Mutations in the gene encoding the inwardly-rectifying renal potassium channel, ROMK, cause the antenatal variant of Bartter syndrome: evidence for genetic heterogeneity

International Collaborative Study Group for Bartter-like Syndromes, consisting of: Group 1: Lothar Karolyi1,2, Martin Konrad1, Arnold Köckerling1, Andreas Ziegler3, Dorthe K. Zimmermann1, Bernd Roth4, Christian Wieg5, Karl-Heinz Grzeschik2, Manuela C. Koch2, Hannsjörg W. Seyberth1, Group 2: Rosa Vargas6, Lionel Forestier6, Genevieve Jean6, Michele Deschaux6, Gian Franco Rizzoni7, Patrick Niaudet6, Corinne Antignac6, Group 3: Delphine Feldmann6, Frederique Lorridon8, Emmanuel Cougoureux8, France Laroze8, Jean-Luc Alessandri10, Louis David11, Pascal Saunier12, Georges Deschenes9, Group 4: Friedhelm Hildebrandt13, Martin Vollmer13, Willem Proesmans14, Matthias Brandis13, Group 5: Lambertus P. W. J. van den Heuvel15, Henny H. Lemmink15, Willy Nillesen16, Leo A. H. Monnens15, Nine V. A. M. Knoers16, Group 6: Lisa M. Guay-Woodford17, Christopher J. Wright18, Gilbert Madrigal19 and Steven C. Hebert20

Inherited renal tubular disorders associated with hypokalemic alkalosis (Bartter-like syndromes) can be subdivided into at least three clinical phenotypes: (i) the hypocalciuric-hypomagnesemic Gitelman variant; (ii) the classic variant; and (iii) the antenatal hypercalciuric variant (also termed hyperprostaglandin E syndrome). Mutations in the Na-CI cotransporter (NCCT) underlie the pathogenesis of the Gitelman variant and mutations in the Na-K-2Cl cotransporter (NKCC2) have recently been identified in the antenatal hypercalciuric variant. We now describe mutations in the gene encoding the inwardly-rectifying potassium channel, ROMK, in eight kindreds with the antenatal variant of Bartter syndrome. These findings indicate that antenatal Bartter syndrome is genetically heterogeneous and provide new insights into the molecular pathogenesis of Bartter-like syndromes.
INTRODUCTION

Bartter syndrome encompasses a set of primary renal tubular disorders associated with hypokalemic metabolic alkalosis; often increased urinary prostaglandin excretion; hyperreninemic hyperaldosteronism with normal blood pressure; and hyperplasia of the juxtaglomerular apparatus (1). Within this relatively rare set of disorders, familial cases occur commonly and inheritance best fits with autosomal recessive transmission (2,3). This set of disorders can be subdivided into at least three clinical phenotypes: (i) the hypocalciuric-hypomagnesemic variant described by Gitelman et al.; (ii) the classic syndrome originally described by Bartter et al.; and (iii) the antenatal hypercalciuric variant associated with severe systemic manifestations (3–6).

Among these disorders, Gitelman syndrome is characterized by a relatively mild course and late age of onset. Profound hypokalemia and hypomagnesemia lead to the major manifestations of fatigue, muscle weakness and recurrent episodes of tetany (7,8). Hypocalciuria and mildly impaired urinary concentrating ability are constant features of this sub-type (9). Recent studies demonstrate that this disorder results from presumptive loss of function mutations in the gene encoding the thiazide-sensitive sodium-chloride (Na-Cl) cotransporter (NCCT) of the distal nephron (10). Genetic homogeneity in Gitelman syndrome has been confirmed in subsequent studies (11,12).

In comparison, patients with classic Bartter syndrome (CBS) generally present during early childhood. These patients fulfill the core criteria of Bartter's original description without associated tetanic episodes or profound hypercalciuria (3,6,13). Typically, urinary calcium excretion is normal or slightly elevated and urinary concentrating capacity is close to normal (9,14). While much attention has been focused on defective chloride transport in the medullary thick ascending limb of the loop of Henle (mTAL) as the proximate abnormality in CBS (15–18), the underlying defect in the classic variant remains to be elucidated.

