebral spinal fluid by Na\(^+\) influx through activated Aqp1. An inward Na\(^+\) current would be expected to decrease the net efflux of Na\(^+\), decreasing water efflux on the apical side.

Although the effects of elevated cGMP have not been extensively studied in the renal epithelia that abundantly express Aqp1, the results of a microfusion study in renal tubules do suggest that localized elevation of cGMP does increase the net reabsorption of Na\(^+\) in the proximal tubules. Figure 1.13 depicts a model in the proximal tubule cell of the kidney in which activation of Aqp1 ion conductance by cGMP increases net Na\(^+\) reabsorption (Yool and Weinstein, 2002). Nitric oxide donor, S-nitroso-N-acetylpenicillamine (SNAP), applied on the luminal side of renal tubules resulted in increased reabsorption of Na\(^+\) and water across the tubules (Wang, 1997) whereas inhibition of NO decreased volume reabsorption by 47% (De Nicola, et al., 1992). This is consistent with a model based calculation which predicted that the activation of even a small subpopulation of Aqp1 ion channels on the luminal side could increase cell Na\(^+\) entry by 45%, translating to an estimated 18% increase in overall epithelial Na\(^+\) reabsorption (Yool and Weinstein, 2002). Activation of Aqp1 on the basolateral side would negate this directional effect.
Data from this dissertation work supports the idea that Aqp1 ion channel activation is dependent on multiple signaling pathways, including the downstream pathways of the tyrosine kinase signaling pathway. Aqp1 expression appears to be highly coordinated with other signaling molecules (Abu-Hamdah, et al., 2004; Cowan, et al., 2000; Schnitzer and Oh, 1996). Considering the importance of signaling complexes of specific intracellular microdomains to protein behavior, perhaps the polarized function of Aqp1 occurs by the polarization of signaling complexes that dictate cGMP-activation of Aqp1 ion channel. The distinct gating modes of Aqp1 ion channel could provide the basis for directional salt and water transport in barrier cells.

All three examples (vascular system, kidney, and choroid plexus) are consistent with the proposal that Aqp1 could be one of the molecular target of cGMP, and perhaps a missing link in the well-known phenomena of cGMP-mediated fluid secretion and absorption.
1.3 GENERAL HYPOTHESIS AND AIMS

Aqp1 was initially described as an exclusive osmotic water channel (Preston and Agre, 1991) but the scope of functional roles for Aqp1 is ever expanding. Aqp1 is a cGMP-gated ion channel (Anthony, et al., 2000; Saparov, et al., 2001) that also mediates CO₂ transport across the plasma membrane (Blank and Ehmke, 2003). Other members of the Aquaporin family also have been identified as ion channels including Aqp0 (Ehring, et al., 1990) and Aqp6 (Yasui, et al., 1999a). Aqp1 is emerging as a sophisticated multi-functional protein shown to form macromolecular protein complexes with other transmembrane proteins interactions interactions (Abu-Hamdah, et al., 2004; Cowan, et al., 2000). Protein-protein interactions regulate major cellular events such as intracellular signaling, trafficking, targeted proteolysis, cytoskeletal organization, and gene expression. Disruption of Aqp1 interaction with a receptor tyrosine kinase protein, EphB2, results in reduced fluid production in the inner ear and improper development (Cowan, et al., 2000). The reduced endolymph fluid production suggests that the water function of Aqp1 is influenced by interaction with EphB2.

Identifying the mechanisms involved in regulating cGMP-activation of Aqp1 ion channels was the central objective of this dissertation. My hypothesis
is that Aqp1 channel proteins are gated convergently by multiple signaling pathways. My experiments have confirmed other work showing that only a small fraction of Aqp1 proteins behave as ion channels whereas the vast majority remain active as water channels when heterologously expressed in *Xenopus* oocytes. The ability of Aqp1 to serve as a cGMP-activated ion channel appears to be tightly regulated. The Aqp1 carboxyl tail encodes a number of putative regulatory domains that could be targeted by molecules of intracellular pathways. The carboxyl tail of Aqp1 contains a PDZ-binding domain (Cowan, et al., 2000), a putative cGMP-binding domain (Anthony, et al., 2000), putative consensus sites for phosphorylation by tyrosine kinases (Songyang and Cantley, 1995) and cGMP-activated protein kinase (PKG) (Kennelly and Krebs, 1991), and a putative Ca^{2+} binding site (Fotiadis, et al., 2002). Aqp1 is subject to phosphorylation by PKA (Han and Patil, 2000) and is able to form macromolecular protein complexes by binding a PDZ domain-containing linker protein, PICK-1, on the PDZ binding domain on the carboxyl tail of Aqp1 (Cowan, et al., 2000).

To identify mechanisms regulating cGMP-activation of Aqp1 ion channels, I used two-electrode voltage clamp recordings of *Xenopus* oocytes expressing Aqp1, manipulated intracellular signaling pathways, and designed selected mutations of Aqp1. Data presented here support the hypothesis that Aqp1 proteins are convergently gated by multiple signaling pathways. Some of these signals may serve as ‘master switches’ governing the ability of Aqp1 to behave as an ion channel. My specific aim was to identify the regulatory domains of
Aqp1 that mediated the potentiating effect of insulin treatment and to characterize the effects of insulin on the properties of Aqp1 ionic current. *Xenopus* oocytes are known to have insulin growth-factor I tyrosine kinase receptor (Scavo, et al., 1991). I have found that insulin enhanced the SNP-induced ion conductances of Aqp1-expressing oocytes. Two distinct cGMP-activated gating modes (rapid and slow) in Aqp1-expressing oocytes were characterized and could reflect multiple gating mechanisms. The roles of the putative regulatory domains of Aqp1 C terminus in both the potentiating effect of insulin and the generation of distinct gating modes were tested by truncation of these sites by site-directed mutagenesis.

Results provided here show that insulin potentiated cGMP-induced activation of Aqp1 ionic current but did not influence the gating mode. The distal C terminus did not appear to mediate the effects of insulin, but the removal of the distal C terminus did result in the selective loss of the slow gating mode, therefore suggests that this region of Aqp1 is required for the slow gating mode. Taken together, these results suggest that Aqp1 ion channel is not solely gated simply by cGMP binding but that cGMP-induced activation involves multiple signaling pathways.
Figure 1.1 Predicted topology of Aquaporin1 subunit as a six transmembrane (TM) spanning protein with intracellular amino and carboxyl termini. TM are connected by Loops A,B,C,D, and E. Loops B and E contain the NPA motif that is the Hallmark signature of all MIP channels. The filled in circles indicate residues that are conserved among all AQPs. *N42 indicates site of glycosolation. Cys189 mediates Hg²⁺ block of water permeability and Tyr 186 mediates external TEA⁺ inhibition of water permeability (Yool and Stamer, 2003).
Figure 1.2 Proposed “hourglass” model of AQP based on functional data. Loops B and E invaginate into the membrane and the NPA motifs interact forming the aqueous pathway. Filled in circles indicate residues that were mutated for functional studies (Jung et al., 1994).
Figure 1.3 Side view of one subunit of Aquaporin 1. Loop B structure is colored in red and Loop E structure is colored in green (Rasmol 2.6; Aqp1 PDB# 1FQY, submitted by K. Murata et al., 7-Sept-2000).
Figure 1.4 Alignment of the Loop B domains of AQPs show conserved patterns across two segments shown in blue and red. See next page for details.
Figure 1.4 Alignment of the Loop B domains of AQPs show conserved patterns across two segments shown in blue and red. In Aqp6 mutation of lys (underline) to glu abolished ion channel function and gained water channel function. Sources of sequences: hAqp0 human P30301, mAqp0 mouse NP_032626, hAqp1 human NM_198098.1, mAqp1 mouse NP_031498, hAqp2 human I64818, mAqp2 mouse P56402, hAqp3 human NP_004916, mAqp3 mouse Q8R2N1, hAqp4 human I39178, mAqp4 mouse P55088, mAqp5 mouse Q9WTY4, hAqp5 human AAH32946, hAqp6 human NP_001643, mAqp6 mouse Q8C4A0, hAqp7 human NM_001170, mAqp7 mouse AB010100, hAqp8 human O94778, mAqp8 mouse P56404, hAqp9 human NP_066190, mAqp9 mouse NP_071309.
Figure 1.5 Alignment of a segment of Loop E in AQPs shows a conserved pattern shown in blue. See next page for details.
Figure 1.5 Alignment of a segment of Loop E in AQPs shows a conserved pattern shown in blue. Sources of sequences: hAqp0 human P30301, mAqp0 mouse NP_032626, hAqp1 human NM_198098.1, mAqp1 mouse NP_031498, hAqp2 human I64818, mAqp2 mouse P56402, hAqp3 human NP_004916, mAqp3 mouse Q8R2N1, hAqp4 human I39178, mAqp4 mouse P55088, mAqp5 mouse Q9WTY4, hAqp5 human AAH32946, mAqp6 human NP_001643, mAqp6 mouse Q8C4A0, hAqp7 human NM_001170, mAqp7 mouse AB010100, hAqp8 human O94778, mAqp8 mouse P56404, hAqp9 human NP_066190, mAqp9 mouse NP_071309, hAqp10 human AL354980.16.
Figure 1.6 Schematic depiction of the selectivity filter of one subunit of Aquaporin and disruption of the single file column of water molecules. The constriction site is the narrowest region of the selectivity filter. The NPA region is proposed as the proton filter because the interaction of water with the Asn (N) of this region contributes to the reorientation of water molecules (Tajkhorshid, et al., 2002).
Figure 1.7 Extracellular view of Aquaporin-1 homotetramer.
Transmembrane domains 1-3 (gold), 4-6 (blue) and Loops B and E (green) are indicated in all subunits. The four-fold axis of symmetry is indicated by red filled in circle and the psuedo two-fold axis is indicated by a dashed white line for one subunit. (Rasmol 2.6; Aqp1 PDB# 1FQY, submitted by K. Murata et al., 7-Sept-2000).
Figure 1.8 Intracellular view of crystal structure of one subunit of AQP1 from bovine (left) and human (right). The four consecutive sequence of arginines in Loop D (R159 – R162) are highlighted in red as well as a lysine in TM2. Circle indicates the location of the central cavity. Models were created with Cn3D (NCBI 3D Structure Viewer) with information from the Molecular Modeling Database for AQP1 (Bovine) (MMDB# 18789, submitted by H.Sui et al., 19-Oct-2001) (Human) (MMBD # 16226, submitted by G.Ren et al., 18-Apr-2001).
Figure 1.9 Differences in reported endogenous whole cell current of HEK cells in a conventional patch clamp mode. **A.** Current traces of Aqp1-expressing HEK cell with cGMP application (Tsunoda *et al.*, 2004). No ion current increase was reported. **B.** Current traces of a cell-attached patch clamp mode of a HEK cell (Birdsell and Yool, unpubl). **C.** Current traces of endogenous channels in a HEK cell recorded in a whole cell patch clamp mode (Zhue *et al.*, 1998). **D.** Current traces of endogenous channels in a HEK cell recorded in a whole cell patch clamp mode (Birdsell and Yool, unpubl) (Figure made by A. Yool).
Figure 1.10  Diagram illustrating the proposed interactions of junctional proteins. These proteins limit paracellular permeability of endothelial cells to water and solutes (Adapted from Huber et al., 2001).
Figure 1.11 Diagram proposing how cGMP activation of Aqp1 ion channels may contribute to the increase of endothelial permeability. See next page for details.
Figure 1.11 Diagram proposing how cGMP activation of Aqp1 ion channels in EC may contribute to the increase of endothelial permeability. A. cGMP is elevated by the Ca$^{2+}$/NO/cGMP signaling cascade activated by angiogenic and inflammatory mediators (Michel, 1999). cGMP mediates increase vascular permeability and cAMP has an opposite effect. Aqp1 ion channel is activated with cGMP and might work in concert with other processes involved in the cGMP-mediated increase of EC paracellular permeability. B. Solute-drag influx of water through cGMP-activated Aqp1 channels may mediate EC swelling leading to compromise of junctional proteins.
Figure 1.12 Schematic diagram of the Choroid Plexus epithelia. This diagram proposes how activation of Aqp1 ion channel contributes to the decrease of Cerebral Spinal Fluid production by creating a pathway for Na⁺ influx from the apical side therefore decreased water flux out of the apical side (simplified by Boassa from Stamer and Yool, 2004).
Figure 1.13 Schematic diagram of solute transport pathways across a proximal tubule cell. Luminal and peritubular membrane express Aqp1 where high water permeability is mediated. Net reabsorption of NaCl is primarily mediated by Na<sup>+</sup>/H<sup>+</sup> exchanger working in parallel with HCO<sub>3</sub>/Cl<sup>-</sup> exchanger on the luminal side. The Na<sup>+</sup>/K<sup>+</sup> ATPase on the peritubular side serves as the Na<sup>+</sup> exit. cGMP stimulation Aqp1 may serve as an additional pathway that may increase Na<sup>+</sup> entry (Simplified version of Yool and Weinstein, 2002).
CHAPTER 2:  AQUAPORIN-1 ION CHANNEL FUNCTION IS REGULATED BY INTRACELLULAR SIGNALING ACTIVATED BY THE INSULIN SIGNALING PATHWAY.

2.1 INTRODUCTION

Aqp1 shows unusual gating properties when compared with other classes of ligand-gated channels. Only a small fraction (0.0001 to 0.002%) of the Aqp1 channels expressed in Xenopus oocytes or reconstituted in lipid bilayers served as ion channels, despite saturating levels of cGMP (Saparov, et al., 2001; Yool and Weinstein, 2002). Furthermore, the magnitude of cGMP-activated current in Aqp1-expressing oocytes varies greatly among oocyte batches despite consistent levels of water permeability. This inconsistency indicates that cGMP is necessary but not sufficient as the signaling pathway regulating the ion channel function of Aqp1. The central question is what regulates the ability of Aqp1 to serve as a cGMP-activated ion channel?

A number of ion channel classes are targeted by intracellular messengers (Nara, et al., 2000; Szellas and Nagel, 2003). The existence of several different putative regulatory domains in the carboxy terminus of Aqp1 supports the idea that Aqp1 ion channel function may be subject to complex modes of regulation by intracellular signaling pathways in addition to cGMP. For example, the carboxyl tail of Aqp1 contains a PDZ-binding ligand domain (Cowan, et al., 2000). Colocalization of Aqp1 and a receptor tyrosine kinase EphB2 is mediated by a linker protein, PICK1, that interacts with the PDZ-binding domain in the Aqp1
carboxyl tail (Cowan, et al., 2000). Aqp1 association with EphB2 is necessary for the proper development of the inner ear (Cowan, et al., 2000). Other candidate regulatory sites in Aqp1 carboxyl tail include consensus sites for phosphorylation by PKG/PKA kinases (Han and Patil, 2000; Kennelly and Krebs, 1991) and tyrosine kinases (Songyang and Cantley, 1995), a Ca\textsuperscript{2+} binding site (Fotiadis, et al., 2002), and putative cGMP-binding domain (Anthony, et al., 2000; Boassa and Yool, 2002).

Tight control of ion channel function by multiple signaling pathways has precedent in the ATP-hydrolyzing protein, CFTR, in which multiple target sites result in a synergistic event of channel activation (Gadsby and Nairn, 1999). CFTR contains two nucleotide binding domains (NBD) as well as 10 sites of phosphorylation that are targeted by PKA, PKC, and PKG as well as phosphatases (Gadsby and Nairn, 1999; Szellas and Nagel, 2003). Phosphorylation of CFTR by PKA is prerequisite for ATP-mediated ion channel activation (Tabcharani, et al., 1991). The binding of ATP to the NBD2 appears to mediate the opening while the hydrolysis of ATP is responsible for channel closure (Basso, et al., 2003). The exact mechanism by which phosphorylation contributes to the function of channel opening is still under investigation.

Big Brain (BIB) is a Drosophila MIP channel that functions in neurogenesis (Rao, et al., 1992). When expressed in X. oocytes, BIB serves as an ion channel that is negatively regulated by tyrosine kinases (Yanochko and Yool, 2002). In oocytes, insulin binds to an endogenous insulin-like growth receptor (IGF) that
functions as a tyrosine kinase (Scavo, et al., 1991). Insulin receptors trigger signaling cascades involved in a wide spectrum of biological processes, including cell growth, glucose uptake, glycogen, lipid and protein synthesis (Taha and Klip, 1999). The biological end points of insulin often involve various serine/threonine kinases, including those in the mitogen-activated protein kinase (MAPK) signaling pathway (Taha and Klip, 1999). The insight that connected the involvement of insulin signaling pathway in the regulation of Aqp1 function stemmed from an anecdotal observation in which I noted that batches of oocytes that yielded high BiB currents yielded low Aqp1 currents and visa versa. This led me to test the hypothesis that Aqp1 serves as a target for intracellular pathways that include tyrosine kinases. The idea that Aqp1 is a convergent target for several intracellular signaling pathways is supported by the fact that Aqp1 also is regulated by PKA kinase (Han and Patil, 2000) and G_{i3}-PLA2 signaling (Abu-Hamdah, et al., 2004).

Data presented in this chapter provide insight into potential mechanisms that may dictate Aqp1 ion channel ability to activate in the presence of cGMP. I show here that insulin positively regulates the cGMP-mediated activation of Aqp1 ion channels when expressed in X. oocytes. Surprisingly, systematic truncation of the putative regulatory sites (PDZ-binding domain, tyrosine phosphorylation site, and cGMP-binding site) resulted in no change in the effect of insulin on enhancing Aqp1 current activation, suggesting that the regulatory region is upstream of the Aqp1 carboxyl tail or mediated by an associated protein.
Although biochemical analysis showed that Aqp1 channels expressed in *Xenopus* oocytes are targeted for phosphorylation by an endogenous serine/threonine kinase, the consequence of this phosphorylation for ion channel function remains unknown. Multiple signaling pathways may serve as ‘master switches’ governing the ability of Aqp1 channels to behave as gated ion channels.

2.2 MATERIALS AND METHODS

(1) *Molecular techniques.* Wild-type Aqp1 subcloned into the 5',3' *Xenopus* β-*globin* UTR plasmid vector was provided by Dr. Peter Agre (John Hopkins, Baltimore MD). A stop codon was introduced at K243, D237, and I260 by site-directed mutagenesis performed with Stratagene QuickChange (La Jolla, CA) using PfuTurbo DNA polymerase. The tyrosine 253 and serine 236 residues identified as potential targets of kinase phosphorylation were mutated to alanine. Complementary pairs of synthetic oligonucleotide primers containing the desired mutation were used (mutation bases underlined):

K243Z

“sense”: 5’- CCT CAC AGA CCG CGT GTA GGT GTG GAC CAG-3’

“antisense”: 5’- CTG GTC CAC ACC TAC ACG CGG TCT GTG AGG-3’
D237Z

“sense”: 5’- GCC CCA CGC AGC AGT TAG CTC ACA GAC CGC-3’
“antisense”: 5’- GCG GTC TGT GAG CTA ACT GCT GCG TGG GGC-3’

I260Z

sense: 5’- CCT GGA TGC CGA CGA CTA GAA CTC CAG GGT GGA G-3’
antisense: 5’-CTC CAC CCT GGA GTT CTA GTC GTC GGC ATC CAG G-3’

Y253A

sense: 5’-GGC CAG GTG GAC GAG GCT GAC CTG GAT GCC GAC-3’
antisense: 5’-GTC GGC ATC CAG GTC AGC CTC CTC CAC CTG GCC-3’

S236A

sense: 5’-GCC CCA CGC AGC GCT GAC CTC ACA GAC C-3’
antisense: 5’-GGT CTG TGA GGT CAG C GC TGC GTG GGG C -3’

K243Z-S236A

sense: 5’-GCC CCA CGC AGC GCT GAC CTC ACA GAC C-3’
antisense: 5’-GGT CTG TGA GGT CAG CGC TGC GTG GGG C -3’

The PCR product of the full length plasmid was treated with DpnI endonuclease to digest the methylated wild-type cDNA template. The plasmid was transformed
and amplified in E. coli XL1-blue supercompetent cells. DNA sequencing, using forward and reverse primers to check the entire Aqp1 sequence, confirmed successful incorporation of the mutations. Capped cRNA transcripts encoding Aqp1 wild type and mutants were synthesized in vitro with T3 RNA polymerase enzyme, using BamHI linearized DNA as a template. Correct RNA size was determined by Northern gels and RNA concentration was determined by UV absorbance spectrophotometry.

(2) Oocyte preparation and injection. Oocytes were harvested from female *Xenopus laevis* and defolliculated by 1-2 hrs treatment with collagenase (1.5 mg/ml Type 1, Sigma) in the presence of a trypsin inhibitor (0.5 mg/ml Type III-); Sigma Chemical Co., St. Louis, MO). One day following isolation, Stage V and VI oocytes were injected with 50 nl of sterile water (control oocytes) or 50 nl of sterile water containing cRNA encoding Aqp1 (1 ng/50nl) or K243Z (20 ng/50nl) or D237Z (20ng/50nl). Injected oocytes were maintained at 18 °C in ND96 culture medium (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 5 mM Hepes, 2.5 mM pyruvic acid, 100 U/ml penicillin, and 100 ug/ml streptomycin, pH 7.6) for 2-5 days to allow protein expression before electrophysiological recording. Oocytes that successfully expressed channels of the Aqp1 wildtype or truncation mutants ruptured in distilled water within 3 minute and 6 minutes, respectively. Control oocytes resisted rupture for over 20 minutes.
(3) Electrophysiological recordings. Two-electrode voltage clamp recording was performed to assess the macroscopic current of Aqp1 wildtype- and mutant-expressing oocytes. Borosilicate electrodes (1-3 MΩ) were filled with 3M KCl and the data were collected with a GeneClamp amplifier (Axon Instruments, Foster City, CA), filtered at 2 kHz digitized at 10 kHz, and stored to hard disk for off-line analysis performed with pClamp software (Axon Instruments, Foster City, CA). Recordings were done at room temperature using isotonic Na+ recording saline (100 mM NaCl, 5 mM MgCl₂, 5 mM Hepes, pH 7.3). Aqp1 ion channels were activated with 8-14 mM Sodium Nitroprusside (SNP) (Sigma Co., St. Louis MO), a nitric oxide donor that stimulates soluble guanylate cyclase to produce cGMP in X. oocytes (Boassa and Yool, 2003). SNP was prepared in recording saline (200-400 mM stock), maintained on ice protected from light. Final working concentration was achieved by adding calculated stock solution to the recording chamber. Oocytes were tested by a single dose of SNP. A subset of oocytes were exposed to the following treatments: The endogenous tyrosine kinase signaling pathway of oocytes was activated by 3-10 hrs preincubation with 1.7-3.4 µM bovine insulin (Sigma Co, St Louis, MO). The 3-10 hrs incubation time window was selected based on the time required for the insulin-mediated enhancing effect observed over several batches of oocytes. The endogenous cGMP-activated protein kinase (PKG) activity was inhibited by 0.5-2 hr incubation with PKG-specific inhibitor, KT5823 (Sigma Co, St Louis, MO). Channel activation was monitored using a voltage protocol that stepped to +40 mV for 50
msec from a holding potential of –40 mV. Magnitude of activation was measured by the net current value at 240 sec post-SNP application, to measure the height of current rise at a predetermined time.

(4) Osmotically-induced swelling assay. Analysis of water permeability was performed by swelling assays in hypotonic saline, following methods published previously (Anthony, et al., 2000). Briefly, oocytes in isotonic Na⁺ saline (200 mOsM) were transferred into a swelling assay chamber containing hypotonic saline (100 mOsM) and the increase in cross-sectional area was captured by a high performance CCD video camera (Cohu, San Diego, CA). Images were captured every 4 seconds for 3 minutes and analyzed using Scion Image Software (Scion Corporation, Frederick, MD). Measured cross sectional areas of the oocytes were used to calculate spherical volume. The relative increase in volume was standardize to the initial volume at time zero and plotted as a function of the time that oocytes were exposed to hypotonic saline. The plotted data were fit with a model-independent second order polynomial, and the initial rates of swelling [(d(V/V₀)/dt)] were calculated from the linear component of the fit. The osmotic water permeability (Pf) values for each oocyte were calculated as: Pf = [V₀ x d(V/V₀)/dt]/[S x V w X (osm in – osm out)], with initial oocyte volume V₀ = 9 x 10^-4 cm³, initial oocyte surface are S = 0.045 cm², molar ratio of water V w = 18 cm³/mol, osmolarity inside the oocyte osm in = 200 mOsM, and osmolarity
outside the oocyte in hypotonic saline osm\textsubscript{out} = 100 mOsM. Pf values (cm/s x 10^{-4}) are given as mean ± SEM.

(5) **Cellular fractionation.** 2-3 days post cRNA (20ng/50nl) injection, the plasma membrane proteins of oocytes expressing Aqp1 and K243Z were extracted using a method adapted from Blondeau et al., 1984. Twenty-five oocytes were lysed at 4°C by trituration in 500 µl of lysis buffer (83 mM NaCl, 1 mM MgCl\textsubscript{2}, 10 mM Hepes pH 7.9, 10 mM PMSF, 1% aproprotinin, 10µg/ml leupeptin, 10 µg/ml pepstatin A, 2 mM sodium orthovanadate). Cell lysates were centrifuged (1000 x g) for 10 minutes at 4°C to remove the yolk fraction. The supernatant was transferred to a new tube and centrifuged (10,000 x g) for 20 minutes at 4°C to pellet the plasma membrane fraction. The plasma membrane enriched pellet was resuspended with 25 µl of Laemmli buffer (10% glycerol, 50 mM Tris HCl, 2% SDS, 5% β-mercaptoethanol, 0.02% bromophenol blue) and resolved on a SDS-PAGE for western blotting. Leupeptin, pepstatin A, PMSF and sodium orthovanadate were purchased from Sigma Chemical Company (St. Louis, MO).

(6) **Immunoprecipitation and Western blotting.** To assess direct phosphorylation of tyrosine and serine/threonine residues, I conducted immunoprecipitation (IP) studies. Oocytes were injected with 20 ng/50nl cRNA encoded with wild-type, Y253A, and S236A, incubated for 48 hrs in ND96 saline to allow protein expression. Wild-type or Aqp1 mutants-expressing oocytes were preincubated
with or without 3.4 μM of bovine insulin (Sigma) and 20 oocytes per treatment group were homogenized on ice in lysis buffer as described in the cellular fractionation methods section. Aqp1 was immunoprecipitated from the lysed supernatant with anti-Aqp1 antibodies raised against a carboxyl tail fusion peptide (Aqp1-COOH). Anti-Aqp1 antibodies were incubated in cell lysates overnight at 4 ºC with rotation. 10 µl of 1:1 slurry of Protein A beads were added to cell lysates containing the anti-Aqp1 antibodies (Pierce, Rockford, IL) for 1-2 hrs at 4 ºC with rotation. The antigen-antibody complexes were pulled down with Protein A Beads. The IP protein samples were resolved on paired sets of 10% SDS-page gels. The resolved proteins from each gel was transferred onto a PDVF membrane (polyvinylidene difluoride; Bio Rad, Hercules, CA) in CAPS buffer (10% MeOH, 10 mM of 3-(cyclohexylamino)-1-propanesulfonic acid, Sigma) at 100 V for 90 minutes on ice. The blot containing Aqp1 IP protein sample was probed with antiphosphotyrosine or antiphosphoserine/threonine antibodies and visualized for presence of bands by horseradish peroxidase chemiluminescence. These blots were then stripped and reprobed with Aqp1-COOH antibodies to visualize immunoreactive bands. Protein pellets from cellular fractionation process were resolved by 10% SDS-PAGE and were transferred on to a PDVF membrane in CAPS buffer at 100 V for 90 minutes on ice. Blots were probed with affinity purified with antibody raised against a C-loop fusion protein at 1:500 dilution (Aqp1-Loop C) (provided by Dr, W.D. Stamer, Tucson, AZ) in 1.5% milk to prevent non-specific binding in TBST (154 mM NaCl,
10 mM Tris-base, 0.2% Tween-20) overnight at 4°C. The PDVF membrane was subjected to three 15 minutes washes in TBST and then exposed for 1 hr at 22°C to donkey anti-chicken horse-radish peroxidase conjugated antibody (1:10,000) (Jackson Immuno Research Labs, West Grove, PA). The blot was subjected to washes with TBST as described above and protein bands were recognized by the primary antibodies visualized by enhanced chemi-luminescence (Pierce, Rockford, IL) and recorded on X-OMAT film (Kodak).

(7) Immunofluorescence of intact oocytes. Intact oocytes, 2-3 days post cRNA injection, were fixed in distilled water with 4% para-formaldehyde for 2-3 hours at 4°C, then briefly washed with 30 mM SSC (300 mM sodium chloride, 20 mM Sodium-Citrate) and 100 mM glycine. Oocytes were permeabilized in 30 mM SSC in 0.1% Triton X-100, followed by overnight incubation with antibody raised against the carboxyl tail of Aqp1 (1:500) in the antibody dilution buffer (30 mM SSC, 1% bovine serum albumin, 0.05% Triton X-100). Oocytes were washed in antibody wash buffer (30 mM SSC, 0.05% Triton X-100) for 1 hour and then incubated with a FITC conjugated anti-rabbit antibody (Jackson Immuno Research Labs, West Grove, PA) for 1 hour in antibody dilution buffer. Labeled oocytes were imaged by a 10x objective Leica TCS-4D laser scanning confocal microscope.
2.3 RESULTS

SNP-induced Aqp1 current response is highly variable across experiments.

The Aqp1 ionic conductance depends on cGMP, but the magnitude of the response was not uniform between experiments. To determine whether this variability in response was due to low levels of Aqp1 expression at the plasma membrane, I correlated amplitudes of ionic current responses with water permeability in the same oocytes, and evaluated the responses with respect to the number of days after cRNA injection, concentration of cRNA injection, month of experiment (seasonal variability), and insulin treatment.

Data presented in figure 2.1 (A-C) demonstrate the variability of Aqp1 current activation by SNP across batches of oocytes. Panel (A) shows the water permeability values of individual Aqp1-expressing oocytes plotted against the corresponding net ion conductance of the same oocyte measured at approximately 15 minutes after SNP application. The presence of ample water permeability (indicator of Aqp1 expression level) but the lack of corresponding SNP induced ionic current in some oocytes demonstrates that the lack of SNP-induced current did not stem simply from poor expression levels of Aqp1 at the plasma membrane. Direct injection of 8-Br-cGMP in Aqp1-expressing oocytes failed to induce current in some batches of oocytes, suggesting that the lack of current induction is not solely due to insufficient cGMP production or low cGMP levels due to robust activity of phosphodiesterases (PDEs) (Data not shown).
The inconsistency of SNP-induced ionic current in Aqp1-expressing oocytes also was evaluated as a function of the number of days after cRNA injection. The mean net current of Aqp1-expressing oocytes (240 sec after SNP stimulation) was plotted as a function of the number of days after cRNA injection (panel B). The results showed a declining trend of the magnitude of current response with time after cRNA injection. In contrast, the water permeability of Aqp1-expressing oocytes from a single oocyte batch increased as a function of days after cRNA injection, suggesting that expression of Aqp1 increased. Taken together, the function of Aqp1 as ion channels appear to decline with time despite ample expression at the plasma membrane. The ion channel function of Aqp1 proteins could depend on the density of channels expressed on the plasma membrane or intracellular components that may decrease over time.

To determine if the magnitude of SNP-induced Aqp1 ion channel activation correlated with protein concentration expressed at the plasma membrane, I plotted the net conductances of SNP-activated current of oocytes injected with different concentrations of Aqp1 cRNA (Panel C). Water permeability was positively correlated with increasing concentration of cRNA injection (data not shown). However, the correlation between Aqp1 cRNA injection and the net ionic conductance depended on the oocyte batch. Some batches showed a direct correlation; other batches showed a bell-shaped profile where intermediate levels of Aqp1 cRNA resulted in highest net conductance. In summary, the
responsiveness of Aqp1 channels to SNP-activation appears to depend on the oocyte batch, and is subject to decline over time.

Amphibian oocytes are dynamic systems that show seasonal changes in endogenous protein expression (Fraser, 1997) and responses to hormones (Morrill and Kostellow, 1998). Some proteins heterologously expressed in oocytes function differently according to season (Stuhmer, 1998) (Sung, et al., 1996). To determine if there was an effect of season on SNP-induced ion channel activation of Aqp1, batches of Aqp1-expressing oocytes were classified based on month and year of recording for responsiveness to SNP (Table 2.1). Oocyte batches that showed no conductance after SNP in all oocytes tested were considered non-responsive, although Aqp1-induced osmotic water permeability was present. Batches that had a mixture of responsive and non-responsive Aqp1-expressing oocytes were considered non-responsive/responsive. Batches that showed consistent cation conductance in responses to SNP in all Aqp1-expressing oocytes, and no response in controls were considered responsive. A subset of oocyte batches that showed endogenous current (SNP-induced current in controls) were classified as “endogenous” and disqualified from further analysis. Results summarized in Table 2.1 showed no obvious pattern of seasonal influence that dictated Aqp1 channel responsiveness to SNP. A variety of independent variables that influence Aqp1 ion channel function could include genetic and hormonal factors.
that differentially predispose oocytes to down regulate the Aqp1 ion channel function through endogenous signaling mechanisms.

cGMP activation of Aqp1 current in oocytes is positively regulated by the activation of insulin-mediated tyrosine kinases.

My hypothesis was that the cGMP-gating of Aqp1 ion channel is regulated by intracellular signaling pathways. A clue arose from an anecdotal observation: I noted that batches of oocytes that yielded high BIB currents yielded low Aqp1 currents, and batches that yielded low BIB currents yielded high Aqp1 currents. The contrasting responsiveness of BIB and Aqp1 channels in the same oocyte batch suggest that endogenous signaling pathways of oocytes that negatively regulated BIB current could positively influence Aqp1 current. Insulin-activated tyrosine kinases negatively regulate BIB ion channel function (Yanochko and Yool, 2002). To test the hypothesis that insulin-activated tyrosine kinases (either directly or via downstream cascades) could positively regulate Aqp1 ion channel function, Aqp1-expressing oocytes were treated with 1.7 – 3.5 µM insulin for ≥ 3 hrs prior to electrophysiological recordings.