Finally, the antenatal variant of Bartter syndrome (ABS) is a life-threatening disorder in which both the renal tubular hypokalemic alkalosis as well as profound systemic symptoms are manifest (5,19,20). The abnormalities begin in utero with marked fetal polyuria that leads to polyhydramnios between 24 and 30 weeks gestation and typically, premature delivery (5,19–21). The amniotic fluid contains high chloride levels but normal concentrations of sodium, potassium, calcium and prostaglandin E2 (22). Affected neonates have severe salt wasting and hypophosphatemia, moderate hypokalemic metabolic alkalosis, hyperprostaglandinuria and failure to thrive. An essential manifestation is marked hypercalcemia and as a secondary consequence affected infants develop nephrocalcinosis and osteopenia (23–26). The fever, vomiting and occasional diarrhea associated with this disorder has been attributed to the stimulation of renal and systemic prostaglandin E2 activity in affected infants and these symptoms are effectively treated with inhibitors of prostaglandin synthesis. Based on these clinical features, the term hyperprostaglandin E syndrome was coined to describe this antenatal variant of Bartter syndrome (27).

<table>
<thead>
<tr>
<th>Families</th>
<th>Polyhydramnios</th>
<th>Gestational age (weeks)</th>
<th>Iso-hyposthenuria</th>
<th>Hypercalciuria</th>
<th>Nephrocalcinosis</th>
<th>Alkalosis</th>
<th>Hypokalemia and/or elevated FEK+</th>
<th>$fK$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB.Paris1</td>
<td>+</td>
<td>32</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AB.Paris2</td>
<td>+</td>
<td>35</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AB.Paris3</td>
<td>+</td>
<td>33</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AB.Paris5</td>
<td>+</td>
<td>35</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AB.Paris6</td>
<td>+</td>
<td>28</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AB.Paris8</td>
<td>+</td>
<td>32</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AB.Paris9</td>
<td>+</td>
<td>24</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AB.Marbl</td>
<td>+</td>
<td>33</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AB.Mar2b</td>
<td>+</td>
<td>31</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AB.Mar3b</td>
<td>+</td>
<td>32</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AB.Mar4b</td>
<td>+</td>
<td>32</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AB.Mar5b</td>
<td>+</td>
<td>30</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AB.CR1</td>
<td>+</td>
<td>35</td>
<td>nd</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AB.CR2</td>
<td>+</td>
<td>34</td>
<td>nd</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sporadic cases</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AB.MarS1</td>
<td>+</td>
<td>28</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AB.MarS2</td>
<td>+</td>
<td>34</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AB.NijS1</td>
<td>+</td>
<td>31</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AB.FreS1</td>
<td>+</td>
<td>34</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AB.ParS1</td>
<td>+</td>
<td>35</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Without indomethacin therapy; $b$ urine osmolality ≤300 mosmol/kg with plasma osmolality >290 mosmol/kg; $c$ renal calcium excretion >0.6 mg/mg creatinine; $s$ renal sonographic evidence of nephrocalcinosis; $d$ serum bicarbonate >26 mmol/l; $e$ serum potassium <3.3 mmol/l; $f$ fractional excretion of potassium >15%.

Sporadic ABS patients are indicated by the suffix, SI.

nd, not done.
Recent studies have demonstrated mutations in the gene encoding the mTAL luminal bumetanide-sensitive Na-K-2Cl cotransporter (NKCC2) in a cohort of five ABS kindreds (28). These genetic data indicate that the antenatal variant of Bartter syndrome results from a primary defect in mTAL chloride transport. This hypothesis is consistent with clinical studies demonstrating that ABS patients have an impaired diuretic response to furosemide (29). However, in applying haplotype analyses with polymorphic chromosome 15 markers tightly linked to NKCC2 in our cohort of 14 antenatal Bartter syndrome families, we excluded NKCC2 as the disease-susceptibility locus in six families.

Transepithelial chloride transport in the mTAL involves an interplay between the apical Na-K-2Cl cotransporter (NKCC2), the luminal, ATP-regulated potassium channel, ROMK (KCNJ1), the basolateral chloride channel (CLC), a basolateral K-Cl cotransporter, and the Na-K-ATPase (reviewed in 30,31). Therefore, defective chloride transport may result from loss of function mutations in any of the genes encoding these channels or cotransporters. We now report the identification of mutations in the ROMK gene (KCNJ1) in three kindreds and five sporadic cases with ABS. These findings indicate that the antenatal variant of Bartter syndrome is genetically heterogeneous.

RESULTS

Antenatal Bartter syndrome kindreds

We have identified a cohort of 14 families with antenatal Bartter syndrome and five sporadic cases for study (Table 1). In nine of these families, the affected children were the products of consanguineous unions. All index cases had a history of polyhydramnios and premature delivery. Postnatally, patients developed hypokalemic alkalosis and all had hypophosphatemia. Prior to the initiation of indomethacin therapy, all affected infants had failed to thrive with growth parameters less than the third percentile for age. Urinary excretion rates of prostaglandin E2 (PGE2) and/or its major metabolite, prostaglandin E-M (PGE-M) were markedly elevated (100% above upper limit of normal) in all patients in whom these measurements were performed. Hypercalciuria with associated sonographic evidence of nephrocalcinosis was demonstrated in all 14 kindreds. Isolated hypercalciuria was detected in one sporadic case who was treated in the immediate postnatal period with indomethacin. Renal biopsies obtained for three unrelated hypercalciuric infants (index cases from families AB.Paris2, AB.Mar5 and AB.Marbs2) revealed medullary calcifications with associated tubular atrophy, interstitial inflammation and fibrosis.

Genotype analysis

Initially, we sought to determine whether the NKCC2 gene was the disease-susceptibility locus in our ABS families. Markers tightly linked to the NKCC2 locus on chromosome 15 were typed in these kindreds. Lod scores for this set of markers were not conclusive (data not shown), which raised the possibility of genetic heterogeneity in our ABS cohort. Haplotypes constructed for these pedigrees demonstrate that six ABS families (AB.Paris2, AB.Paris5, AB.Paris6, AB.Mar3, AB.Mar4 and AB.Mar5) are not linked to the NKCC2 locus either because two affected siblings do not share the same haplotypes or an affected child from a consanguineous union is not homozygous for markers of the NKCC2 gene interval (Fig. 1). Of note, there were no clinical or laboratory features that distinguish the group linked to NKCC2 and the group unlinked to this locus (Table 1).

In those families where the NKCC2 gene is excluded as the disease-susceptibility locus, we evaluated whether ABS is linked to the gene encoding ROMK, the inwardly-rectifying, ATP-regulated, potassium channel. Fluorescence in situ hybridization has mapped the ROMK gene, KCNJ1, to chromosome 11q24-25 (32,33). Therefore, we typed these families for a set of five microsatellite markers that spanned a 14 cM candidate interval on

---

Figure 1. Pedigrees and haplotypes of antenatal Bartter syndrome families not linked to the chromosome 15 NKCC2 interval. For each child, the paternal chromosome is represented on the left and the maternal chromosome on the right. The thin lines indicate uninformative markers.
chromosome 11q24–q25. The haplotype data for these six families suggested linkage between ABS and the KCNJ1 gene (Fig. 2). In two of the consanguineous families, (AB.Paris2 and AB.Paris6), the affected children are homozygous for the 9 cM interval between D11S912 and D11S1320. Since a homozygous mutation has been found in family AB.Marb3 (see below), the region of homozygosity defined by this family allows us to position the KCNJ1 gene in the genetic interval flanked by D11S912 and D11S910. Given the haplotype data, we initiated a search for mutations in the KCNJ1 gene in these six families and the five sporadic cases.