Electrophysiological recordings of SNP-induced macroscopic currents from Aqp1-expressing oocytes demonstrated that insulin is a positive regulator of Aqp1 ion channel function. Figure 2.2 panel (A) shows representative initial and net response current traces and panel (B) shows the current-voltage relationship of traces shown in panel A. Net response was calculated by subtraction of the
initial current from the SNP-activated current. A high concentration of insulin (20 µM) was shown previously to activate endogenous channels in oocytes that display outward rectification and a reversal potential of –19 ± 4 mV (Yanochko and Yool, 2002). However the doses of insulin used in my study (1.7- 3.4 µM) did not induce these endogenous currents in control oocytes in the absence or presence of SNP. Panel C shows the time course of activation of SNP-induced current. Panel D summarizes the normalized net current (at 240 seconds after SNP) in control oocytes and in wild-type oocytes. Data for each group were normalized to the mean current of insulin-treated Aqp1 group in the same oocyte batch. Pre-incubation of oocytes expressing Aqp1 in 1.7-3.4 µM of insulin for > 3.5 hours resulted in a significant enhancement of SNP-induced ion current as compared to non-insulin treated wild-type group and the insulin-treated control group (two-tailed Student’s t-test; Aqp1 *P<0.002; ** P<0.00005) (Figure 2.2 D).

Insulin alone did not induce ionic response, as indicated by the lack of current in Aqp1-expressing oocytes in initial recordings before SNP. Therefore insulin does not activate channels independently, but facilitates the cGMP-dependent gating of Aqp1 ionic currents.

No effect of season on insulin potentiation of SNP-induced Aqp1 ion current.

To determine if the positive effect of insulin on SNP-activation of Aqp1 current was seasonal, the percent of wild-type oocytes (with and without insulin)
that responded to SNP (percent response) was plotted as a histogram by month (Figure 2.3). 50-75% of Aqp1 oocytes treated with insulin responded to SNP independent of month during which they were tested, indicating that the potentiating effect of insulin was not seasonal. The basal activity of intracellular pathways is known to vary in oocytes (Sadler and Maller, 1987; Savchenko, et al., 2001). It is possible that conditioning with insulin could generate a more uniform level of activity that is conducive to Aqp1 ion channel function.

**Insulin treatment does not enhance Aqp1 expression level.**

To determine the mechanism of insulin on enhancing SNP-activation of Aqp1 ion channel function, we tested if insulin enhanced Aqp1 osmotic water permeability used as a measure for protein expression level. Water permeability of Aqp1-expressing oocytes with and without insulin treatment was tested using hypotonic swelling assays. Figure 2.4 summarizes the normalized mean water permeabilities of wild-type-expressing oocytes two batches. Normalized values were calculated by dividing individual water permeability per oocyte by the mean water permeability of non-insulin treated Aqp1 group in the same oocyte batch. There was no difference between the insulin-treated and non-treated groups. Thus the potentiation of ion current by insulin is unlikely to result from increased expression or membrane insertion of Aqp1 proteins.
Insulin activated signaling pathways may converge on potential intracellular targets of Aqp1.

Figure 2.5 panel A shows potential phosphorylation sites for PKG (Kennelly and Krebs, 1991), PKA (Han and Patil, 2000) and tyrosine kinase (Songyang and Cantley, 1995), as well as the putative cGMP-binding domain (Anthony, et al., 2000; Boassa and Yool, 2002) and putative Ca$^{2+}$ binding site (Fotiadis, et al., 2002). The PDZ ligand consensus site is necessary for Aqp1 interaction with PDZ-domain proteins (Cowan, et al., 2000). Panel B schematically depicts the carboxyl half of the Aqp1 protein and the intracellular carboxyl tail. To test if any of these domains mediated the potentiating effect of insulin on Aqp1 ion channel function, several regions of the Aqp1 carboxyl tail were targeted for point mutation or truncation by site-directed mutagenesis. Potential serine and tyrosine phosphorylation sites were mutated to alanine (S236A and Y253A). The PDZ-binding domain was truncated at I260 (I260Z) by substitution with a stop codon. The putative cGMP-binding region was truncated at K243 and D237 (K243Z and D237Z respectively). Aqp1 channels truncated at the D237 position showed no current activation by cGMP when expressed in the lipid bilayer system (Saparov, et al., 2001). Yet when expressed in oocytes treated with insulin, D237Z channels showed cGMP-mediated ionic responses. The lack of channel function when reconstituted in the bilayer system indicates that in the absence of intracellular signaling pathways, the distal C terminus is required for
cGMP ionic response. These results suggest multiple mechanisms of cGMP gating of Aqp1 ion channels.

In the absence of insulin, Aqp1 is not appreciably tyrosine phosphorylated.

Figure 2.6 displays a western blot containing plasma membrane-enriched fractions from control oocytes (lane 1), Aqp1-expressing oocytes (lane 2) and K243Z-expressing oocytes (lane 3). The blot was probed with anti-phosphotyrosine antibodies (anti-PY), stripped, and re-probed with antibody raised against loop C structure of Aqp1. Bands that immunoreacted with anti-Aqp1-LoopC antibodies on lanes 2 and 3 (right panel) had molecular weights consistent with the predicted sizes of Aqp1 and K243Z. This result confirmed that the K243Z truncation mutant was successfully translated into protein at levels equivalent to wild-type. The lack of immunoreactivity with anti-PY antibodies on lanes 2 and 3 at the predicted molecular weights indicates that Aqp1 is not appreciably phosphorylated at a tyrosine residue under the conditions tested. Since these results were collected from oocytes not treated with insulin, we cannot rule out the possibility that Aqp1 is appreciably phosphorylated at a tyrosine residue if the endogenous tyrosine-kinase pathway is activated by insulin. The key experiment that needs to be done is to test the effect of insulin on possible tyrosine phosphorylation of Aqp1. The antiphosphotyrosine signals in the control lane (lane 1) were not found in lanes 2
or 3 (Aqp1 and K243Z lanes) and indicate that oocytes express native tyrosine-phosphorylated protein, whose expression is reduced with heterologous expression of Aqp1, perhaps because exogenous RNA out-competes endogenous RNA for translation. To eliminate this possibility an additional control is required, where oocytes are injected with another exogenous cRNA. To eliminate the possibility that the negative result was due to poor binding of antiphosphotyrosine antibodies, a positive control (protein with known tyrosine phosphorylation) needs to be included. However, the positive bands in the control oocytes suggests this explanation is less likely.


Swelling assays were used to demonstrate the expression of Aqp1 mutants at the oocyte plasma membrane. Figure 2.7 panel (A) shows representative traces of relative volume increases of oocytes as a function of time in hypotonic saline. Panel (B) summarizes the normalized mean water permeability of oocytes injected with 50 nl water (control) or 1 ng/50 nl of cRNA encoding Aqp1, I260Z, or Y253A. Differences in water permeabilities of wild-type expressing oocytes between batches suggest that the levels of expression of heterologous proteins vary with oocyte batch or cRNA preparation. Therefore, water permeability values were normalized to the mean water permeability of Aqp1 wild-type in the same oocyte batch. The water permeabilities of Aqp1 mutants I260Z and Y253A
were not significantly different from wild-type and were significantly greater than water controls (two-tailed student t-test; *P < 0.0003).

The Aqp1 carboxyl tail truncation at the D237 position was reported not to express at the plasma membrane in oocytes (Jung, et al., 1994). To determine expression of our mutant channels at the plasma membrane, intact oocytes injected with K243Z and D237Z were fixed and immunostained with anti-Aqp1-Loop C antibodies that bound to the extracellular loop C of Aqp1. Figure 2.8 panel (A) shows immunolabeling of intact oocytes expressing wild-type or truncation mutants, K243Z and D237Z, at the plasma membrane. The oocytes were labeled with antibodies raised in chicken against Aqp1-Loop C and FITC-conjugated anti-chicken secondary antibody. Control oocytes showed no labeling. K243Z and D237Z showed labeling but the intensities were dramatically less than that of wild-type, suggesting that the expression at the plasma membrane was decreased as compared to wild-type. Panel B shows relative volume increases of single oocytes expressing Aqp1, K243Z, or water control, in 50% hypotonic saline. Oocytes expressing K243Z showed permeability greater than water controls but less than wild-type. Panel C summarizes the calculated mean water permeabilities (Pf) of control, Aqp1 (1ng/50nl or 20ng/50nl), K243Z (1ng/50nl or 20ng/50nl), and D237Z across several oocyte batches. Panels B and C show that the truncation mutants have water permeabilities significantly greater than water controls (one tailed Student’s t-test; Aqp1 (1ng/50nl) *P < 10^-17; K243Z (1ng/50nl) *P < 10^-6; (20ng/50nl) *P <
0.003; D237Z *P< 0.003) but dramatically less than wild-type ( K243Z (1ng/50nl) #P< 10^{-16}; (20ng/50nl) #P< 10^{-10};D236Z #P<0.0001). Fainter intensities of immunolabels of mutants when compared to wild-type in confocal pictures suggest that the reduced water permeability is due to decreased number of channels expressed at the plasma membrane rather than compromised water function caused by the truncation. Although, previous studies reported that the D237Z does not express at the plasma membrane (Jung, et al., 1994), here we report that it does. The basis for these conflicting reports could be due to different expression levels of D237Z proteins at the plasma membrane. Jung and colleagues injected 10 ng of cRNA encoding D237Z per oocyte while I injected 20 ng. Perhaps injection of 10 ng of cRNA encoding D237Z results in a protein expression level that falls below ranges detectable for confocal analysis. When 20 ng of cRNA encoding K243Z and D237Z were injected into oocytes, analyses by confocal and swelling assays confirmed the presence of the mutant channels at the plasma membrane.

**The potentiating effect of insulin is not mediated by the PDZ-binding domain or the putative tyrosine phosphorylation site.**

Aqp1-mutants expressed in oocytes were treated with 1.7 – 3.5 µM insulin for ≥ 3 hrs prior to electrophysiological recording. Electrophysiological recordings of SNP-induced macroscopic currents from Aqp1 wildtype and mutant channels demonstrated that insulin also had positive regulatory effects on Aqp1-mutant
channels I260Z and Y253A as compared to wild-type (Figure 2.9 A &B). Panel (A) shows representative initial and net response current traces. Panel (B) summarizes the normalized net current (at 240 seconds post-SNP application) in control oocytes or in oocytes injected with cRNA encoding Aqp1, I260Z, and Y253A channels pretreated or not treated with insulin. Data for each group were normalized to the mean current of insulin-treated Aqp1 group in the same oocyte batch. The normalized net currents of I260Z and Y253A-expressing oocytes without insulin were not significantly different from each other or from wild-type without insulin. Pre-incubating oocytes expressing I260Z in 1.7-3.4 uM of insulin for ≥ 3.5 hours resulted in a significant enhancement of SNP-induced ion current as compared to non-insulin treated groups and the insulin treated control group (two-tailed Student’s t-test; I260Z *P<0.03, **P<0.05) (Figure 2.9 B).

Although there was a trend after treatment with insulin toward enhancement of the current response of Y253A, the results were not significant perhaps because of the small sample size. Therefore conclusions for the Y253A mutant cannot be drawn. Insulin significantly enhancing the ion channel function of Aqp1 mutants I260Z suggests two ideas. First, the putative PDZ binding domain is not involved in mediating the potentiating effect of insulin. Second, this regulatory domain does not constitute the structural region responsible for inhibiting Aqp1 proteins from functioning as a cGMP-gated ion channel.
With insulin pretreatment, Aqp1 mutants lacking the putative cGMP-binding domain activate with SNP.

Figure 2.10 panel (A) shows SNP-activated current traces of individual oocytes injected with water, or cRNA encoding Aqp1, K243Z, or D237Z, and treated with 1.7-3.4 µM of insulin for greater than 3 hrs. Mutant-expressing oocytes (K243Z or D237Z) not treated with insulin failed to activate with SNP, but wild-type expressing oocytes were responsive under identical conditions. The SNP-induced current activation of mutant-expressing oocytes treated with insulin was not uniform; only a subpopulation of oocytes were responsive to SNP. Panel A shows traces from selected oocytes that were strongly responsive to SNP after insulin pretreatment. Panel (B) summarizes normalized data of net current (240 sec post-SNP) for all oocytes tested. Normalization was done as described for data in Figure 2.2 panel (B). Insulin treatment significantly increased SNP-activated ion current of K243Z and D237Z-expressing oocytes as compared to insulin treated control oocytes (one-tailed Student’s t-test; Aqp1 *P< 10^-4 and **P<0.003; K243Z *P< 0.03 and ** P<0.02; D237Z *P< 0.002 and ** P<0.001). These results indicate that the function of Aqp1 as an cGMP-activated ion channel does not require the putative cGMP-binding region, if insulin-mediated signal cascades are activated. However, in the absence of insulin signaling, the SNP-dependent ionic conductance in the truncation mutants is not evident.
Aqp1 is phosphorylated in vivo by an endogenous ser/threonine kinase in oocytes.

Aqp1 proteins from oocytes purified by immunoprecipitation (IP) were probed with monoclonal antiphosphoserine antibody. To mimic experimental conditions used for electrophysiology, Aqp1-expressing oocytes were exposed to 12 mM SNP for 8 minutes. The Aqp1 proteins were isolated by using anti-Aqp1-COOH antibodies raised against the carboxyl tail of Aqp1. The antigen-antibody complexes were pelleted by protein A beads and were stringently washed to remove non-specific proteins (see methods). As negative control for non-specific protein interactions an IP extraction was done without anti-Aqp1-COOH antibodies but included protein A beads. Figure 2.11 (A-B) shows a western blot containing purified Aqp1, with each image representing different probes of the western blot with specific antibodies. Aqp1 proteins were loaded on a SDS-PAGE gel using a range of protein concentrations (doubling the amounts in each subsequent lane). Lanes 2-5 were loaded with Aqp1 proteins. In order to rule out the possibility that the antiphosphoserine immunoreactive bands were contributed by the light and heavy chains of anti-Aqp1-COOH antibodies used in the IP method, antibodies alone were loaded on Lane 7. To rule out the possibility that the immunoreactive bands were contributed by non-specific proteins derived from interaction with protein A beads, protein eluted from protein A beads without antibodies were run on a separate gel and showed no Aqp1 proteins were pulled down with protein A beads alone (data not shown). Panel
(A) shows the western blot probed with anti-phosphoserine antibodies, stripped, and reprobed with anti-Aqp1-COOH antibodies. A ~28 KDa immunoreactive visualized with anti-phosphoserine antibody indicates that a protein at the molecular weight expected for Aqp1 was phosphorylated at a serine or threonine residue. This 28 KDa immunoreactive band was not observed in the lane with anti-Aqp1-COOH antibodies (lane 7) or in lanes loaded with non-specific proteins (data not shown). This result indicates that the ~28 KDa immunoreactive band was not contributed by the light chain of anti-Aqp1-COOH antibodies alone or non-specific proteins co-isolated from the IP process. The Aqp1 protein at ~28 KDa was confirmed with anti-Aqp1-COOH antibodies. The ~60 KDa immunoreactive band detected on the anti-Aqp1-COOH probed blot represents the heavy chain of the Aqp1-COOH antibodies used in the IP process. The presence of a ~60 KDa immunoreactive band in Lane 7 further confirms this conclusion.

To confirm if the anti-phosphoserine ~28 KD immunoreactive band colocalized with the anti-Aqp1-COOH ~28 KD immunoreactive band, the images with anti-Aqp1 and anti-phosphoserine antibodies were superimposed. Panel (B) shows a cropped view of data in panel A displayed individually and then superimposed. Taken together, the data support the idea that Aqp1 is phosphorylated at a serine or threonine residue. Due to technical difficulties, I could not conclude if insulin had an effect on serine phosphorylation or on Aqp1 expression levels from this experiment. If protein expression is to be quantified
an additional internal control to serve as a benchmark for protein loading is required. Further experiments testing the effects of serine phosphorylation on ion channel activation are of interest for future work.

2.4 DISCUSSION

Summary of findings

The novel finding of my work is that insulin is a positive regulator of Aqp1 ion channel function. This finding provides the first evidence that Aqp1 is targeted by insulin-activated intracellular signaling pathways and the consequence of this targeting is enhanced Aqp1 ion channel function in the presence of cGMP. Prior studies have shown that only a small subpopulation of Aqp1 functions as cGMP-gated ion channels, while the vast majority function as water channels. SNP and other methods of cGMP delivery inconsistently activated ionic current in some batches of Aqp1-expressing oocytes. Even direct injection of 8-Br-cGMP in Aqp1-expressing oocytes failed to induce current in some batches of oocytes, suggesting that the lack of current induction is not solely due to insufficient cGMP production. Based on these observations, I hypothesized that although cGMP gates Aqp1 ion channels, the ability of Aqp1 to function as an ion channel is regulated by other intracellular signaling pathways converging on Aqp1. Data presented here show that insulin-activated tyrosine kinases positively regulate
cGMP-mediated activation of Aqp1 ion channels when expressed in X. oocytes. The idea of Aqp1 serving as a convergent target for several intracellular signaling pathways is supported by the reports that Aqp1 is phosphorylated by PKA kinase (Han and Patil, 2000) and that the water channel function of Aqp1 is gated G_{\alpha i3}-PLA2 signaling pathway (Abu-Hamdah, et al., 2004).

Three lines of evidence demonstrate that SNP-activated Aqp1 ion channel function is positively regulated by insulin-sensitive signaling cascades: (1) control oocytes treated with insulin lacked SNP-induced current; (2) insulin treatment increased the magnitude of current induced by SNP in Aqp1-expressing oocytes; and (3) insulin treatment did not alter Aqp1 expression level at the plasma membrane, as indicated by water permeability.

**Insulin positively modulates cGMP-regulated Aqp1 ion channel function.**

The insulin-activated signaling pathways positively modulate the ion channel function of Aqp1, but do not directly cause channel activation. The intermediate signaling pathways involved in coupling the insulin activated receptor tyrosine kinase IGF with enhanced Aqp1 ion channel function are unknown. The insulin-activated signaling pathway is not sufficient alone for activation of Aqp1 ion channels, since insulin treatment alone did not induce ionic current in Aqp1-expressing oocytes. Ion channel activation was gated by cGMP. Through reconstitution in lipid bilayer system, Aqp1 channels were activated by
cGMP (Saparov, et al., 2001) in the likely absence of any intracellular signaling kinase pathways. These observations suggest that insulin-activated signaling pathways enhance, rather than directly gate, Aqp1 ion channels.

The mechanism of insulin potentiation of Aqp1 ion current is not known. Insulin treatment of Aqp1-expressing oocytes did not increase water permeability, thus suggesting that insulin did not increase channel density. Instead, enhanced ion channel functionality could be attributed to a modal gating pattern, recruiting ion channel activity within existing pools of Aqp1 protein by insulin-activated signaling pathways. Unexpectedly, I found that Aqp1 mutant channels lacking major portions of the distal C terminus functioned as cGMP-activated ion channels when oocytes were treated with insulin, and showed current amplitudes equivalent to that of wild-type channels. This C-terminus truncation mutant channels did not respond to cGMP when reconstituted in the lipid bilayer (Saparov, et al., 2001), a system lacking tyrosine signaling pathways. These results provided two conclusions: 1) that the effect of insulin treatment was not mediated by domains on Aqp1 distal C terminus; and 2) the putative cGMP-binding domain in the distal C-terminus is not required for cGMP-gating of Aqp1 channels in the presence of insulin-sensitive intracellular signaling pathways. These conclusions suggest that regions of the intracellular loops or amino terminus (or a combination) could mediate the effects of insulin. The mechanism of cGMP-gating in the absence of the distal C-terminus requires the signaling input from intracellular signaling pathways. In addition, these results
suggest insulin-sensitive signaling pathways might change how cGMP gates Aqp1 ion channels. Aqp1 mutants lacking the distal C-terminus had reduced water permeability that could be the result from reduced channel expression at the plasma membrane. However, assuming that the mutation did not alter single channel conductance, the equivalent current amplitudes indicate similar numbers of channels contribute to the signal. This suggests that the distal C terminal domain may be involved in regulating the water channel (rather than the ion channel) function of Aqp1.

**Aqp1 domains that mediate the enhancing effect of insulin is proximal to the distal C terminus.**

Aqp1 is phosphorylated by an endogenous serine/threonine kinase in the oocyte system, but the consequence on ion channel activity is not known. The involvement of insulin-activated intracellular pathways to the phosphorylation state of Aqp1 remains to be determined. The putative PKG phosphorylation site (S236) located at the proximal C-terminus of Aqp1 remained intact in the C-terminus mutant channels, and could have a role in the response to cGMP following insulin treatment. Insulin stimulation activates both protein kinases and protein phosphatases therefore simultaneously phosphorylating some proteins and dephosphorylating others (Taha and Klip, 1999). The downstream consequences of insulin stimulation include activation of serine/threonine kinases (Sakanoue, et al., 1988; Taha and Klip, 1999).
The idea of direct phosphorylation as the mechanism mediating the effect of insulin is attractive. Phosphorylation of Aqp1 proteins isolated from mouse kidneys was demonstrated in vitro using the catalytic subunit of PKA (Han and Patil, 2000). The authors proposed that the direct phosphorylation of Aqp1 by PKA was the basis for forskolin-mediated enhanced water permeability, but this study did not test the effects of phosphorylation on ion channel function. Direct injection of the catalytic subunit of PKA did induce cationic current (Yool, et al., 1996), demonstrating that PKA signaling pathway is involved in regulating the function of Aqp1 ion channels. It has not been determined if the effect of PKA activity on Aqp1 ion channel activation is through direct phosphorylation or indirect effects on other signaling molecules that could regulate Aqp1. Nevertheless, these observations demonstrate that Aqp1 ion channel activity is modulated by multiple intracellular pathways.

Conclusion

cGMP-activated Aqp1 ion channel function can be positively modulated by signaling pathways activated by IGF stimulation in oocytes. Tyrosine kinases or these other signals may serve as ‘master switches’ governing Aqp1 ion channel ability to behave as ion channels. Given the large abundance of Aqp1 in native tissues, and the fact that altering membrane potential requires the net movement of only a very few number of charges (Hille, 2001), the discovery of Aqp1 ion channel function being tightly regulated by intracellular signaling pathways
makes sense for biological systems. Synergistic regulation may allow channels to integrate multiple signals to permit finer regulatory control to meet biological demands.
Figure 2.1 SNP-induced Aqp1 current response is highly variable across experiments. See next page for details.
Figure 2.1 SNP-induced Aqp1 current response is highly variable across experiments. Panel (A) shows the water permeability values of individual Aqp1-expressing oocytes plotted against the corresponding net ion conductance of the same oocyte measured at approximately 15 minutes after SNP application. Panel (B) shows mean net current (240 sec post SNP stimulus) and the mean water permeability plotted against the corresponding number of days after cRNA injection ( ◆ indicates mean net current; ■ indicates water permeability). Panel (C) shows the net conductances of SNP-activated current of oocytes injected with different concentrations of Aqp1 cRNA across different oocyte batches.
Number of oocyte batches showing Aqp1 ion channel function, by month and year

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Table 2.1 No pattern of seasonal influence affecting Aqp1 channel response to SNP. Batches of Aqp1-expressing oocytes were classified based on month/year of recording and ionic current response to SNP (no-response, mixed response, response, or had endogenous currents).
Figure 2.2 The function of Aqp-1 as a cGMP-regulated ion channel is enhanced by insulin-activated tyrosine kinases in the *Xenopus* oocyte expression system. See next page for details.
Figure 2.2 The function of Aqp-1 as a cGMP-regulated ion channel is enhanced by insulin-activated tyrosine kinases in the *Xenopus* oocyte expression system. Panel (A) shows representative initial and net response current traces with insulin or without insulin (scale bar 2 µA/50 msec). Panel (B) shows current-voltage relationship of traces shown in panel A (+ indicates insulin treatment). Ionic current in Aqp1-expressing oocytes (but not in control oocytes) is activated by SNP, a compound that elevates intracellular cGMP in oocytes. Net response was calculated by subtraction of the initial current from the SNP-activated current. Panel (C) shows the development of current after SNP application without insulin or with insulin treatment (* indicates insulin treatment). Panel (D) summarizes the normalized net current (at 240 seconds post-SNP). Induction of SNP-activated ionic current in Aqp1-expressing oocytes treated with insulin is significantly greater than that seen for Aqp1-expressing oocytes with no insulin exposure (two-tailed student t-test; *P<0.002; ** P<0.00005). Normalized net current + SEM reflects data of each group normalized to the mean current of insulin-treated Aqp1 group in the same oocyte batch. A double asterisk (**) indicates value that is significantly different from control incubated in insulin; an asterisk (*) indicates values under the bracket are significantly different.
Figure 2.3 No effect of season on insulin potentiation of SNP-induced Aqp1 ion current. Histogram of the percent of wild-type oocytes (with or without insulin) that responded to SNP for specific months sampled.
Figure 2.4 Insulin preincubation does not enhance water permeability of Aqp1-expressing oocytes. The water permeabilities of Aqp1-expressing oocytes with or without insulin treatment were tested by swelling assay (see methods). Due to the batch to batch variability of water permeability, individual data sets were normalized values were calculated by dividing individual water permeability per oocyte by the mean water permeability of non-insulin treated Aqp1 group in the same oocyte batch. There was no statistical difference between the two treatment groups; n values are given in parentheses.
Figure 2.5  Putative regulatory domains of Aqp1 along the carboxyl tail. See next page for details.
Figure 2.5  Putative regulatory domains of Aqp1 along the carboxyl tail.
Panel (A) shows the location of potential regulatory sites that include PDZ-ligand binding domain as well as the putative PKG/PKA phosphorylation site, putative tyrosine phosphorylation site, putative cGMP-binding domain, and putative Ca$^{2+}$ binding site. Panel (B) shows a schematic cartoon of the COOH half of Aqp1. The putative site of PKG and PKA Phosphorylation ($\ddagger$ *) remains on both carboxyl truncation mutants (Kennelly and Edwin, 1991; Yool et al., 1996). The putative site of tyrosine kinase Phosphorylation (*) (Songyang et al., 1995). Lightning symbols indicate regions of carboxyl tail ablated to test the contribution of these domains for Aqp1 ion channel function.
Aqp1 is not phosphorylated at a tyrosine residue. The identical western blot containing plasma membrane enriched fractions from oocytes injected with water (lane 1), Aqp1 cRNA (lane 2) and K243Z cRNA (lane 3) proteins was probed with anti-PY, stripped, and re-probed with anti-Aqp1-LoopC antibodies. Bands that immunoreacted with anti-Aqp1-LoopC antibodies on lane 2 and 3 fell at a molecular weight consistent with Aqp1 and K243Z, indicating wild-type and mutant channel protein presence. The lack of immunoreaction with anti-PY antibodies on lanes 2 and 3 at the predicted molecular weights indicates that Aqp1 is not phosphorylated at a tyrosine residue.
Figure 2.7 The lack of Aqp1 PDZ binding domain or mutation of putative tyrosine phosphorylation site does not interfere with water channel function when expressed in *Xenopus oocytes*. See next page for details.
Figure 2.7 The lack of Aqp1 PDZ binding domain or mutation of putative tyrosine phosphorylation site does not interfere with water channel function when expressed in *Xenopus* oocytes. Aqp1 mutants I260Z and Y253A show equivalent water permeabilities as to wild-type when injected with equal cRNA concentrations (1ng/50nl). Panel (A) summarizes the mean water permeability of oocytes injected with water, Aqp1, I260Z, and Y253A and panel (B) show average representative traces of increase relative volume of oocytes over time in hypotonic saline. Water permeabilities of Aqp1 mutants I260Z and Y253A were not significantly different from wild-type, but were significantly greater than water controls (*P<10^{-9}). An asterisk (*) indicates a value significantly different than water controls; A pound sign (#) indicates values significantly different than wild-type.
Figure 2.8 Carboxyl tail truncation mutants express at the plasma membrane and functions as water channels. See next page for details.
Figure 2.8  Carboxyl tail truncation mutants express at the plasma membrane and functions as water channels. (A) Intact oocytes were immunolabeled with an Aqp1 antibody to confirm the expression of the truncation mutants, K243Z and D237Z, at the plasma membrane. The oocytes were labeled with antibodies raised against Aqp1-Loop C and FITC-conjugated anti-chicken secondary antibody. Control oocytes show no labeling. K243Z, and D237Z show labeling but the intensities were dramatically less than wild-type, suggesting that the expression at the plasma membrane is severely compromised compared to wild-type. (B) Relative volume increase, measured by increase cross sectional area, of a single oocyte expressing Aqp1, K243Z, or water control, in response to 50% hypotonic saline recorded by videomicroscopy. Oocytes expressing K243Z mutants increase in volume in hypotonic saline more than water controls but less than wild-type. (C) Summary of calculated mean water permeabilities (Pf) (See methods) of water, Aqp1 (1ng/50nl or 20ng/50nl), K243Z (1ng/50nl or 20ng/50nl), and D237Z across several oocyte batches. Pf values (cm/s x 10^-4) are mean ± SEM ; n = values are shown in parentheses. The truncation mutants have water permeabilities significantly greater than water controls (One-tailed student t-test; Aqp1 (1ng/50nl) *P<10^-17; K243Z (1ng/50nl) *P< 10^-6; (20ng/50nl) *P< 0.003; D237Z *P< 0.003) but dramatically less than wild-type (K243Z (1ng/50nl) **P<10^-16; (20ng/50nl) **P<10^-10 ;D236Z **P<0.0001) under identical testing conditions. An asterisk (*) indicates a value significantly different than water controls; A pound sign (#) indicates values significantly different than wild-type.
Figure 2.9  Aqp1 PDZ binding domain does not mediate the potentiating effects of insulin-preincubation. See next page for details.
Figure 2.9 Aqp1 PDZ binding domain does not mediate the potentiating effects of insulin-preincubation. Panel (A) show representative averaged current traces of each group treated with or without insulin treatment in response to a series of voltage steps (scale bar 2 µA/ 50 msec). Panel (B) show a summary of normalized net current at 240 seconds post-SNP application of oocytes injected with water, Aqp1, I260Z, and Y253A mutants. Due to large variability between oocyte batches, individual data sets were normalized/ divided by the mean net current at 240 sec post-SNP stimulus of Aqp1 of each respective batch. Insulin treatment of oocytes expressing Aqp1 and I260Z resulted in a significant enhancement of SNP-induced ion current compared to non-insulin treated groups or insulin treated water injected control group (student t-test; Aqp1 *P< 10^-5, **P<0.002; I260Z *P<0.03, **P<0.05). An asterisk (*) indicates a value significantly different than water controls; A pound sign (#) indicates values significantly different than wild-type. Double asterisk (**) indicate significant difference between insulin and non-insulin treatments.
Figure 2.10 SNP activates ionic current in Aqp1 lacking the putative cGMP-binding domain when expressed in oocytes. See next page for details.
Figure 2.10  SNP activates ionic current in Aqp1 lacking the putative cGMP-binding domain when expressed in oocytes. Panels (A-B) show current increase of SNP-activated oocytes injected with water or cRNA encoding Aqp1, K243Z, and D237Z. Panel (A) shows current traces over a series of voltage steps of individual oocytes expressing water control, Aqp1, K243Z, or D237Z treated with insulin and activated by SNP in isotonic NaCl saline. Panel (B) summarizes normalized data of net current 240 sec post-SNP stimulus. Due to large variability between oocyte batches, individual data sets were normalized/divided by the mean net current at 240 sec post-SNP stimulus of Aqp1 of each respective batch. Preincubation in insulin had a dramatic potentiating effect on currents of all Aqp1 groups that was statistically significant compared to water controls (one-tailed Student's t-test; Aqp1 *P< 10^-4 and **P<0.003; K243Z *P< 0.03 and ** P<0.02; D237Z *P< 0.002 and **P<0.001). An asterisk (*) indicates a value significantly different than water controls; A pound sign (#) indicates values significantly different than wild-type. The pound-asterisk sign (**) indicates significant differences between non-insulin and insulin treatments.
Figure 2.11 Aqp1 is phosphorylated at a serine residue. See next page for details.
Figure 2.11 Aqp1 is phosphorylated at a serine residue. (A) Oocyte plasma membrane fractions from Aqp1-expressing oocytes were immunoprecipitated (IP) with anti-Aqp1-COOH antibodies. Blank (lane 1), Aqp1 0.5 µl (lane 2), Aqp1 1 µl (lane 3), Aqp1 2 µl (lane 4), Aqp1 4 µl (Lane 5), blank (lane 6), and Aqp1-COOH antibody 1 ul (lane 7). The blot was probed with anti-phosphoserine, stripped, and reprobed with anti-Aqp1-COOH antibodies to show the presence of Aqp1. An asterisks (*) indicates the molecular weight of 32.2 KDa. Lane 7 was loaded with anti-Aqp1-COOH antibodies to show that the ~ 28 KD band immunoreactive to antiphosphoserine antibodies was not the light chain of anti-Aqp1-COOH antibodies. Loading of non-specific proteins that interacted with the protein A beads (see methods) showed no phosphoserine reactive band (data not shown). (B) A ~ 28 Kda immunoreactive band on the anti-phosphoserine probed blot (shown in panel A) was colored yellow. A ~28 Kda immunoreactive band on the anti-Aqp1-COOH probed blot was colored green. The blot images shown in panel (A) were superimposed to determine if the immunoreactive phosphoserine bands had identical molecular weight as Aqp1 proteins.
CHAPTER 3: DIVERSE GATING MODES OF cGMP-ACTIVATED CURRENT IN AQP1-EXPRESSING OOCYTES.