**Mutation analysis of the human KCNJ1 gene in antenatal Bartter syndrome kindreds**

Previous studies by Shuck *et al.* have demonstrated that the gene encoding human ROMK produces five distinct transcripts by differential splicing of its five exons (34). All five transcripts share a common 3′ exon (exon 5) that encodes the majority of the channel protein. The largest product, ROMK-1, is initiated at a start codon in exon 4, contains 391 amino acid residues, and is the most efficiently translated isoform (34). Based on the sequence of ROMK-1, sets of primer pairs were used to amplify the coding sequence for exon 4 and exon 5 from genomic DNA of our test families (Table 2). The amplified products were analyzed by single-strand conformation polymorphism analysis (SSCA). Aberrant SSCA patterns were detected for exon 5 in three consanguineous families (AB.Paris2, AB.Marb3 and AB.Marb4) and the five sporadic cases. The affected children in the consanguineous families were homozygous for these aberrations. None of these aberrant SSCA patterns has been observed in analyses of at least 50 normal unrelated Caucasian controls (100 test alleles).

In total, 11 mutations were identified and four of these mutations were homozygous (Table 3; Fig. 3). Sequence analysis reveals that these mutations are distributed throughout the ROMK-1 protein (Fig. 4). Nine mutations are missense, causing substitutions of residues which are highly conserved in wild-type human, rat and *Xenopus laevis*. One mutation introduces a stop codon that deletes the terminal 63 residues of the channel protein and one 4 bp deletion causes a frameshift that results in a truncated, 351 amino acid protein. All of these mutations were inferred to alter the ROMK protein structure.

Mutations in the KCNJ1 gene have not been detected in families AB.Paris5, AB.Paris6 or AB.Marb5. It is possible that these families have KCNJ1 gene mutations involving the exon–intron splice junctions of exon 4 or exon 5 or in an as yet unexamined exon. In addition, we cannot exclude the possibility that another gene may be the disease-susceptibility locus in these families.

**DISCUSSION**

Antenatal Bartter syndrome is a phenotypically distinct variant of the Bartter-like syndromes characterized by polyhydramnios with preterm delivery, severe salt wasting, hyperthennuria, and hypercalciuria (Table 1). Our identification of 11 independent mutations in the gene KCNJ1 that segregate specifically with ABS and result in either non-conservative amino acid substitutions in important functional domains or premature truncation of the channel protein indicate that defects in KCNJ1 cause the antenatal variant of Bartter syndrome. These findings demonstrate that ABS is genetically heterogeneous, involving mutations in KCNJ1 as well as NKCC2 (28).
Table 2. PCR primers for SCA analysis of the human KCNJ1 gene exons 4 and 5 (5'→3')

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>hROMKex4</td>
<td>TTTTTCAGATGCTCACTTTA</td>
<td>TTGATGTGTTTTCAGATCA</td>
</tr>
<tr>
<td>hROMKex5.5</td>
<td>GATAGAAAGACCAACAA</td>
<td>GGAAGGTCTTGTGAAATGTA</td>
</tr>
<tr>
<td>hROMKex5.4</td>
<td>AAGTGGAGATACAAAATGACC</td>
<td>CAGAAAAATGGCAGTGG</td>
</tr>
<tr>
<td>hROMKex5.3</td>
<td>TATGGATACGTTGTCGTTG</td>
<td>AAAATAATGCTTTCCCT</td>
</tr>
<tr>
<td>hROMKex5.2</td>
<td>CTATTGGCCAGTACATTTA</td>
<td>TATTTCCCTTCTTCTCCT</td>
</tr>
<tr>
<td>hROMKex5.1</td>
<td>CTACGGTTTTGCCTCCTTTA</td>
<td>ACTTGCTTTACTCTGGTAA</td>
</tr>
</tbody>
</table>

Based on the sequence previously reported by Shuck et al. (34), a single primer pair was designed to amplify exon 4 and a set of nested primer pairs were designed to amplify the coding region of exon 5.

Table 3. Mutations in the human ROMK gene (KCNJ1) in antenatal Bartter syndrome

<table>
<thead>
<tr>
<th>Ethnic origin</th>
<th>Mutation</th>
<th>Nucleotide</th>
<th>Consequence</th>
<th>Mutation #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Families</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AB.Paris2</td>
<td>North Africa</td>
<td>A198T</td>
<td>G→A at 1153</td>
<td>Ala→Thr at 198</td>
</tr>
<tr>
<td>AB.Marb3</td>
<td>India</td>
<td>G167E</td>
<td>G→A at 1062</td>
<td>Gly→Glu at 167</td>
</tr>
<tr>
<td>AB.Marb4</td>
<td>Turkey</td>
<td>D108H</td>
<td>G→C at 883</td>
<td>Asp→His at 108</td>
</tr>
<tr>
<td>Sporadic cases</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AB.MarbS1</td>
<td>Germany/Croatia</td>
<td>P110L</td>
<td>C→T at 890</td>
<td>Pro→Leu at 110</td>
</tr>
<tr>
<td>AB.MarbS2</td>
<td>Germany</td>
<td>V315G</td>
<td>T→G at 1505</td>
<td>Val→Glu at 315</td>
</tr>
<tr>
<td>AB.Nijs1</td>
<td>Netherlands</td>
<td>W99C</td>
<td>G→T at 858</td>
<td>Trp→Cys at 99</td>
</tr>
<tr>
<td>AB.FreiS1</td>
<td>Belgium</td>
<td>A198T</td>
<td>G→A at 1153</td>
<td>Ala→Thr at 198</td>
</tr>
<tr>
<td>AB.ParisS1</td>
<td>France</td>
<td>V122E</td>
<td>T→A at 926</td>
<td>Val→Glu at 122</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D74Y</td>
<td>G→T at 781</td>
<td>Asp→Tyr at 74</td>
</tr>
</tbody>
</table>

Sense strand sequences are shown. Amino acids are numbered from the first Met in exon 4. The mutation # refers to the mutation position in Figure 4. Nucleotides are numbered as in Shuck et al. (34).

The ROMK gene is a disease-susceptibility gene in antenatal Bartter syndrome

KCNJ1 encodes the luminal, ATP-sensitive, potassium channel in the distal nephron (35–37). This channel belongs to a family of inwardly rectifying potassium channels (Kir family; ROMK is Kir1.1) and is characterized by little-to-no voltage dependence, inward rectification, exquisite pH-sensitivity and modulation by ATP (31,38–40). The ROMK channel is involved in potassium secretion in the TAL and the distal nephron. In the mTAL, this secretory function recycles potassium across the apical membrane, thus ensuring that adequate luminal potassium is available for the efficient functioning of the Na-K-2Cl cotransporter (41). Inhibition of luminal potassium channels reduces NaCl reabsorption across the apical membrane, thereby maintaining the gradient needed for sodium reabsorption. In human kidney, differential splicing produces five distinct transcripts of ROMK. While the relative distribution of these isoforms in distal nephron segments has not been established, exon 5 is common to all of these isoforms and encodes the majority of the channel protein (33,34).

The detection of 11 independent mutations involving this common exon predicts that ROMK activity is disrupted in our cohort of ABS patients.

We have identified mutations involving the amphipathic M0 segment; the M1 and M2 transmembrane segments; the M1–H5 linker segment that comprises the channel pore; the putative ATP-binding domain; and the C-terminus of the ROMK channel protein (Fig. 4). Two non-conservative missense mutations within the M0 region of the N-terminus have been identified, V72G (mutation #5 in Table 3, Fig. 4) and D74Y (mutation #11). This region of ROMK has been implicated in internal pH regulation of ROMK (43). Since this channel is exquisitely sensitive to reductions in cytosolic pH, these mutations in M0 could significantly alter ROMK channel function. The homozygous missense mutation, W99C (mutation #8), that involves the substitution of a polar cystine for Trp-99 in the Ml domain is likely to alter the configuration of the channel pore and thus, would be predicted to disrupt channel function. The missense mutations in AB.Marb4 (mutation #3), AB.MarbS1 (mutation #4) and AB.ParisS1 (mutation #10) all cause non-conservative amino acid changes in the M1–H5 linker region. Experimental studies have demonstrated that this segment is important in external pH regulation of the inwardly-rectifying potassium channels, HIR (44) and ROMK. Mutation of the glycosylation site, Asn-117, in this region of ROMK has been shown to
dramatically decrease channel function (45). In addition, this region is conserved in different classes of potassium channels and in voltage-gated channels, single amino acid substitutions within this segment result in loss of channel function or alterations in single channel conductance and ion selectivity (37). Within the M2 transmembrane segment, prior \textit{in situ} mutagenesis studies have indicated that residue Gly-167 is oriented so that it faces the channel pore (46). Therefore, the homologous mutation, G167E (mutation \#2), that substitutes a glutamic acid with its large polar R group for City-IC would be predicted to alter the pore conformation and thus, be deleterious for channel function. The homologous missense mutation, A198T (mutation \#1), causes a non-conservative substitution within a 63 amino acid segment that lies just 3' to the M2 domain and is involved in ROMK channel regulation. This regulatory region contains important phosphorylation sites [two protein kinase C (PKC) and one protein kinase A (PKA) sites] and a Mg$^{2+}$-ATP-binding motif (Walker A site) that have been shown to be important in the non-hydrolytic Mg$^{2+}$-ATP regulation of ROMK (37). Both PKA (35,47) and PKC (37,48) are important regulators of ROMK function and structural alterations due to non-conservative amino acid substitutions within this region may disrupt channel regulation. Of note, the same A198T mutation was found in a non-related compound heterozygote (AB.FreiS1). That this