3.1 INTRODUCTION

Diverse mechanisms of channel gating exist across all the major classes of ion channels and are the subject of intense investigation. Strategies to achieve gating diversity include direct phosphorylation/ dephosphorylation (Levitan, 1994), cytoplasmic proteins that interact with specific intercellular domains through protein-protein interaction (Wang, et al., 2000), voltage-sensitivity (Benndorf, et al., 1999), direct ligand-binding (Altenhofen, et al., 1991), and indirect consequences of ligands as secondary messengers (Fukao, et al., 1999). These mechanisms are thought primarily to influence the stability of channel open-state by altering protein conformation, and may act in combination. For example, cyclic nucleotide-gated channels (CNG), which belong in the broad super family of ion channels that includes voltage-gated K⁺ channels, are directly gated by cAMP and cGMP, but a number of factors regulate the stability of the open-state. Ligand-gated CNG channels are composed of α and β subunits, where only the α subunits are able to form homomeric channels when expressed in heterologous expression systems (Biel, et al., 1999). β subunits are considered to function as modulatory subunits of CNG channels (Biel, et al., 1999). Sensitivity to ligand binding is dependent on subunit combination and protein phosphorylation. Tyrosine phosphorylation of CNG channels of the rod photoreceptors decreases cGMP affinity (Savchenko, et al., 2001) while
phosphorylation by CaM Kinase II of CNG channels in bipolar cells leads to voltage-dependent decrease of conductance (Shiells and Falk, 2001). Calcium directly regulates the gate of CNG channels by interacting with high affinity binding sites and indirectly regulates by activating other proteins like calmodulin (Lucas, et al., 2000). The amino and carboxyl domains of the olfactory CNG channels interact thus increasing cGMP sensitivity (Varnum and Zagotta, 1997).

My work supports the hypothesis that aquaporins also have diverse mechanisms of regulatory control. Aquaporin proteins belong to the Membrane Intrinsic Protein (MIP) family. Although more ostensibly known as water channels, a subset of Aquaporins (Aqp0, Aqp1, Aqp6) have been demonstrated to function as ion channels with diverse modes of regulation. In addition to water channel function, Aqp0 reconstituted in a lipid bilayer shows that a large conductance channel mediates a voltage-sensitive current that is dependent on phosphorylation (Ehring, et al., 1992). Aqp1 expressed in oocytes is a cGMP-gated cation channel (Anthony, et al., 2000). Aqp6 expressed in oocytes functions as a Cl⁻ channel gated by pH and Hg²⁺ (Yasui, et al., 1999a). Another channel that belongs to the MIP family Big Brain (BIB), shows ion channel function that is regulated by tyrosine kinase signaling (Yanochko and Yool, 2002). Possible ion channel functions in other aquaporins remain to be evaluated.

In chapter 2, I presented data demonstrating the positive effects of insulin on Aqp1 ion channel function, and thus provided the first insight to one of the
potential mechanisms that could dictate Aqp1 ion channel availability. Data presented in chapter 3 are the first to show two distinct types of gating based on kinetic properties of SNP-induced activation and deactivation of current in Aqp1-expressing oocytes. I hypothesized that these different gating kinetics reflect distinct gating mechanisms of Aqp1 ion channels, and tested the role of the carboxy terminal by deletion mutations.

Data presented here show SNP-activated currents display two distinct modes of gating kinetics in Aqp1-expressing oocytes that differ in properties of activation and deactivation, voltage sensitivities, and reversal potentials. One mode displays rapid SNP-dependent activation and rapid deactivation after SNP removal. This gating mode is voltage-independent, as indicated by the linear current-voltage relationships. The second mode of gating displays slow SNP-dependent activation and slow deactivation after SNP removal. This gating mode is voltage-sensitive, showing outward rectification in the current-voltage relationship. My work is the first to indicate diversity in cGMP-mediated mechanisms of gating of Aqp1 ion channels, that suggest activation is more complex than direct cGMP-binding.
3.2 MATERIALS AND METHODS

(1) Molecular techniques. Aqp1 mutations were generated by site-directed mutagenesis with Stratagene QuickChange (La Jolla, CA) as described in chapter 2.

(2) Oocyte preparation. Stage V- VI oocytes were harvested from Xenopus laevis oocytes and prepared for electrophysiological recording as described in methods section of chapter 2.

(3) Electrophysiological recordings. Two-electrode voltage clamping was used to measure whole cell conductance of Aqp1-expressing oocytes after SNP stimulation as described in methods section in chapter 2. The endogenous tyrosine kinase signaling pathway of oocytes was activated by 3-10 hrs preincubation with 1.7-3.4 µM bovine insulin (Sigma Co, St Louis, MO). Aqp1 currents were activated by 8-14 mM of SNP and inactivated by perfusing the 200 or 700 µl volume in the bath chamber with 10 ml of fresh saline. A perfusion calibration test with a dye marker showed complete bath exchange within 30 seconds after approximately 2 mls perfusion. Channel activation was monitored using a voltage protocol that stepped to +40 mV for 50 msec from a holding potential of –40 mV. Magnitude of activation was measured as the net current value at 240 sec post-SNP application. The percent of current recovery after
SNP wash out was calculated as $\left[ \frac{(C_o - C_x)}{C_o} \right] \times 100$; where $C_o$ is the peak current amplitude after SNP activation, and $C_x$ is the amplitude of the sustained current after SNP wash out measured after a interval equal to that allowed for SNP-activation. For example, if peak current measurement ($C_o$) was made 500 sec after SNP-activation, then the amount of sustained current ($C_x$) was measured 500 seconds after SNP removal.

The voltage sensitivity of the current was quantified as the magnitude of relative outward rectification of the current-voltage plot calculated as a ratio of the absolute values of current amplitudes measured at +30 mV and –70 mV. These two points were selected as being approximately equidistant from the reversal potential for a cation current (-17 mV). An outward rectification ratio of 1 indicated linear current voltage relationship; ratio of greater than > 1 indicated outward rectification.

The pattern of current activation after SNP was measured by using an index referred to here as a slope ratio. Slope ratios were calculated by calculating the slope values fit from 5 sample points at two windows of time (point A = 300-325 sec; point B = 500-525 sec). Slope ratio was calculated as slope A divided by slope B. Rapid responses to SNP corresponded to slope ratios > 1, reflecting a plateau phase by 500 seconds, while slow responses had slope ratios of ≤ 1 showed and lacked an obvious plateau phase by 500 s. Statistical analyses were done with student’s t-test and simple regression as specified in the results.
3.3 RESULTS

Results from previous studies supported a model in which ion channels of Aqp1 are directly gated by cGMP binding to the carboxyl tail (Anthony, et al., 2000; Boassa and Yool, 2003). Data presented in this chapter show the properties of rapidly gated mode of cGMP-dependent current are consistent with the findings of published work. But unexpectedly, I also found populations of Aqp1-expressing oocytes that showed a slowly gated mode of SNP-activated current that persisted after SNP removal, as well as oocytes with a mixture of both modes. The slow mode was similar to the rapid mode in being dependent on cGMP, but contrasted from the rapidly gated current in several ways: (1) non-plateauing pattern of current activation; (2) presence of a sustained current after removal of SNP; (3) voltage-sensitivity (outward rectification); and (4) a more negative reversal potential. Both rapid and slow gated SNP-currents were observed in Aqp1-expressing oocytes incubated with or without insulin, thus indicating that an increase in tyrosine kinase activity and downstream signaling cascades did not promote a specific type of current gating mode. The relative frequency of the two modes appeared to vary with different batches of oocytes (even from the same frogs), suggesting seasonal or hormonal factors influence the regulatory signaling pathways.
Rapid mode of gating.

Figure 3.1 (A) shows SNP-induced current activation and, current deactivation after SNP washout as a function of time in an Aqp1-expressing oocyte typifying the rapid mode. Current deactivation occurred over a time course similar to that for current activation. Panel (B) shows the current-voltage relationships at time points indicated as a-e on Panel A. Data are plotted in Panel (C). Linear current-voltage relationships indicate voltage-independent properties of the cGMP-activated channels. 8 mM of NaCl was used as a sham application to control for effects of 8 mM SNP application. The lack of current activation after the application of 8 mM NaCl indicates that the response to SNP was not an artifact of osmotic shock or mechanical disturbance.

Slow mode of gating.

In contrast, the plot in figure 3.2 (A) shows a SNP-induced current with a mixture of fast and slow modes. The amplitude of the fast component is reflected by the rapid partial deactivation immediately after SNP removal. The rapid or slow gated SNP-activated current compositions were distinguished by perfusion studies that separated deactivated currents from sustained currents by SNP washout. The slow component seen as a sustained current after washout of SNP was reversibly blocked by Cd$^{2+}$ (an Aqp1 ion channel blocker) and the
current gradually decreased over a longer time course, indicating indirect dependence on SNP. The near instantaneous relief of Cd$^{2+}$ block by bath exchange supports the results of prior calibration studies that showed efficient exchange of the bath saline by perfusion, thus the slow recovery phase is unlikely to be due to residual SNP in the bath, but instead suggests a rate-limiting conformational change. Panel (B) shows the current voltage relationships at different time points (a-e on Panel A). Data plotted in Panel (C) show that sustained currents (after SNP washout) have a voltage-dependent conductance characterized by outward rectification. Panel (D) shows current traces of (a) the slow mode visualized, as the sustained current after washout of SNP (same trace as Panel B (c) and of (b) the rapid mode generated by subtracting (c) from (b) (Panel B). From the relative amplitudes, I calculate that this oocyte showed approximately 20% fast and 80% slow modes. Panel (E) shows the current-voltage relationship of component current traces in panel (D). The sustained current was characterized by outward rectification, while the rapidly deactivated current showed a linear current-voltage relationship, indicating voltage-independence. Single channel studies in excised patches from Aqp1-expressing oocytes have shown examples of cGMP-activated channels that deactivated slowly after cGMP removal, and were not seen in control oocytes (Yool, unpubl). This finding corroborates the results of Fig 3.2 A. The rapid and slow gated currents observed in Fig 3.2 reflect the activation of either two separate
populations of channels or a single population of channels with distinctly different gating modes.

Rapid and slow gated currents have different reversal potentials

Mean reversal potentials of rapidly deactivating (fast) and sustained currents (slow) components are shown in Fig 3.3 panel A. The mean reversal potential of sustained current component was statistically different than that of rapidly deactivating current component (student’s t-test; P< 0.03; rapid mode –16.28 mV ± 2.57; slow mode –25.14 mV ± 2.52). Data sets were taken from insulin and non-insulin treated Aqp1-expressing oocytes that displayed the slow gated SNP-activated current. The reversal potentials of both current components are within the reversal potential range predicted for either a mixed cation or predominantly Cl\(^-\) current. Ion substitution experiments in which 60 mM Cl\(^-\) ions are replaced with 60 mM gluconate would be one approach to answer if the current is carried partially or predominantly by Cl\(^-\).

Differences in reversal potentials indicate differences in ion selectivity, and classic theory would suggest that the current is carried by separate channels. However, no SNP-induced ionic conductance was observed in control oocytes and both current types are sensitive to Cd\(^{2+}\) block. These data support the possibility that the sustained and rapidly deactivated currents may represent two different cGMP-gating modes of Aqp1, resulting in differences of ionic selectivity.
This would be unusual but the idea does have a precedent. Purinergic receptors are ATP-gated ion channels that alter ion selectivity over a prolonged exposure to the ligand (Khakh, et al., 1999; Virginio, et al., 1999). Mutagenesis studies have confirmed that the selectivity filters for different ions arise from the same structure which is predicted to be the pore pathway (Virginio, et al., 1999). Alternatively, Aqp1 may associate with and facilitate the activation of the associated endogenous channels. In the secretory granule, Aqp1 co-immunoprecipitates with other proteins, that include K⁺ channels (IRK-8), that may serve as part of a regulatory complex (Abu-Hamdah, et al., 2004). Interestingly, reducing the water channel function of Aqp1 appears to decrease K⁺ conductance when Aqp1 and the co-isolated regulatory complex were reconstituted in lipid bilayer, suggesting that the activation of K⁺ channel depended on Aqp1 water channel activity (Abu-Hamdah, et al., 2004).

Slow gated SNP currents showed voltage-dependent decrease in conductance indicated by outward rectification.

Figure 3.4 shows that there is a correlation between the value of outward rectification and the percentage of the slow gated current component for Aqp1-expressing oocytes (linear regression analysis; P ≤ 0.002). Rapidly gated currents had little outward rectification with values near 1 (1.10 ± 0.09.) which indicates a linear voltage-insensitive current. Slowly gated currents showed outward rectification values of typically > 1 (2.36 ± 0.31).
involvement of endogenous channels because I observed no SNP-induced current activation in the control group in the same batch of oocytes. Batches of oocytes with endogenous currents were disqualified from further analysis.

To test the possibility that voltage-sensitivity is due to open-channel block by divalent ions, dose-dependent studies of the effects of divalent ions (Ca^{2+}, Mg^{2+}, Ni^{2+}) must be performed. As well, a gain of voltage-sensitivity in calcium activated K\(^+\) channels can be mediated by direct phosphorylation (Levitan, 1994). Other ion channels such as MIP ion channel Aqp0 (Ehring, et al., 1992) as well as CNG channels (Benndorf, et al., 1999; Shiells and Falk, 2001) gain voltage-sensitivity once phosphorylated by kinases. Although my data show insulin does have a potentiating effect on cGMP-activated ionic response of Aqp1, the slow component displayed voltage-sensitivity regardless of treatment with insulin. This suggests that the signaling pathways activated by insulin stimulation did not induce a gain of voltage-sensitivity on cGMP-activated Aqp1 current.

Aqp1 PDZ-binding domain is not required for rapid or the slow gating modes of SNP current

Ion channel gating is influenced by accessory proteins binding to regulatory domains located at the cytosolic facing protein structures of the channel (Roosild, et al., 2004). Aqp1 contains a PDZ-binding ligand domain that interacts with the linker protein PICK1 which mediates the association of Aqp1 to a receptor tyrosine kinase EphB2 and other signaling molecules (Cowan, et al., 2000). To
test the contribution of the PDZ-binding domain to the gating of Aqp1, Aqp1-mutant channels with a truncation that removed the PDZ-binding domain (I260Z) were expressed in oocytes and activated with SNP. Both the rapid and slow gating currents were observed. Figure 3.5 panel (A) shows that SNP induces a rapid rise of current and rapid decay after removal of SNP as a function of time in an I260Z-expressing oocyte. Panel (B) shows the current traces sampled at different time points (indicated as a-c on Panel A). Panel (C) shows a linear current-voltage relationship of the traces given on panel B, indicating that the channels mediating the current were insensitive to voltage. Panel (D) shows the current-voltage relationship of the rapidly deactivating net current after SNP washout. Figure 3.6 illustrates another I260Z-expressing oocyte showing primarily the slow gating mode. Panel (A) shows that SNP induces a slower rise of current and slower decay after removal of SNP as a function of time in an I260Z-expressing oocyte. Panel (B) shows the current traces sampled at different time points (indicated as a-c on Panel A). Panel (C) shows a outward rectifying current-voltage relationship of the traces given on panel B, indicating that the channels mediating the current were sensitive to voltage. The relatively small rapidly deactivating component, even though small in amplitude, showed the voltage-independent properties characteristic of the rapid gating mode, indicating (as in wild type) both modes can co-occur in the PDZ-truncated form. These data indicate that the PDZ-binding ligand domain was not required for either gating mode, in the oocyte expression system. Future studies using coexpression of
known interacting proteins may reveal a modulatory function for the PDZ-ligand domain.

**Aqp1 putative cGMP-binding domain is required for the slow gating mode of SNP current**

Published work supports the idea that the ion channel of Aqp1 might be activated by the direct binding of cGMP, and could involve the putative phosphodiesterase-like cGMP-binding domain in the carboxyl tail (Anthony, et al., 2000; Boassa and Yool, 2003). I tested this hypothesis using two truncation mutants, K243Z and D237Z, that have portions of the putative cGMP-binding domain ablated (see Figure 2.4 for location of truncations). Unexpectedly, truncated Aqp1 still showed cGMP-dependent activation. However, the response differed from wild type in that oocytes expressing D237Z or K243Z showed the rapidly gated mode, but not the slowly gated mode (n = 5). Figure 3.7 panel (A) shows SNP-induced current activation and immediate deactivation after washout of SNP in a K243Z-expressing oocyte. Panel (B) shows the current traces sampled at different time points (indicated as a-c on Panel A). Panel (C) shows the linear current-voltage relationship of the traces given in panel B, indicating that the channels mediating the current were insensitive to voltage. Panel (D) shows the current-voltage relationship of the current traces of net current deactivated after washout of SNP (inset is the current trace of Panel (D)). The preliminary data presented here are the first to show that the putative cGMP-
binding domain is not required for the rapidly gated current activated by cGMP in Aqp1-expressing oocytes, but it could be required for the slowly gated current. I would speculate that the rapid gating mode must be mediated by the proximal region of the C-terminus (not removed by D237Z truncation) or by other regions of Aqp1 such as the intracellular loops or N-terminus, or both. The fact Aqp1 mutants lacking the distal C-terminus function as cGMP-activated ion channel indicate that there are other domains proximal to the C-terminus that mediate the effects of cGMP.

The unexpected functionality of the K243Z and D237Z mutants suggested that the Aqp1 ion channel function may be convergently regulated by multiple pathways. It is not yet clear if the actions depend on direct activation, indirect activation, removal of chronic inhibition, or a combination. The selective loss of the slow gating mode in Aqp1 lacking the distal C-terminus suggests the rapid and slow gated modes are mediated by a structurally distinct mechanism. These preliminary data are clues that suggest further work could identify specific regions of Aqp1 that give rise to the distinct gating modes.

**Slow gated SNP-activated current has a linear non-pleateauing activation pattern**

Data described above provide evidence for two types of activation patterns. One activation pattern resulted in a rapid current rise that plateaued within the set recording duration (8 minutes) (Figure 3.8), a finding consistent with what was described in previous publications (Boassa and Yool, 2003). The second
activation pattern resulted in a steady rise in current that did not plateau within 8 minutes of recording (Figure 3.8). The steady rise seen in the slow mode could result from: (1) an increased number of channels of Aqp1 (by new insertion, or recruitment of initially silent channels); (2) an increase in single channel conductance; (3) an increased probability of channel opening; (4) post-translational modification (phosphorylation, dephosphorylation); (5) increased rate of cGMP synthesis as compared to the rate of degradation resulting in progressive increases in the intracellular concentration; or (6) a combination of mechanisms. All these proposed mechanisms are postulated on post-translational events delivered by the microenvironment. Since the intracellular cytoplasm is a highly compartmentalized environment this may be the basis for the co-occurrence of the two current types in a single oocyte.

To compare the activation patterns of SNP-induced Aqp1 currents, I quantified the relative profile of activation by calculating a “slope ratio” as illustrated in Figure 3.8. The ratio (A/B) was calculated as the linear slope measured at point A (300-325 sec) divided by the measured slope at point B (500-525 sec). Slope ratios > 1 represent a plateau response (characteristic of rapid activation) while slope ratios < 1 represent activation kinetics characteristic of slow activation. Data are shown for two Aqp1-expressing oocytes chosen to illustrate the rapid pattern (slope ratio of 3.3), and the slow pattern (slope ratio of 0.31), after stimulation with SNP.
To determine if there was a difference in slope ratios between the slow and rapid current types I first had to select a parameter to classify current types. Figure 3.9 A shows a frequency histogram of outward rectification values (see methods for calculation of outward rectification). I noted a bimodal distribution of the data and therefore classified observations that were within bins of 0.8 – 1.4 as linear and bins within 1.6 – 2 as rectifying. The linear group represented the rapid current and the rectifying group represented the slow current. I sorted the slope ratio data according to their corresponding rectification grouping (linear or rectifying). Figure 3.9 B shows the mean slope ratio of linear and rectifying groups. The slope ratio of the linear (rapid current) was on average 3.14, indicating a plateau kinetics. The slope ratio of the rectifying (slow current) was on average 0.75, indicating non-plateau kinetics. The slope ratio of the two groups differed significantly (*P< 0.05; linear 3.15 ± 0.93; rectifying 0.75 ± 0.31).

Wild-type properties and the effects of insulin

Insulin treatment significantly increased the amplitude of the Aqp1 current activated by SNP (Figure 3.10 panel A), but did not influence the characteristic properties or relative frequencies of occurrence of the slow and rapid gating modes (Figure 3.10 panels B-C). These results suggest that although insulin-induced signaling leads to enhanced availability of Aqp1 ion channels, it does not preferentially enhance either mode of gating.
3.4 DISCUSSION

Summary of findings

Data presented in this chapter are the first to show that two distinct modes of cGMP-gating of SNP-induced current co-occur in Aqp1-expressing oocytes. Rapidly gated SNP-induced currents displayed kinetic properties of rapid activation and deactivation after the addition and washout of SNP, respectively, a plateau phase after current activation, voltage-insensitivity and an average reversal potential of –16 mV. In contrast, slowly gated SNP-induced currents displayed kinetic properties of slow activation rate without a plateau phase and with incomplete deactivation after SNP during the time periods defined for the recordings. The slowly gated current also differed in having properties of voltage-sensitivity (outward rectification) and an average reversal potential of –25 mV.

Two patterns of activation kinetics (plateau and non-plateau) were observed in SNP-activated Aqp1-expressing oocytes. The slowly gated currents consistently exhibited a steady rise of current (non-plateau kinetics). The rapidly gated currents exhibited both steady state (plateau) and steady rise conditions.

My results show that both the rapid and slow gating modes can co-occur and can be individually isolated in Aqp1-expressing oocytes (in preparation). The data suggest that the differences in macroscopic current properties in SNP-
stimulated Aqp1-expressing oocytes could reflect the activation of two distinct channel populations or a single channel population with multiple activation modes. It is noteworthy that oocytes expressing an Aqp1 truncation mutant lacking the putative cGMP-binding domain showed only the fast gating mode. This result suggests that the slow mode involves the distal C terminus of Aqp1, and may be a clue to possible mechanisms of regulation (below). The differences in activation kinetics and reversal potentials between the fast and slow gating modes by convention appear to support the idea of distinct channel populations; however other possibilities exist. This discussion section explores rational possibilities that could explain the data presented in this chapter and describes experiments that would test each proposed possibility.

**The molecular basis for gating differences**

The ionic response of Aqp1 has been described as a cGMP-dependent voltage-insensitive cation current (Anthony, et al., 2000) with a reversal potential of ~ -15 mV in Na+ recording saline, properties associated with the current I have classified as rapidly gated. Four lines of evidence suggest that the slow gating mode reflects the activation of a distinct ion channel population: 1) difference in reversal potential; 2) voltage-dependence (outward rectification); 3) slower activation and deactivation kinetics; and 4) a linear rate of activation within the recording period. Nonetheless, the presence of this mode appears to be
dependent on the expression of Aqp1 which could mediate the conductance directly, or indirectly by enhancing endogenous currents. The obligatory involvement of Aqp1 is supported by the binding domain truncation mutants. If endogenous channels in combination with Aqp1 cause the slow gating mode, it could be mediated by oocyte channels regulated by cGMP, by volume regulatory ion channels activated by osmotic gradients induced through the water permeable feature of Aqp1, or by ion channels that are regulated by direct interactions with Aqp1 channels.

Four lines of evidence indicate that the slow gating as well as the rapid modes require the involvement of Aqp1 channels: (1) Very little SNP-induced current response is seen in control oocytes; (2) Both modes of currents are dependent on cGMP; (3) Both modes of gating are blocked by Cd^{2+} (an Aqp1 ion pore blocker); and (4) The slow gating mode is selectively lost with truncation of part of the C terminus, including the putative cGMP binding domain. The selective loss of the slow gating mode in Aqp1 carboxyl tail mutants suggests that this mode is dependent on regulatory sites on the distal C terminus. The distal C terminus of Aqp1 contains a number of putative regulatory sites including a putative cGMP-binding domain (Anthony, et al., 2000), PDZ binding domain (Cowan, et al., 2000), and putative Ca^{2+} binding domain (Fotiadis, et al., 2002). Possible regulatory mechanisms that mediate the slow gating mode include additional cGMP regulatory sites (direct or indirect) in the putative cGMP-binding region, protein-protein interaction with accessory proteins via PDZ binding
domain, or interaction with Ca$^{2+}$. Thus, experiments are needed to test if the currents are derived from distinct channel populations or from a single population.

**Two channel population hypothesis**

Could the slow gating mode be co-mediated by additional ion channels regulated by cGMP directly or indirectly? Expression of heterologous proteins has been shown to induce the expression of endogenous channels otherwise not expressed in control oocytes (Tzounopoulos, et al., 1995). To test this possibility, oocytes injected with an equal concentration of mRNA encoding Aqp1 mutant Y186N which was shown to be expressed in the oocyte plasma membrane but lacks ion permeability (Anthony, et al., 2000) would be a useful control.

Could the slow gating mode be mediated by endogenous volume regulatory ion channels activated by osmotic changes facilitated by Aqp1? *Xenopus* oocyte express a number of volume regulatory ion channels activated by changes in osmotic pressure (Ackerman, et al., 1994; Koomoa, et al., 2002). The water channel of Aqp1 has been proposed to serve an osmotic or turgor biosensor that in turn activates other signaling pathways, including osmo-regulating ion channels (Hill, et al., 2004). However increasing water permeability of oocytes alone by the expression of Aqp5 showed no cGMP-
activated currents (Anthony, et al., 2000). These results suggest that the ionic response is not an indirect effect of water permeability. I showed that increasing the extracellular osmotic tonicity by 10-15 mM in Aqp1-expressing oocytes did not alone induce any appreciable current, suggesting that the ionic response is not simply due to osmotic changes facilitated by Aqp1. Additionally, results from experiments that eliminate the osmotic factor also support this idea. Single channel recordings in excised patches of Aqp1 expressed in oocytes showed cGMP-activated currents with deactivation kinetics after cGMP removal that were consistent with both the rapid and slow mode of gating observed in macroscopic recordings (Yool and Karpen, unpubl). Since the osmotic gradient was not altered in these excised patch environments, this result argues against the idea that the ionic response is an indirect effect of water permeability.

Could the slow gating mode be mediated by endogenous ion channels that are regulated by interaction with distal C terminus of Aqp1 channels? Observations in the literature hint of the existence of cross-regulatory mechanisms where activity of one type of channel affects the functioning of another type of channel. The water channel function of Aqp1 has been suggested to be regulated by activity of Aqp1 associated proteins which include potassium channels IRK-8 and proteins involved in the $G_{\alpha3}$-PLA2 signaling pathway in the Zymogen granules (Abu-Hamdah, et al., 2004). Macromolecular signaling complexes that include Aqp1, anion exchangers, and receptor tyrosine kinase EphB2 are networked by interaction with the PDZ-containing protein
PICK-1 (Cowan, et al., 2000). Disruption of these macromolecular complexes give rise to abnormal development of the inner ear in the mouse (Cowan, et al., 2000). In light of the results from these studies, it is reasonable to propose the idea that Aqp1 could also influence the behavior of other signaling proteins, possibly activating other ion channels. The most direct way of testing the involvement of Aqp1 C-terminus in the activation of endogenous channels is by expressing the Aqp1 C terminus alone in oocytes and then record the cGMP-activated ionic response.

My data indicate that the PDZ domain is not necessary for the ionic response of cGMP-activated Aqp1 channels expressed in oocytes, but this result needs to be confirmed by additional experiments. The results of my preliminary data showed that the slow gating mode occurs in Aqp1 mutants that lack the PDZ-binding domain, but not in Aqp1 mutants that lack the putative cGMP and Ca\(^{2+}\) binding sites. This finding rules out the molecular interaction that occur at PDZ ligand binding domain (at least in oocytes), but cannot eliminate possible interactions at the other putative binding domains. If expression of the Aqp1 C-terminus alone in oocytes induced the slow gating mode, then the likelihood of a contribution of endogenous channels to the slow gating mode would be high.

Currents with different reversal potentials traditionally are considered to indicate channels with different ion selectivity pathways, therefore by convention would suggest the parallel contributions of distinct channel populations. The assumption of an immutable ionic selectivity for a single channel must be
reconsidered after the discovery of the purinergic receptor (P2X), family of ligand-gated cation channels. Upon activation by ATP-binding, P2X receptors mediate cation flux that undergoes changes in ion selectivity which is dependent on time and prior ATP exposure (Khakh, et al., 1999; Virginio, et al., 1999). The basis of the ion selectivity change is thought to be due to changes in pore diameter (Khakh, et al., 1999; Virginio, et al., 1999). The change in ion selectivity correlates with a change in activation kinetics (Khakh, et al., 1999), thus lending support to the idea current kinetics alter when ion permeability is altered in a single channel population. Although the mechanistic basis for this phenomenon is not well defined, regions of the carboxyl tail and pore domain were shown to influence the ability to alter ion selectivity (Khakh, et al., 1999; Surprenant, et al., 1996). The unconventional behavior of purinergic channels sets a precedent that supports the idea that the rapid and slow current modes in Aqp1-expressing oocytes may reflect a single channel population with state-dependent ion permeability. The slow current mode of Aqp1 had distinct characteristics from that of the rapid current mode (e.g. voltage-sensitivity, non-plateau activation profile, and sustained current after SNP removal), thereby providing a means of detecting the modes of current. Unlike the P2X receptor, however, a possible mode change in Aqp1 would not be directly time dependent, (or if it is, would be very slow) since I did not see conversion from one mode to the other within the recordings.
**Single channel population hypothesis:**

Could the existence of different Aqp1 gating modes indicate that the ionic function of Aqp1 is regulated by cGMP by more than one mechanism and sensitive to the environment of the expression system? I show that the cGMP-mediated ionic response is still retained in Aqp1 mutants lacking major portions of the distal C-terminus (D237Z and K243Z) when expressed in the oocyte system. In contrast, the ionic response was not seen when the C-terminal truncated Aqp1 channels were reconstituted in the bilayer system (Saparov, et al., 2001). This difference suggests that a molecular component expressed endogenously in the oocyte system is necessary for cGMP-mediated ionic response in Aqp1 mutants that lack the distal C-terminus, but not necessary to induce cGMP-mediated ionic response in wild-type channels. This unexpected result indicates that direct application of cGMP is sufficient and necessary for the activation of the wild type Aqp1 ion channel, but is not sufficient for Aqp1 channels lacking the distal C terminus, and argues for more than one mechanism of activation.

The rapid gated current of truncated Aqp1 (retained in oocytes and lost in bilayers), thus appears to require an endogenous component from the oocytes. Among many possibilities, these could be kinases or phosphatases gating the rapid current of Aqp1 by direct protein phosphorylation or dephosphorylation. A PKG consensus site (Kennelly and Krebs, 1991) is found in Aqp1 near the
putative cGMP-binding domain, and is retained in the C-terminal truncation
mutants (D237Z and K243Z). Results in chapter two showed that Aqp1 is
phosphorylated by unknown serine/threonine kinases in oocytes. Gating of ion
channels by protein phosphorylation and dephosphorylation is a common
mechanism in physiology (Fukao, et al., 1999; Levitan, 1999; Nara, et al., 2000;
Yanochko and Yool, 2002). cGMP activates PKG which in turn phosphorylates a
number of other ion channels (Fukao, et al., 1999; Nara, et al., 2000) as well as
some classes of phosphatases (Hofmann, et al., 2000; Wooldridge, et al., 2004).
To date, experimental evidence argues against the idea of PKG serving as the
link to Aqp1 ion channel activation in the presence of an intact C-terminus.
Experiments from Anthony et al showed that inhibition of PKG still allowed
appreciable current activation in cGMP-stimulated Aqp1-expressing oocytes;
whereas pharmacological activation of PKG by the drug Sp-8-Br-PET-cGMPS
failed to induce Aqp1 current in oocytes without cGMP stimulation (Anthony, et
al., 2000), suggesting that phosphorylation alone is insufficient to drive activation
in the wild-type channels. These results were interpreted as showing that PKG is
not involved in cGMP-activation of Aqp1 channels. However, we cannot rule out
the idea that phosphorylation by other kinases is a prerequisite for cGMP
activation and may even be sufficient in the absence of the distal C-terminus.
Phosphorylation of the anion channel Cystic Fibrosis Tranport Regulator (CFTR)
by PKA is a prerequisite for ATP-mediated ion channel activation (Tabcharani, et
al., 1991).
An alternative model is that Aqp1 is gated by dephosphorylation mediated by an unknown serine/threonine phosphatase that is activated by cGMP signaling. This predicts that phosphorylated Aqp1 is the non-activating channel state. Preliminary data show that the putative serine phosphorylation site (S236) is not necessary for SNP-induced Aqp1 channel activation since mutation to alanine does not prevent a cGMP-induced current (data not shown). However, these data would fit with the idea that the dephosphorylated state is capable of ion channel activation. Pharmacological inhibition of phosphatases in Aqp1-expressing oocytes would test the dephosphorylation hypothesis.

Another possibility is that the endogenous component required for the rapid gating mode could be an accessory protein interacting with cytoplasmic facing intracellular loops of Aqp1 or the N-terminus or a combination. The cGMP gating of the rapid current could be mediated by direct binding to the associated protein or could enhance cGMP binding to Aqp1. As an example, the α-subunit of the rod CNG channels interacts with a tightly associated protein that binds cGMP analogs and mediates activation of the CNG channel (Brown, et al., 2000), perhaps influencing the stability of ligand binding to the CNG channels. Using a similar mild treatment with trypsin on excised patch from an Aqp1-expressing oocyte would test if tightly associated protein(s) also are involved in the ionic response of Aqp1.
Mechanisms of the slow gating mode

The selective loss of the slow gating mode in C terminus truncation mutants (D237Z and K234Z) indicates this region governs the rate of channel activation, but does not directly couple cGMP signaling to opening of the pore.

There are a number of putative regulatory domains in this region of Aqp1 including putative cGMP-binding domain (Anthony, et al., 2000), putative PDE-like domain (Boassa and Yool, 2002), putative Ca$^{2+}$ binding site (Fotiadis, et al., 2002), a putative tyrosine phosphorylation site (Songyang and Cantley, 1995), and a protein interaction ligand (PDZ) domain (Cowan, et al., 2000). Each of these potential regulatory mechanisms will be considered.