![Diagram](image-url)

**Figure 4.** Structural model of the ROMK channel protein and the positions of mutations identified in antenatal Hunter syndrome patients. The H5 region contains the putative channel pore and is located between the M1 and M2 membrane-spanning domains. The M0 segment is an amphipathic region. The protein kinase A (PKA) phosphorylation sites (open circles with 'P') and kinase C (PKC) phosphorylation sites (closed circles with 'P') are indicated. The singe possible N-linked glycosylation (N-glycosyl) site is shown. Each symbol represents a single amino acid. Mutations are indicated by numbered arrows and each number corresponds to a mutation listed in Table 3.

**Figure 3.** Mutational analysis with the \textit{AKT1} gene in antenatal Hunter syndrome patients. Aberrant band patterns were identified by SSCA and evaluated by DNA sequence analysis. Patients are identified as in Table 1. A representative sample of eight mutations in six patients are shown. Each composite panel shows the sequence data from a control individual and the affected individual on the left and a representative autoradiograph of the SSCA pattern on the right. The sequence of the sense strand is shown unless otherwise indicated. (A) In index case AB.Harb3, the first base of codon 198 is mutated from G to A causing the A198T substitution. (B) In index case AB.Mars2, the second base of codon 167 is mutated from G to A resulting in the G167E change. (C) In index case AB.MarW, a G to C change in the first base of codon 167 cause the substitution, DHSS1 antisense strand data shown. (D) In the compound heterozygote, AB.MarS2, the first mutant allele has a T to G mutation in exon 14 resulting in the V115G substitution. In the second mutant allele, a 4 bp deletion produces a frameshift in codon 334, which causes the premature termination of the protein. Sense strand data shown. (E) In the compound heterozygote, AB.FreiS1, the first mutant allele has the same G to A change in codon 198 as AB.Pars2, in the 198T substitution (antisense strand data shown). In the second mutant allele, a C to T change in the first base of codon 334 introduces a stop codon Tryptophan in AB.NahS1, a homologous G to T mutation in the last base of codon 99 causes the W99C change.
specific point mutation occurs in two unrelated families and is not evident in 100 test chromosomes provides further supporting evidence that this mutation is pathogenic and not simply a polymorphism. Finally, we have identified three mutant alleles that alter the C-terminus of the ROMK protein. Since the C-terminus has been shown to be important in determining pore properties (49), mutations in this region could affect channel function. In one mutant allele, a 4 bp deletion (mutation #7) causes a frameshift mutation at codon 334 and results in the introduction of a termination codon 36 amino acids downstream. In another mutant allele, a stop codon is substituted at R338 (mutation #9), thereby deleting the terminal 53 residues of the carboxy tail. These two mutations could alter phosphorylation at the tyrosine kinase site at Y337, which is conserved in rat and human ROMK. In the third mutant allele, a glycine for Val-315 substitution (mutation #6) lies adjacent to the cyclic AMP-dependent PKA phosphorylation site in the carboxy tail which has been shown to be critical for channel function (47). In all affected individuals, these mutations would be predicted to cause loss of function in the channel protein.