Does the C-terminus directly bind cGMP? Prior work has shown that carboxyl tail mutations of conserved amino acid residues of Aqp1 that show sequence homology with other cGMP-binding protein resulted in decrease in the amplitude cGMP-activated Aqp1 currents expressed in oocytes (Boassa and Yool, 2003). Based on sequence similarities between Aqp1 C-terminus, and the subdomains of PDE and CNG proteins, residues D237 and K243 were targeted for point mutation and the effects of the mutations were tested. Not all the amino acid substitutions tested caused a functional impairment, thus the idea these specific residues are directly involved in forming the cGMP-specific binding domain are not supported by these results. In addition, a comparison of the putative binding domain with the binding domains of CNG channels shows that
much of the necessary CNG structure is not obvious in Aqp1. These mutagenesis results suggest that the specific targeted residues may influence the structure of the C terminal and hence affect its modulatory function indirectly. Point mutations of D237A and K243C resulted in decreased cGMP-induced current amplitudes. The effects of a double mutation D237A K243C were tested. The result of the double mutation was one of further decrease in current amplitude, indicating the additive modulatory effect of these residues to cGMP-activation of Aqp1 current. It would be interesting to reanalyze data analyzed by Boassa and Yool to test the prediction that the slow gating component was selectively lost in the D237A K243C C-tail mutants. Additional experiments using competitive inhibitors could test if there is direct binding of cGMP to the C-terminus, to better understand the nature of cGMP involvement in Aqp1 activation. One approach would be to add purified fusion protein of Aqp1 C terminus into the recording bath of an excised patch to compete with the cGMP binding with wild-type Aqp1 channels. Another approach using an excised patch would be to add anti-Aqp1 antibodies raised against the distal C-tail with the idea of mimicking or antagonizing the possible cGMP binding site.

Does the slow gating mode reflect a rate limiting enzymatic activity? The gating of Aqp1 ion channels by a cGMP-hydrolyzing mechanism is suggested by the conserved patterns of amino acids in key sequences similar to those of phosphodiesterase (PDE) selectivity domains. Other channels are gated by enzymatic activity intrinsic to the protein. Cystic Fibrosis Transport Regulator
(CFTR) functions as an ATP-activated ion channel and an enzyme that hydrolyzes ATP (Basso, et al., 2003; Berger, et al., 2005). The binding of ATP to the nucleotide binding domain 1 and 2 (NBD1 and NBD2) of CFTR appears to mediate the opening while the hydrolysis of ATP bound to NBD2 is responsible for channel closure (Basso, et al., 2003; Berger, et al., 2005). It is reasonable to propose that the slow gated current of Aqp1 could arise through an analogous mechanism. cGMP could directly bind to the PDE-like putative cGMP-binding region of Aqp1, though is unlikely to be the only step initiating channel opening since the deletion of the domain still allows for rapid cGMP-dependent activation. However, channel closure could be dependent on cGMP hydrolysis, thus explaining the sustained current observed after SNP washout. The amino acids that are conserved in the Aqp1 C terminus and in the cGMP binding domain of phosphodiesterase (PDE) (Boassa and Yool, 2002) are critical in PDEs for substrate selectivity between cAMP and cGMP, and for mediating the binding of 3-isobutyl-1-methylxanthine (IBMX), a PDE inhibitor (Huai, et al., 2004). Preliminary experiments in Aqp1-expressing oocytes show that incubation with IBMX ablates Aqp1 current activation by SNP (Boassa and Yool, unpubl), suggesting that IBMX can interfere with the cGMP-induced activation of Aqp1. IBMX might act by competing with cGMP binding to Aqp1 and preventing activation, or by occupying the PDE site and trapping the channel in a closed state. To test this possibility that Aqp1 can cause cGMP hydrolysis, I would
propose that a fusion peptide carrying the C terminus of Aqp1 should be tested for its ability to hydrolyze cGMP.

Does Ca$^{2+}$ binding at the EF hand regulate the availability of Aqp1 channels to be gated by cGMP? The distal C-terminus of Aqp1 contains a putative Ca$^{2+}$ binding site (Fotiadis, et al., 2002) suggested by sequence homology with an EF-hand motif belonging to a superfamily of Ca$^{2+}$ binding proteins that include Calmodulin (Fotiadis, et al., 2002). Ion channel function is commonly regulated by direct Ca$^{2+}$ binding. Depolarization drives voltage-sensitive Na$^{+}$ channels into an inactivated state, therefore rendering them functionally unavailable (Wingo, et al., 2004). Direct binding of Ca$^{2+}$ ion to an EF-hand binding domain on the C-terminus of Human cardiac Na$^{+}$ channel (hH1) modulates hH1's voltage-dependent inactivation (Wingo, et al., 2004). Experiments that would test the effects of mutations of the Aqp1 putative Ca$^{2+}$ binding site would provide insight into its possible role in the slow gating mode. Experimental manipulation of internal Ca$^{2+}$ concentrations on cGMP-activated Aqp1 channels in excised patches would be a logical first step. To test if Ca$^{2+}$ directly interacts with Aqp1 distal C tail, a Ca$^{2+}$ binding assay using radiolabeled Ca$^{2+}$ to label immunoprecipitated Aqp1 wild-type and Aqp1 mutant proteins would be valuable.

Could the slow gating mode be mediated by phosphorylation or dephosphorylation at the putative tyrosine phosphorylation site in the Aqp1 distal C terminus? This idea was tested by site-directed mutagenesis of the putative
tyrosine residue to Alanine (Aqp1 mutant Y253A). Oocytes expressing the Y253A channels had SNP-induced ionic currents characteristic of the rapid gating mode, a finding consistent with the observation that the rapid gating mode does not require regulatory domains in the distal C terminus. However, I cannot conclude if the effects of the Y253A mutation include the selective loss of the slow gating mode because of the sample size tested (n of 2).

Do endogenous components of oocytes interact with the Aqp1 distal C terminus to give rise to the slow gating mode? Preliminary data show that the slow gating mode does not depend on PDZ-binding domain of Aqp1 in oocytes, but this requires further testing due to small sample size. Several studies have shown that Aqp1 channels exist in macromolecular signaling complexes (Abu-Hamdah, et al., 2004; Cowan, et al., 2000) required for proper development of the inner ear in mice (Cowan, et al., 2000) or GTP-induced swelling in Zymogen vesicles in exocrine pancreas (Abu-Hamdah, et al., 2004). While it is unknown how Aqp1 interacts with the GTP-activated signaling complex in Zymogen vesicles, it is known that Aqp1 interacts with the PDZ-containing linker protein PICK-1 at the PDZ-ligand binding domain in the distal C terminus (Cowan, et al., 2000). Colocalization of Aqp1 and the receptor tyrosine kinase EphB2 in the inner ear is suggested to occur through PICK-1 (Cowan, et al., 2000). The involvement of Aqp1 channels in regulatory complexes and the disruption of these complexes leading to physiological dysfunction suggest that the function of Aqp1 water or ion channel may be regulated by endogenous proteins.
Basis of non-plateauing activation kinetics

Two patterns of activation kinetics (rapid and slow) were observed in SNP-activated Aqpl-expressing oocytes. The steady rise of current seen in the slow pattern did not reach a plateau in the window of time chosen for my recordings. The possible bases for the steady rise of current could be: (1) an increased number of channels of Aqpl (by new insertion, or recruitment of initially silent channels); (2) an increase single channel conductance; (3) an increased probability of channel opening; (4) post-translational modification (phosphorylation, dephosphorylation); or (5) increased rate of cGMP synthesis as compared to the rate of degradation resulting in progressive increases in the intracellular concentration. To ask if the steady current rise is due to increased channel density, testing the effects of cyclohexamide (protein synthesis inhibitor) or Leupeptin (prevents protein degradation) treatment on the cGMP-ionic response in Aqpl-expressing oocytes would be of great interest. This approach would not detect an increase in Aqpl ion channel availability. To directly test this possibility, experiments at the single channel level must be performed. To ask if the observed steady rise is due to an increase of cGMP level, intracellular cGMP could be measured using a biosensor (cGMP gated CNG channel). Parallel measurement of cGMP and ionic current levels in real time would provide information as to how cGMP levels influence activation kinetics and current
amplitudes. cGMP assays have confirmed that SNP elevates cGMP levels in X. oocytes (Boassa and Yool, 2003), but to match cGMP levels with properties of current activation, deactivation, and plateau rate of activation would show whether the current amplitude shows a dose dependent response. To ask if the steady rise is due to post-translational modification (mainly phosphorylation), testing the effects of kinase blockers or Aqp1 mutations of putative phosphorylation sites could answer this possibility. Single channel experiments would be required to determine if single channel conductance or increase channel open times are the bases for the steady rise of macroscopic current observed. Each experimental approach could provide insight to the basis of the observed steady current rise. However, it should be noted that the steady rise in the current I observed may not be due to a “single” mechanism, but rather reflect several simultaneously occurring mechanisms. If the latter is the case, this will be more difficult to dissect.

Summary

In conclusion, my data show two distinct types of SNP-activated currents in Aqp1-expressing oocytes that differ in several properties, but both show dependence on cGMP. The effects of distal C-terminus truncations showed that the slowly gated current requires the presence of the Aqp1 distal C terminus while the rapidly gated currents do not. In the batches of oocytes selected for
analysis, the assessment criterion was that control oocytes showed no SNP-sensitive currents. Thus, Aqp1 expression is necessary for both SNP-sensitive gating modes. Additional experiments are required to determine if the distinct current types are mediated solely by Aqp1 channels or involve accessory proteins such as endogenous channels. The rapid and slow gating modes appear to be regulated by cGMP through separate mechanisms. I speculate that the rapid gating mode is regulated by a direct binding of cGMP to an unidentified site, based on the rapid time course of cGMP-mediated current activation and immediate deactivation after SNP washout. In addition, I speculate that the slow gating mode is regulated by a process that has a slow-rate limiting step such as cGMP hydrolysis, that would allow the cGMP-activated current to be sustained after SNP washout. Additional experiments could reveal that there are multiple mechanisms in Aqp1 ion channels that sculpt the cGMP-dependent gating.
Figure 3.1 The rapid gating mode of SNP-activated Aqp1 current displays properties of rapid deactivation after SNP removal and a linear current-voltage relationship. See next page for details.
Figure 3.1 The rapid gating mode of SNP-activated Aqp1 current displays properties of rapid deactivation after SNP removal and a linear current-voltage relationship. A. Ion current profile at +40 mV as a function of time after SNP in an Aqp1-expressing oocyte. Holding potential was −40 mV. Symbols (a-e) indicate the points of time when current-voltage recordings were taken. Boxes indicate bath perfusion; arrows show bolus applications, with calculated final concentrations. B. Current traces evoked from +60 mV to −120 mV at 10 mV increments from a holding potential of −40 mV (scale bar 2 µA/ 50 msec). Current traces were taken at time point (a) initial current; (b) after SNP-stimulation; (c) after bath perfusion; (d) after SNP-stimulation; and (e) after bath perfusion. C. Current-voltage relationships of the traces shown in panel B at time points (a-e). D. Net current trace of inactivated current after SNP removal, calculated by subtracting the current traces at time point (c) from the current traces at time point (b). E. The net current-voltage relationship of current traces from panel D.
Figure 3.2 The slow gating mode of SNP-activated Aqp1 current displays properties of delayed deactivation after SNP removal and outward rectification. See next page for details.
Figure 3.2  The slow gating mode of SNP-activated Aqp1 current displays properties of delayed deactivation after SNP removal and outward rectification.  

A. Ion current amplitude at +40 mV as a function of time after SNP in an Aqp1-expressing oocyte. Symbols (a-e) indicate the points of time when the current-voltage recordings were taken. (Boxes and arrows as described for Fig 3.1) indicate bath perfusion events and duration.  

B. Current traces evoked from +60 mV to –120 mV at 10 mV increments from a holding potential of –40 mV (scale bar 2 µA/ 50 msec). Current traces were taken at time point (a) initial current; (b) after SNP-stimulation; (c) after bath perfusion; (d) after Cd$^{2+}$ block; and (e) after bath perfusion. Scale bars are 2000 nA and 50 msec.  

C. Current-voltage relationships of the traces shown in panel B at time points (a-e).  

D. Current traces mediated by the slow gated and rapid gated modes comprised the total SNP-activated current in an Aqp1-expressing oocyte. The current trace of the rapid gating mode was obtained by subtracting the current sustained after SNP removal with SNP-activated current (time points c-b).  

E. The current-voltage relationships of current traces from panel D.
Figure 3.3 Rapid and slow SNP gated currents have reversal potentials that are significantly different. Reversal potentials of recovered and sustained current components from SNP-activated currents calculated from SNP-activated current that showed that showed properties of delayed inactivation after SNP removal.
Figure 3.4  Outward rectification of current positively correlates with the percent rapidly deactivating current in the absence of SNP. Relative outward rectification values of SNP-activated currents in Aqp1-expressing oocytes plotted over their corresponding percent current recovery in the absence of SNP (Simple regression; P < 0.002). The percent current recovery was calculated as [(C₀ - Cₓ) / C₀] * 100; where C₀ is the highest current value after SNP activation, and Cₓ is the current value after SNP wash. The magnitude of relative outward rectification was calculated as a ratio of the absolute values of current amplitudes measured at +30 mV and −70 mV. These two points were selected as being approximately equidistant from the reversal potential for a cation current (−17 mV).
Figure 3.5  The PDZ-binding domain does not mediate the rapid gating properties observed in SNP-activated Aqp1 current. See next page for details.
Figure 3.5 The PDZ-binding domain does not mediate the rapid gating properties observed in SNP-activated Aqp1 current. 

**A.** Ion current amplitude of an I260Z-expressing oocyte sampled at +40 mV from a holding potential of –40 mV, as a function of time in response to SNP stimulation. SNP removal was by bath perfusion. Symbols (a-c) indicate the points of time when current-voltage recordings were made. Boxes indicates bath perfusion. 

**B.** Current traces evoked from +60 mV to –100 mV at 10 mV increments from a holding potential of –40 mV (scale bar 2 µA/ 50 msec). Current traces were taken at time point (a) initial current; (b) after SNP-stimulation; and (c) after bath perfusion. 

**C.** Current-voltage relationships of the traces given on panel B at time points (a-c). 

**D.** The current-voltage relationship of current deactivated after SNP removal. Deactivated current was calculated by subtracting the current trace at time point (c) with the current trace at time point (b) (insert is the net current trace of (D)).
Figure 3.6 The PDZ-binding domain does not mediate the slow gating properties observed in SNP-activated Aqp1 current. See next page for details.
Figure 3.6 The PDZ-binding domain does not mediate the slow gating properties observed in SNP-activated Aqp1 current. A. Ion current amplitude of an I260Z-expressing oocyte sampled at +40 mV from a holding potential of −40 mV, as a function of time in response to SNP stimulation. SNP removal was by bath perfusion. Symbols (a-c) indicate the points of time when current-voltage recordings were made. Box indicates bath perfusion. B. Current traces evoked from +60 mV to −100 mV at 10 mV increments from a holding potential of −40 mV (scale bar 2 µA/50 msec). Current traces were taken at time point (a) initial current; (b) after SNP-stimulation; and (c) after bath perfusion. C. Current-voltage relationships of the traces given on panel B at time points (a-c).
Figure 3.7  The putative cGMP-binding domain does not mediate the rapid gating observed in SNP-activated Aqp1 current. See next page for details.
Figure 3.7 The putative cGMP-binding domain does not mediate the rapid gating observed in SNP-activated Aqp1 current. A. Ion current amplitude of a K243Z-expressing oocyte sampled at +40 mV from a holding potential of –40 mV, as a function of time in response to SNP stimulation. SNP removal was by bath perfusion. Symbols (a-c) indicate the points of time when the current-voltage recordings were made. The box indicates bath perfusion. B. Current traces evoked from +60 mV to –100 mV at 10 mV increments from a holding potential of –40 mV (scale bar 2 μA/ 50 msec). Current traces were taken at time point (a) initial current; (b) after SNP-stimulation; and (c) after bath perfusion. C. Current-voltage relationships of the traces given on panel B at time points (a-c). D. The current-voltage relationship of current deactivated after SNP removal. Deactivated current was calculated by subtracting the current trace at time point (c) with the current trace at time point (b) (insert is the net current trace of (D)).
Figure 3.8 The activation rates of SNP-stimulated Aqp1 current display kinetic properties reflecting either a plateau or a linear/exponential trend by 500 seconds after SNP stimulus. The slope ratio was calculated as (slope A/slope B). The slope A and slope B (point A = 300-325 sec; point B = 500-525 sec) were calculated by the rise over run over 5 time points within two windows of time. Current development with slope ratios > 1 indicate plateau (or saturating) kinetic properties (Black activation curve = 3.33) while slope ratios of ≤ 1 indicate linear or exponential kinetic properties (gray activation curve = 0.31) by 500 seconds after SNP-stimulation.
Figure 3.9 Rapidly gated currents have plateau activation profile and slowly gated currents have a non-plateau activation profile. See next page for details.
Figure 3.9  Rapidly gated currents have plateau activation profile and slowly gated currents have a non-plateau activation profile.  

A. Frequency histogram of the outward rectification values calculated from slow and rapid responding currents.  A bimodal distribution of the data were observed and classified as linear (0.8 - 1.4) and rectifying (1.6 - 2).  