Proposed pathogenesis of antenatal variant of Bartter syndrome

In the mTAL, the functional coupling of ROMK and the luminal Na-K-2Cl cotransporter (NKCC2) is crucial for NaCl reabsorption. Therefore, loss of function in ROMK as well as NKCC2 (28) would be predicted to disrupt electrogenic chloride reabsorption in the mTAL.

A primary defect in mTAL chloride transport is consistent with the complex constellation of features evident in ABS patients. Several lines of evidence support this proposed pathogenic model. Chronic inhibition of salt reabsorption in the mTAL by long-term furosemide treatment mimics the clinical and biochemical findings in ABS, i.e. hypokalemic hypochloremic alkalosis, hypo- or iso-osmohemia, and impaired renal conservation of both calcium and magnesium (5,50,51). Furthermore, ABS patients have impaired diuretic, saluretic and hormonal responses to furosemide (29). In contrast, furosemide sensitivity is maintained in Gitelman syndrome (52), which is caused by mutations in the thiazide-sensitive Na-Cl cotransporter of the distal convoluted tubule (10).

In patients with either KCNJ1 or NKCC2 mutations, defective mTAL chloride transport results in increased NaCl delivery to the distal nephron with consequent salt wasting, loss of concentrating capacity and volume contraction. In addition, impaired electrogenic chloride transport in mTAL also inhibits the voltage-driven, paracellular reabsorption of calcium and magnesium (53), thus leading to hypercalciuria and in some patients, hypermagnesuria.

In ABS, stimulation of renal and systemic PGE2 formation is likely to be a secondary phenomenon, at least in those patients with KCNJ1 or NKCC2 mutations. The elevated PGE2 levels play a central role in disease pathogenesis (16,27). Experimental data demonstrate that elevated PGE2 activity stimulates the renin-angiotensin-aldosterone axis (54), specifically inhibits ROMK channel activity (48,55) and impedes NaCl transport in the mTAL (56,57), as well as vasopressin-induced water reabsorption in the collecting duct (58). Therefore, stimulation of PGE2 activity exacerbates the primary defect in mTAL chloride transport, contributes to the aldosterone-mediated hypokalemic metabolic alkalosis and promotes postnephronuria. As would be predicted, suppression of PGE2 formation with cyclo-oxygenase inhibitors, such as indomethacin, attenuates the salt wasting and hypokalemic alkalosis and converts postnephronuria to isohypoamia (29).

In summary, our identification of molecular defects in the KCNJ1 gene, coupled with the previously reported mutations in NKCC2 (28), indicate that antenatal Bartter syndrome is genetically heterogeneous. Together, these data support the hypothesis that ABS involves a primary mTAL transport defect (29). While we cannot exclude the possibility that there may be additional gene defects that cause ABS, these data provide new insights into the complex, interrelated mechanisms involved in the pathogenesis of antenatal Bartter syndrome. In addition, our findings expand the genetic basis for distinguishing Bartter-like syndromes from one another. While specific disorders within the spectrum of Bartter-like syndromes can usually be differentiated by applying rigorous clinical criteria, confounding presentations are not unusual. Further identification of the basic genetic defects in Bartter-like syndromes will provide potential molecular tools for distinguishing specific disorders within this often confusing spectrum, and for dissecting the complicated pathophysiology involved in each disorder.

MATERIALS AND METHODS

Bartter syndrome families

Index cases from families, AB.Paris1, AB.Marb2, AB.Marb3 and AB.Marb4, have been previously reported (5,19,24,29). The index cases of the other 10 families and the five sporadic cases were identified either in Neonatal Intensive Care Units or Pediatric Nephrology Clinics at the collaborating institutions. These studies were approved by local Ethics Committees and Informed Consent was obtained from the parents.

Genotype analysis and haplotype construction

Using standard methods, genomic DNA was extracted from whole blood lymphocytes of all family members studied. Markers tightly linked to the NKCC2 locus on chromosome 15 were typed in all of our families. The markers, D15S132 and D15S209 have been previously reported (28). The markers, D15S132—D15S143—D15S123 are all located on the YAC 956E3 (CEPH library) (59). PCR analysis using a primer set derived from the human NKCC2 cDNA sequence amplified a 1200 bp fragment from genomic DNA and from YAC 956E3, confirming that this YAC contains the NKCC2 gene. Additional tightly linked markers (D15S982, D15S126 and D15S121) were selected from the Genethon database (accessible at http://www.genethon.fr/genethon_en.html). Highly polymorphic markers from the 14 cM interval on chromosome 11q24—25 that contains the KCNJ1 gene were selected from the Genethon database (60). The following marker order for the 14 cM interval between D11S934 and D11S1320 has been established by radiation hybrid mapping (Genome Data Base; accessible at http://gdwebwww.gdb.org): centromere—D11S934—D11S912—D11S874—D11S910—D11S1320—telomere.

Microsatellite polymorphisms were amplified by the polymerase chain reaction using forward primers labeled at the 5’-end either with fluorescent dye or 32P. PCR reactions were performed in a 15 μl volume containing 50 ng of genomic DNA, 1.5 mM MgCl2, 5 mM Tris (pH 8.3), 50 mM KCl, 10 pmol of each primer and 0.5 U of Taq polymerase. After an initial denaturation step at 94°C for 5 min, PCR was conducted for 30 cycles with
denaturation at 94°C for 45 s, annealing at 55°C for 45 s and extension at 72°C for 45 s. The reaction was completed with a final elongation step at 72°C for 10 min. Amplified products were separated on 6% polyacrylamide gels run under denaturing conditions. The gels were analyzed either using the Genscan 672 software (Ver 1.2) or autoradiography. Linkage analysis was performed using the LINKAGE programs (61). Haplotypes were constructed from the genotype data. The most likely haplotypes were inferred by minimizing the number of crossover events in each sibship.

SSCA and DNA sequencing

Aberrant band patterns for the KCNJ1 gene were sought using single-strand conformation polymorphism analysis (SSCA) (62). Based on the sequence for the human KCNJ1 gene previously described (34), an overlapping set of primer pairs were designed (Table 2) and used to amplify the coding sequence for exons 4 and 5 from genomic DNA of our test families as well as 50 unrelated controls. PCR was performed as described above except that the products were labeled by inclusion of 1 μCi of [α-^32P]dCTP or [α-^33P]dCTP in the reaction mixture. Amplified products were separated either at room temperature or at 4°C by electrophoresis at 40 W constant power for 6-8 h on 0.5x MDE gels (AT Biochem) prepared in 0.6x TBE 154 mM Tris borate (pH 7.8). Following autoradiography, conformational variants were excised from the gel and the DNA was isolated by centrifugation through a MicroPure 0.22 filter (Amicon, Inc.). The isolated product was then re-amplified with the same primer pair and subjected to direct DNA sequence analysis using an ABI 373A automated DNA sequencer (Applied Biosystems). Alternatively, non-radioactive PCR was performed and gels were silver-stained as previously described (63). Direct sequencing was performed after reamplification of the remaining PCR product using a direct blotting electrophoresis sequencing system (GATC 1500, GATC, Konstanz, Germany). In all cases, DNA sequences were confirmed by sequencing both strands.

ACKNOWLEDGEMENTS

The authors are indebted to the participating families for their cooperation. LK is a recipient of a fellowship grant from the Deutsche Forschungsgemeinschaft (Ka 1252/1-1). Group I was supported in part by grants from the Deutsche Forschungsgemeinschaft (HWS: 5e 263/11-1) and from the Kempe Stiftung Marburg. Group 4 was supported by the Zentrum Klinische Forschung, A1 Freiburg. Financial support for Group 5 was provided by the Dutch Kidney Foundation (Grant C96-1540). Funding was also provided by grants from the University of Alabama Health Services Foundation and the Alabama Kidney Foundation (LG-W). The Collaborative Study Group is supported by a Concerted Action ‘Identification of the gene defects in Bartter syndrome and Gitelman syndrome’ funded by the European Economic Community (EEC) within BIOMED II program PL950260. This study was performed in cooperation with the International Study of Genetic Renal Disease (ISGRD).

REFERENCES