B. Mean slope ratio values of data classified as linear and rectifying (*P< 0.05; linear 3.15 ± 0.93; rectifying 0.75 ± 0.31).  Asterisk indicates significant difference between the slope ratio of linear and rectifying groups.
Figure 3.10  Summary histogram of the effect of insulin treatment on SNP-induced current properties displayed in Aqp1-expressing oocytes.  
A. Mean current amplitudes by 240 seconds after SNP-stimulation (student’s t-test; P<0.002 (insulin = 817 nA ±103.19 (mean ± SEM; n = 31); no insulin = 389 nA ± 77.47 (mean ± SEM; n = 18).  
B. Mean relative outward rectification value (student’s t-test; (insulin = 2.25 ± 2.29 (mean ± SEM; n = 7); no insulin = 2.66 ± 0.89 (mean ± SEM; n = 2).  
C. Mean slope ratio values (student’s t-test; (insulin = 1.24 ± 0.23 (mean ± SEM; n = 31); no insulin = 1.66 ± 0.21(mean ± SEM; n = 18).  
D. Mean percent sustained current after SNP removal (student’s t-test; (insulin = 43.4 ± 8.53 (mean ± SEM; n = 5); no insulin = 35.9 ± 10.19 (mean ± SEM; n = 10) in Aqp1-expressing oocytes treated with or without insulin. Asterisk indicates significant difference between insulin and non-insulin treated groups.
Aqp1 functions as a cGMP regulated ion channel when expressed in *Xenopus* oocytes or reconstituted in lipid bilayers. The inconsistency of cGMP activation across oocyte batches, indicated that cGMP is not the exclusive intracellular signaling pathway regulating the ion channel function of Aqp1. Prior experiments showed that only a small subpopulation of Aqp1 proteins function as cGMP-activated ion channel. Identifying intracellular pathways that may dictate ion channel responsiveness to cGMP stimulation became the focus of my dissertation. I found that tyrosine kinase signaling pathways activated by insulin binding to the IGF receptors in the oocyte positively modulate cGMP-activated Aqp1 current. I tested the involvement of insulin-activated signaling pathway based on the observation that oocyte batches that produced large BIB current (another MIP ion channel) inversely produced very little SNP-activated Aqp1 current. Prior experiments showed that suppression of endogenous levels of tyrosine kinase activity in oocytes increased BIB current. Conversely, my work has shown that insulin-activated signaling pathways regulate the ion channel activity of Aqp1 in a positive manner. This finding supports the idea that multiple intracellular signaling pathways converge on Aqp1 protein, regulating the cGMP-activated ionic current response.

The insulin-activated signaling pathways appear to positively modulate the ion channel function of Aqp1, and not directly gate channel activation. The intermediate signaling pathways involved in coupling IGF activation with
enhanced Aqp1 ion channel function are unknown. It does appear that the insulin-activated signaling pathway is not sufficient for cGMP activation of Aqp1. Insulin treatment alone did not induce ionic current in Aqp1-expressing oocytes, thus indicating that the ion channel activation was dependent on cGMP. Through reconstitution in lipid bilayer system, Aqp1 channels were activated by cGMP in the likely absence of an intracellular tyrosine kinase pathway. These observations suggest that insulin-activated signaling pathways positively modulate, rather than directly gate, the ion channel function of Aqp1.

The mechanism of insulin potentiation of Aqp1 ion current is not known. Insulin treatment of Aqp1-expressing oocytes did not increase water permeability, thus suggesting that insulin did not increase channel density. Unexpectedly, Aqp1 mutant channels lacking major portions of the distal C terminus functioned as cGMP-activated ion channels when oocytes were treated with insulin. This C-terminus truncation mutant channel did not respond to cGMP when reconstituted in the lipid bilayer, a system lacking tyrosine signaling pathways. These results provided two conclusions: 1) that the effect of insulin treatment was not mediated by domains on Aqp1 distal C terminus; and 2) the putative cGMP-binding domain in the distal C-terminus is not required for cGMP-gating of Aqp1 channels in the presence of activated tyrosine kinase pathways. Aqp1 is phosphorylated by an endogenous serine/threonine kinase in the oocyte system, but the consequence of this phosphorylation to ion channel activity is not known. The putative PKG phosphorylation site located at the proximal C-
terminus of Aqp1 remained intact in the C-terminus truncation mutant channels that were tested and showed responses to cGMP after insulin pre-treatment. The effects of mutating this putative phosphorylation site remain to be determined. Despite the requirement for additional experiments, it is clear that the cGMP-activated Aqp1 ion channel function can be positively modulated by signaling pathways activated by IGF stimulation in oocytes.

I have found that SNP stimulation in Aqp1-expressing oocytes generated two distinct gating modes of current that co-occur from a single oocyte. Rapidly gated SNP-induced currents displayed kinetic properties of rapid activation and deactivation after the addition and washout of SNP, respectively, a plateau phase after current activation, voltage-insensitivity and an average reversal potential of –16 mV. In contrast, slowly gated SNP-induced currents displayed kinetic properties of slow activation rate without a plateau phase and with incomplete deactivation after SNP during the time periods defined for the recordings. The slowly gated current also differed in having properties of voltage-sensitivity (outward rectification) and an average reversal potential of –25 mV. Current from both gating modes are dependent on SNP and blockable by Cd^{2+}. The removal of the Aqp1 distal C-terminus resulted in the selective loss of the slow gating mode, thus suggesting that the regions of the distal C-terminus give rise to the slow gating mode.

Data presented in these chapters showed three distinct gating modes (off, slow, and rapid) in Aqp1-expressing oocytes relatively stable during the time
frame of recording. The diversity of response implicates the involvement of other signaling pathways in the cGMP regulation of Aqp1 channels. This idea is supported by the observation that Aqp1 still functions as a cGMP gated ion channel despite the removal of the putative cGMP-binding domain, but only in insulin treated oocytes. In addition, the slow gating mode is selectively lost with the removal of regions of the distal C terminus in Aqp1 channels. Additional experiments are required to determine if the distinct gating modes are mediated through a single channel population or a separate channel population that is cross regulated by the interaction with the carboxyl tail of Aqp1. Data presented in this dissertation demonstrate the diversity of Aqp1 ion channel behavior and that insulin-activated signaling pathway enhances Aqp1 channel function. These findings are consistent with the idea that Aqp1 is a target for convergent, intracellular signaling pathways that include tyrosine kinases whose synergistic actions result in Aqp1 ion channel activation in the presence of cGMP. Tyrosine kinases and other signals may serve as ‘master switches’ governing Aqp1’s ability to behave as an ion channel.

There are several issues that are currently unresolved by the experiments performed in this dissertation. Where is the site of serine phosphorylation detected in the western blot of Aqp1 proteins expressed in oocytes? Currently, this site is hypothesized to be located at position 236. This hypothesis is based on the observations that the serine 236 is within a PKG consensus site and the domain is exposed to the cytosol of the oocyte. A direct way of testing this
hypothesis is by point mutating the ser236 residue and then asking if the effect of mutation includes elimination of serine phosphorylation normally observed in wild-type proteins. Does Aqp1 phosphorylation have an impact on ion channel function? Preventing the occurrence of channel phosphorylation would allow insight as to what the contribution of phosphorylation is. Assuming ser236 is the site of phosphorylation, the question of what the functional consequence of phosphorylation can be answered by studying the effects of S236 mutation on ion channel function. Another issue left unresolved is the effect of insulin treatment on Aqp1-expressing oocytes on Aqp1 phosphorylation. Does insulin treatment affect the serine phosphorylation status of Aqp1? The hypothesis is that insulin-sensitive signaling pathways affect the phosphorylation status of Aqp1 at the ser236 position. Biological end points mediated by insulin often occur through a phosphorylation/dephosphorylation mechanism. Insulin treatment alone is not sufficient to induce Aqp1 ion current. The next logical question would be are tyrosine kinases necessary to induce cGMP-dependent Aqp1 current? This question could be answered by testing the effects of inhibiting tyrosine kinases in oocytes on Aqp1 ion current.

The next big questions prompted from the findings of this dissertation include what domains of Aqp1 mediate the gating effects of cGMP? What is the intermediate pathway that couples the effects of insulin with Aqp1 ion channel function? How might insulin change cGMP binding?
Understanding the regulatory mechanism of Aqp1 channels not only requires identifying the signaling pathways that modulate channel function, but identifying structural domains of the channels that serve as the substrate for the signaling pathway. Sequence alignment of the amino and carboxyl termini of Aqp1 proteins across vertebrate taxa show that domains are highly conserved (Figure 4.1 A-B). Aqp1 proteins contain a PDZ-interacting ligand binding site (Figure 4.1 A Box 3) in the distal carboxyl terminus and a number of putative regulatory domains have been identified. While the PDZ-ligand and the putative regulatory domains of the distal carboxyl terminus do not appear to be necessary for the rapid mode of cGMP activation of Aqp1 current in X. oocytes, the contributions of these domains may be revealed when Aqp1 functions are studied in mammalian systems.

Do the domains of the amino terminus contain regulatory sites? The Aqp1 amino terminus (Figure 4.1 B) shows three consecutive lysine residues that confer a basic isoelectric value of 9.7 (ExPASy-Compute pI/Mw tool). Actin interacts with proteins with highly basic isoelectric values. The highly basic isoelectric value of the Aqp1 amino terminus supports the possibility that this domain could serve as a putative actin interacting site. Actin interaction has been shown to regulate a number of ion channels. It has been proposed that a major function of Aqp1 is to serve as an osmosensor for the regulation of cell volume (Hill, et al., 2004). Perhaps the ion channel function of Aqp1 is intimately coupled to microcellular structure and actin may serve as the coupling
component. Activation of Aqp1 ion channels in response to changes in actin organization could be a basis for how Aqp1 contributes to the regulation of cell volume.

A number of studies show insulin-sensitive signaling pathways of the oocyte system regulate the function of heterologously expressed ion channels through diverse mechanisms. The function of the non-specific cation channel, Big Brain, is reduced by insulin-mediated phosphorylation of tyrosine residues (Yanochko and Yool, 2002). The function of the rod CNG channel is enhanced by IGF-mediated dephosphorylation of a tyrosine residue on the \( \alpha \) subunit (Savchenko, et al., 2001). The function of voltage-activated \( \text{Kv}_{1.3} \) channel is decreased by insulin-mediated downregulation of channel expression at the plasma membrane (Henke, et al., 2004). As shown in Figure 4.2, auto-phosphorylation of the insulin receptor activates a myriad of downstream signaling pathways which mediate a wide spectrum of biological processes, including cell growth, glucose uptake, glycogen, lipid and protein synthesis (Taha and Klip, 1999). Although the signaling cascade is initiated by tyrosine kinase activity of the insulin receptor itself, much of the downstream phosphorylation/ dephosphorylation events occur through the activity of serine/ threonine kinases and phosphatases.

What are the intermediate pathways involved in coupling the effect of insulin with enhanced Aqp1 ion channel activity? Insulin signals the reorganization of actin in rat liver plasma membrane (Balbis, et al., 2004) and muscle cells (Tsakiridis, et al., 1999). IGF-I (insulin-like growth factor-I) mediates actin
reorganization via PI3-K pathway (Lynch, et al., 2005). Actin cytoskeletal organization is critical for cAMP-dependent activation of CFTR channels (Prat, et al., 1999) and as well as other classes of channels (Shimoni, et al., 1999). Cytoskeletal actin modulates the function of a wide class of ion channels (Ahmed, et al., 2000; Copeland, et al., 2001; Hattan, et al., 2002; Prat, et al., 1999). This modulation occurs either by direct interaction with actin (Ahmed, et al., 2000) or an adaptor protein (Hattan, et al., 2002; Prat, et al., 1999). Although the idea of direct actin involvement in the regulation of Aqp1 ion channel function has yet to be tested, Aqp1 proteins have been described to be inserted in the plasma membrane of cholangiocytes in a microtubule-dependent manner (Marinelli, et al., 1999), thus suggesting that these channel proteins are coupled to cytoskeletal filaments. Could actin organization be the intermediate pathway that couples the effect of insulin with enhanced Aqp1 ion channel function? This idea is attractive for two reasons: 1) a putative actin binding site was identified in the amino terminus of Aqp1, and 2) insulin treatment positively regulated cGMP-ionic response in oocytes expressing Aqp1 C-tail truncation mutants lacking domains of the distal carboxyl terminus, in which the amino terminus remained intact. Testing the role of actin in the regulation of Aqp1 could provide a link between the effects of insulin and the behavior of cGMP-activated Aqp1 channels.

It is clear from the bilayer studies that cGMP is able to activate Aqp1 ion channels in the absence of intracellular components (insulin-sensitive signaling
pathways and actin). The question remains how might insulin-sensitive signaling pathways change or preserve the cGMP-sensitive ion channel function of Aqp1 channels lacking the distal C terminus? A possibility could be that cGMP may activate Aqp1 ion channels by modulating actin binding to the amino terminus of Aqp1 through a cGMP-PKG pathway. PKG is known to regulate two classes of molecules, Ena/VASP and Rho GTPases (Wang, et al., 2005) that in turn regulate actin. Ena/VASP proteins regulate actin polymerization and actin filament branching and turnover (Kwiatkowski, et al., 2003). Rho GTPases are a family of GTP binding proteins that regulate actin cytoskeleton that induce different types of morphological changes (Etienne-Manneville and Hall, 2002). This proposed mechanism is testable and could provide a new view of Aqp1 ion channels and volume regulation.

Fluid balance is a highly regulated event and dysfunction leads to serious pathophysiological states. Tissues directly involved in high fluid transport express high levels of different types of Aqp proteins. Diverse mechanisms regulate Aqp protein expression and differential facilitation of fluxes of water or ion molecules (Table 4.1). These mechanisms include direct phosphorylation by kinases, protein translocation, protein-protein interaction, and regulation by intracellular molecules (cGMP or cAMP) or physiological pH. A physiological consequence of diverse signaling mechanisms may be the coordinated function of Aqp channels resulting in vectorial transport of fluid. The predominant mechanism of vectorial transport is thought to be mediated by polarized
localization of transporters and ion channels. For example, Aqp2 is translocated and inserted in the apical membrane of the kidney epithelia in response to hormone-elevated cAMP. A number of epithelial or endothelial cells shown to be involved in fluid transport (Verkman, et al., 2000) do not show polarized expression pattern for Aqp1. How does a non-polarized expressing channel contribute to vectorial transport of fluid? Perhaps the diverse mechanisms regulating Aqp1 functions result in polarized function of Aqp1 despite non-polarized expression pattern. To date, signaling pathways of cAMP and Gαi3-PLA2 mediated pathways regulate Aqp1 water channel function (Abu-Hamdah, et al., 2004; Han and Patil, 2000; Patil, et al., 1997; Yool, et al., 1996). In addition, data from this dissertation indicate that cGMP-activated ion channel function of Aqp1 is modulated by downstream signaling pathways of insulin. Polarization of Aqp1 function may be conferred by polarized expression of the signaling machinery that regulate Aqp1 water and ion channel functions. Aqp1 associates with regulatory complexes through protein-protein interaction (Abu-Hamdah, et al., 2004; Cowan, et al., 2000). This idea of polarized function of Aqp1 channels is rational when considering the importance of signaling complexes of specific intracellular microdomains to channel behavior. In summary, one possible physiological consequence of diverse regulatory mechanism of AQP proteins is polarization and coordination of channel function despite non-polarized expression pattern. This level of coordination may aid in obtaining vectorial transport of fluid necessary to meet physiological demands.
A. Alignment of the amino terminus

<table>
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B. Alignment of the carboxyl terminus

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<tr>
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<tr>
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<td>frog</td>
<td>FILSPRTSDLTDRVKVWTSGQVEEYLDADDINSRVEMKPK</td>
</tr>
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Homology Domains
- CNG (Anthony et al., 2000)
- PDE (Boassa and Yool, 2002)
- EF-Hand Motif (Fotiadias et al., 2002)

Figure 4.1 Alignment of domains in the amino and carboxyl termini of Aqp1 channel across the vertebral taxa. See next page for details.
Figure 4.1 Alignment of domains in the amino and carboxyl termini of Aqp1 channel across the vertebrae taxa. A. The amino terminus has a basic isoelectric value (9.7) strongly influenced by the three consecutive lysines encoded in red. B. Domains of carboxyl terminus show putative PKG phosphorylation site (box 1), putative tyrosine phosphorylation site (box 2), and PDZ-ligand binding domain (box 3). Underline symbols indicate conserved residues (solid underline = CNG channels, dotted underline = PDE, double underline = Ca\textsuperscript{2+} binding proteins with EF-hand motif). Alignments were generated from clustalx 1.8.
Figure 4.2 The insulin-activated signaling pathway. Insulin peptide binding leads to activation by autophosphorylation of the insulin receptor. The activated insulin receptor phosphorylates insulin receptor substrate-1 (IRS) and Shc resulting in the activation of Grb2/Sos and Ras/Raf/MEK/MAPK pathway. Phosphorylated IRS proteins activate PI 3-Kinase and downstream target PKB, mTOR, p70 S6 kinase and atypical PKCs (Adapted from Taha et al., 1999).
Table 4.1 Diverse mechanisms that regulate AQP expression and facilitation of solute permeation. References: Aqp0 (Ehring et al., 1991; Nemeth-Cahalan and Hall, 2000), Aqp1 (Anthony et al., 2000; Yool et al., 1996; Patil et al., 1997; Abu-Hamdah et al., 2004; Marinelli et al., 1999), Aqp2 (Kuwahara et al., 1995; Klussmann et al., 1999), Aqp3 (Zelenina et al., 2003), Aqp4 (Han et al., 1998; Neely et al., 2001), Aqp5 (Sidhaye et al., 2005), Aqp6 (Yasui et al., 1999; Ikeda et al., 2002), Aqp8 (Huebert et al., 2002), Aqp9 (Branes et al., 2005).
REFERENCES:


