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Molecular and topological characterization of the rat parotid Na^+-K^+-$2Cl^-$ cotransporter[1]

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Abstract

Na^+-K^+-$2Cl^-$ cotransporters play a central role in driving salt and water movements across secretory and absorptive epithelia. We report the cloning of the rat parotid secretory Na^+-K^+-$2Cl^-$ cotransporter, rtNKCC1. The predicted amino acid sequence of this protein is highly homologous to a previously cloned NKCC1 from the shark rectal gland and to mammalian NKCC1s cloned from several cultured cell lines, confirming the presence of the NKCC1 isoform in a naturally occurring mammalian secretory epithelium. In contrast to previously published NKCC1 clones, our sequence also includes an apparently complete 2680 bp 3′-UTR. Hydropathy analyses of rtNKCC1 predicts that this protein consists of large hydrophilic N and C termini (approx. 30 kDa and 50 kDa, respectively) flanking a central hydrophobic transmembrane region consisting of ten to 12 membrane spanning domains. In addition, we report the results of confocal immunofluorescent microscopic studies using rat parotid acini and antibodies directed against specific regions of the predicted N- and C-terminal portions of rtNKCC1. These studies demonstrate that the epitopes recognized by these antibodies are exposed in permeabilized but not in unpermeabilized cells, indicating that the predicted N and C termini of rtNKCC1 are intracellular.

Keywords: Secretory isoform of Na^+-K^+-$2Cl^-$ cotransporter; Exocrine fluid secretion; Antipeptide antibody

1. Introduction

Salt and water movements across a number of secretory and absorptive epithelia are driven by net transepithelial Cl^- transport [1,2]. In many of these tissues the first step in this process, cellular Cl^- entry, is mediated by a Na^+-K^+-$2Cl^-$ cotransporter. This transport protein couples the electroneutral movement of one sodium, one potassium and two chloride ions into the cell and thereby causes the accumulation of intracellular Cl^- against its electrochemical gradient. In the renal thick ascending limb of Henle's loop, an absorptive epithelium, the Na^+-K^+-$2Cl^-$ cotransporter is localized to the apical membrane where it is the site of action of the commonly prescribed loop diuretics furosemide and bumetanide. In secretory epithelia, on the other hand, the cotransporter is localized to the basolateral cell membrane. In both cases a Cl^- channel found on the opposite cell surface provides the pathway for cellular Cl^- exit. Recent molecular cloning studies have demonstrated that there are, in fact, two isoforms of

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the Na^+-K^+-$2Cl^-$ cotransporter referred to as NKCC1 and NKCC2, or alternatively as BSC2 and BSC1, respectively [2,3]. These two isoforms are approx. 65% identical at the amino acid level. The experimental evidence gathered to date indicates that NKCC2 is only present in the kidney and represents the absorptive Na^+-K^+-$2Cl^-$ cotransporter in the thick ascending limb of Henle's loop, while NKCC1 is more widely distributed and corresponds to the basolateral Na^+-K^+-$2Cl^-$ cotransporter of secretory tissues [2,3].

Because of their experimental accessibility and rich hormonal responsiveness salivary glands have proven to be a particularly useful model system for studying the function of secretory epithelia. Studies from several laboratories, including our own, have provided convincing evidence for the involvement of Na^+-K^+-$2Cl^-$ cotransport in the secretion of salivary fluid [4]. Indeed, these tissues are one of the richest sources of this transporter identified to date. In addition, in studies of intact rat parotid acini we have shown that the activity of the Na^+-K^+-$2Cl^-$ cotransporter is up-regulated in response to a variety of physiological and potentially physiologically relevant stimuli and that many of these up-regulatory events are associated with direct phosphorylation of the cotransporter itself [5–8].

In the present paper we report the cloning of the rat parotid Na^+-K^+-$2Cl^-$ cotransporter. This protein is highly homologous to a previously cloned NKCC1 from the shark rectal gland ([9]; 79% amino acid identity) and to mammalian NKCC1s cloned from mouse [10], human [11] and bovine [12] cultured cell lines (approx. 95% identity), thus confirming the presence of the NKCC1 isoform in a naturally occurring mammalian secretory epithelium. Hydropathy analyses of the rat NKCC1, as well as other NKCC1s, predict that this protein consists of large hydrophilic N and C termini (approx. 30 kDa and 50 kDa, respectively) flanking a central hydrophobic transmembrane region consisting of ten to 12 membrane spanning domains. Using antibodies directed against specific regions of the predicted N- and C-terminal portions of rtNKCC1 we have carried out confocal immunofluorescent microscopy on permeabilized and unpermeabilized rat parotid acini. These studies, also reported here, demonstrate that the epitopes of these antibodies are exposed in permeabilized but not in unpermeabilized cells, indicating that the predicted N and C termini of rtNKCC1 are intracellular.

2. Materials and methods

2.1. General

Total RNA was isolated from rat parotid glands, which had been snap frozen in liquid nitrogen, using TRIzol Reagent (Gibco BRL, Gaithersburg, MD). Messenger RNA was isolated using Oligo(dT) Cellulose Columns from Gibco BRL according to the manufacturer's instructions. All RNA was stored at −80°C until use. RT-PCR was carried out with the GeneAmp RNA PCR kit from Perkin Elmer Cetus (Norwalk, CT). DNA sequencing was done on an automated sequencer (ABI Prism Model 377) and/or by manual sequencing using the T7 Sequenase kit 2.0 from Amersham (Arlington Heights, IL). The ZAP Express cDNA library used in these studies was custom synthesized for us by Stratagene (La Jolla, CA) using rat parotid mRNA generously provided by Dr. Keerang Park (University of Rochester).

Sequence analyses were carried out with programs from the Wisconsin Sequence Analysis Package, Version 8 (Genetics Computer Group, Madison, WI).

2.2. Cloning of rtNKCC1

In preliminary experiments we established that the RT-PCR product generated using rat parotid mRNA and the degenerate oligonucleotide primers 5′-TG(C/T)ATG(T/C)TAAA(C/T)ATCTGGGG-3′ (sense) and 5′-TGAGCCTT(A/G)G(A/C)(C/T)TCCCATTCCAT-3′ (antisense), corresponding to bp 799–818 and 1198–1220 of the coding region of the shark NKCC1 [9], respectively, was highly homologous to NKCC1s previously cloned from the shark [9], mouse [10] and human [11]. This (^{32}P-labeled) RT-PCR product was used to probe a rat parotid ZAP Express cDNA library (see above) under stringent conditions. A number of positive clones were isolated from the library by two rounds of plaque purification and excised with Exassist helper phage (Stratagene) into the plasmid pBK-CMV (Stratagene). Sequencing

of the 5′-ends of these clones showed that most contained a cDNA with high homology to previously cloned NKCC1s but that none appeared to contain the 5′-end of the coding region. Further analyses of these incomplete clones by sequencing and restriction digestion indicated that they fell into two groups with 3′-UTRs of 294 bp and 2685 bp, respectively (see Section 3). Two clones, one from each of these groups (nkcc1/S and nkcc1/L, respectively), were sequenced from end to end and found to code for the same protein.

Since additional extensive screening of the ZAP Express library failed to yield a clone containing sequence homologous to the expected 5′-end of the coding region of the rat NKCC1 we employed the upstream walking strategy of the Clontech Rat GenomeWalker Kit (Clontech Laboratories, Palo Alto, CA) to complete our sequence. Briefly stated, the GenomeWalker kit contains five 'libraries' each consisting of rat genomic DNA digested with a single restriction enzyme. These DNA fragments are ligated to an adaptor containing the binding sites for two nested PCR primers AP1 and AP2 supplied with the kit. In our experiments we carried out nested PCR using the primers 5′-AGTACTGCTGGTGGTGTCCACTG-3′ (outer) and 5′-TTCTCGGAAACCGGGTCCACCTGGAA-3′ or 5′-CCCTTGGCCACGCCGCTCTCAG-3′ (inner), derived from nkcc1/S and nkcc1/L, along with the outer and inner sense primers AP1 and AP2. PCR conditions were as recommended by the manufacturer except that Advantage GC Tth Polymerase Mix (Clontech) was used in place of the supplied polymerase buffer. PCR products approx. 500 or 600 bp in length (depending on the inner primer used) were found in the reaction from the *Dra*I GenomeWalker library. These products were subcloned using the TA Cloning Kit (Invitrogen) and multiple clones from two independent nested PCR reactions were sequenced. The resulting consensus sequence overlapped with the 5′-end of clone nkcc1/S and when combined with it yielded an open reading frame for a membrane protein 1203 amino acids in length (rtNKCC1; see Section 3). One of these subcloned PCR products (clone 5C2) was ligated with nkcc1/S using a convenient *Bgl*I restriction site to yield the complete coding sequence of rtNKCC1 in Bluescript SK. Comparison of clones nkcc1/S and nkcc1/L with the complete sequence of rtNKCC1 indicated they began at bp 204 and bp 583, respectively, of the coding region.

2.3. Northern blotting

Northern blotting was carried out using 7.5 μg mRNA glyoxalated as described by Sambrook et al. [13] and separated on 1% agarose gels. The gels were semi-dry blotted onto Nytran Plus nylon membranes (Schleicher and Schuell, Keene, NH) and crosslinked using a UV Stratalinker 1800 (Stratagene). The blots were probed with the same probe used for screening the cDNA library.

2.4. Antibodies

Antibody LL232 [14] was generously provided by Dr. X. He. This antibody was raised in rabbits against a peptide corresponding to amino acids 965–986 of rtNKCC1 (Fig. 1). Antibody α-NT was raised in rabbits against a peptide corresponding to amino acids 238–261 of rtNKCC1; peptide synthesis and conjugation to KLH as well as antibody production were carried out by Biosynthesis (Lewisville, TX). Both antipeptide antibodies were affinity purified against their respective antigens. Antibody α-wCT was raised in rabbits against a 6×His fusion protein corresponding to amino acids 750–1203 of rtNKCC1. This fusion protein was produced as follows. A *Bam*HI/*Xho*I fragment containing the above coding region of rtNKCC1 and the complete 3′-UTR of clone nkcc1/L was isolated by restriction digestion and cloned into the expression vector pQE9 (Qiagen, Santa Clarita, CA) 'in frame' with a 6×His N-terminal tag. Subsequent procedures were essentially as recommended by Qiagen for the pQE9 vector. Briefly, DH5α cells (Gibco BRL, Gaithersburg, MD) were transformed with the recombinant plasmid and screened for 6×His protein expression. A suitable clone was chosen for production of recombinant protein. Cells were grown for 3 h in the presence of 2 mM IPTG then lysed in 6 M guanidine HCl, 0.01 M NaH_2PO_4, 0.01 M Tris, pH 8.0. The lysate was passed over a Ni-NTA affinity column and the column was subsequently washed with several column volumes of lysis buffer. The 6×His tagged recombinant protein was then eluted with

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   1  MEPGPAAPSS GAPRPARDGD APLTAAGVDL PGTAVLSGRE DATAGSQAGG

  51  GVRGEGTPAA GDGLGKPLGP TPSQSRFQVD PVSENAGRAA AAAAAAAAAA

 101  AAGAAGKETP AAGKAGTESG VAKGSEEAKG RFRVNFVDPA ASSSADDSLS

 151  DAAGVGGDGP NVSSQNGGDT VLSEGSSLHS GGGSGHHQQY YYDTHTNTYY

 201  LRTFGHNTMD AVPRIDHYRH TAAQLGEKLL RPSLGEFHDE LEKEPFEDGF

 251  ANGEESTPTR DAVVTYTAES KGVVKFGWIK GVLVRCMLNI WGVMLFIRLS
                                                          m1
 301  WIVGQAGIGL SVLVIAMATV VTTITGLSTS AIATNGFVRG GGAYYLISRS
                          m2
 351  LGPEFGGAIG LIFAFPNAVA VAMYVVGFAE TVVELLKEHS ILMIDEINDI
                               m3
 401  RIIGAITVVV LLGISVAGME WEAKAQIVLL VILLLAIADF VIGTFISLDS
                    m4                                m5
 451  KKPKGFFGYK SEIFSENFGP DFREEETFFS VFAIFFPAAT GILAGANISG
                                                       m6
 501  DLADPQSAIP KGTLLAILIT TVVYIGIAVS VGSCVVRDAT GNVNDTITTE
                                     m7
 551  LTNCTSAACK LNFDFSYCES NTCSYGLMNN FQVMSMVSGF APLISAGIFS

 601  ATLSSALASL VSAPKIFQAL CKDNIYPAFQ MFAKGYGKNN EPLRGYILTF
        m8
 651  LIALGFILIA ELNVIAPIIS NFFLASYALI NFSVFHASLA KSPGWRPAFK
                    m9-m10
 701  YYNMWISLTG AILCCIVMFV INWWAALLTY VIVLGLYIYV TYKKPDVNWG
                           m11-m12
 751  SSTQALTYLS ALQHSIRLSG VEDHVKNFRP QCLVMTGSPN SRPALLHLVH

 801  DFTKNVGLMI CGHVHMGPRR QAMKEMSIDQ AKYQRWLIKN KMKAFYAPVH

 851  ADDLREGAQY LMQAAGLGRM KPNTLVLGFK KDWLQADMRD VDMYINLFHD

 901  AFDIQYGVVV IRLKEGLDIS HLQGQEELLS SQEKSPGTKD VVVNVDYSKK

 951  SDQDAFKASG EKPITQKDEE EDGKTSTQPL LKKESKGPVA PLNVADQKLL

1001  EASTQFQKKQ GKNTIDVWWL FDDGGLTLLI PYLLTTKKKW KDCKIRVFIG

1051  GKINRIDHDR RAMATLLSKF RIDFSDIMVL GDINTKPKKE NIVAFDDMIE

1101  PYRLHEDDKE QDIADKMKED EPWRITDNEL ELYKTKTYRQ IRLNELLKEH

1151  SSTANIIVMS LPVARKGAVS SALYMAWLEA LSKDLPPILL VRGNHQSVLT

1201  FYS
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Fig. 1. Amino acid sequence of rtNKCC1. Predicted transmembrane regions are underlined. Potential *N*-glycosylation sites (Asn-161, Asn-497, Asn-544, Asn-553, Asn-681), protein kinase C consensus sites (Ser-37, Ser-450, Thr-741, Ser-765, Ser-948, Thr-965, Thr-1035, Thr-1036, Thr-1137) and the only protein kinase A consensus site on the molecule (Ser-982) are indicated in bold. Also shown in bold are two conserved threonines (Thr-208 and Thr-1126) that correspond to known phosphoacceptors in the shark NKCC1.

8.0 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris pH 4.5 (with HCl). Rabbit polyclonal antiserum was raised against this eluted protein by Biodesign International (Kennebunk, ME).

2.5. Tissue preparation, immunocytochemistry and confocal microscopy

Rat parotid acini were prepared from the glands of male Wistar strain rats by collagenase digestion as previously described [8]. The final acinar suspension from two rats was filtered through a 450 μm nylon screen to remove large clumps then suspended in 10 ml PSS (135 mM NaCl, 5.8 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 0.73 mM NaH_2PO_4, 20 mM HEPES, 11 mM glucose, 2 mM glutamine pH 7.4) containing 0.01% BSA and kept gassed with 100% O_2 at 30°C until use. The following steps were carried out at room temperature. Glass coverslips were preincubated in 0.5% poly L-lysine for 2 h then washed with several rinses of water and allowed to dry. The coverslips were placed in a 10 cm culture dish, covered with the acinar suspension in BSA-free PSS and centrifuged for 5 min at $500 \times g$. The coverslips and adherent acini were then fixed by incubation in 3% paraformaldehyde for 30 min, washed and incubated in PBS containing 100 mM glycine for 20 min and washed several times with PBS. Acini were permeabilized by incubation in methanol at −80°C for 6 min then washed 5 times with 1×PBS. In some cases (see figure legends) an additional incubation in 0.01% SDS for 20 min was also carried out. Unpermeabilized control acini were treated identically except for the incubation in methanol.

Immunocytochemistry was carried out as follows. All solutions were in PBS containing 0.2% BSA unless otherwise stated. Acini were blocked for 1 h in 5% donkey serum then incubated for 1 h with the anti-Na^+-K^+-$2Cl^-$ cotransporter antibodies described above (see figure legends for dilutions), washed 6 times for 5 min and incubated for 20 min with fluorescein (FITC)-conjugated affi-pure Goat anti rabbit IgG (H+L) from Jackson ImmunoResearch (West Grove, PA) diluted 1/40 000. Following six washes in PBS plus 0.2% BSA, two washes in PBS plus 0.3% Triton X-100 and two washes in PBS alone the cells were mounted on silanated glass slides (Digene, Beltsville, MD) in citofluor glycerol/PBS mixture (Ted Pella, Redding, CA). Confocal microscopy was carried out as previously described [14].

3. Results and discussion

The nucleotide sequence obtained here from the rat parotid (see Section 2) contains an open reading frame beginning with a Kozak consensus sequence ([15]; GCGATGG) for translation initiation and coding for a membrane protein 1203 amino acids in length. The predicted amino acid sequence of this protein is highly homologous to the previously cloned mouse [10], human [11], bovine [12] and shark [9] NKCC1s (95%, 94%, 94% and 79% identity, respectively). As has been previously noted for NKCC1s the greatest degree of diversity among these proteins is in their N-terminal regions, particularly in the first approx. 140 amino acids [2,3]. Even here, however, the mouse, human and bovine NKCC1s and our rat sequence align well and exhibit ≥75% amino acid identity. Based on this high degree of homology as well as other similarities with known NKCC1s discussed below, we denote our rat parotid sequence as rtNKCC1. Given the well established involvement of a Na^+-K^+-$2Cl^-$ cotransporter in fluid secretion from the rat parotid [4–8] we suggest that this transporter and rtNKCC1 are one and the same.

The amino acid sequence of rtNKCC1 is illustrated in Fig. 1. The molecular mass of the protein predicted from this sequence is 130.4 kDa, which agrees well with that of enzymatically deglycosylated NKCC1s from several tissues [1], including the rat parotid (S.I. Lee and R.J. Turner, unpublished observations). The underlined regions labeled m1 through m12 in Fig. 1 are those predicted to form transmembrane helices by the program PHDhtm [16]; essentially identical predictions for membrane spanning regions are obtained from the method of Kyte and Doolittle [17] using a 19 amino acid window. The segments labeled m9–m10 and m11–m12 are rather long for a typical transmembrane spanning helix of approx. 20 amino acids. In analyses of other NKCC1s, where very similar hydrophobicity patterns are seen, it has been suggested that the regions corresponding to m9–m10 and m11–m12 consist of two transmembrane helices plus a short connecting extramembrane loop [9–12]. However, they could equally well represent single transmembrane helices set at an angle to the perpendicular to the

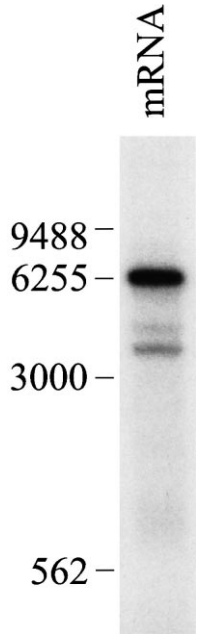

Fig. 2. Northern blot analysis of rtNKCC1 transcripts in rat parotid. Northern blotting was carried out as described in Section 2 using a rtNKCC1-specific probe.

membrane or some more complex structure. Additional experimental work will be required to clarify this aspect of the topology of these proteins.

Also indicated in Fig. 1 are a number of potential *N*-glycosylation sites, consensus sites for phosphorylation by protein kinase C, and the only consensus site for phosphorylation by protein kinase A on the molecule (see legend to Fig. 1). Most of these sites, including the protein kinase A consensus site, are conserved in other mammalian NKCC1s. In addition, we identify two conserved threonines (Thr-208 and Thr-1126) that correspond to known phosphoacceptors in the shark NKCC1 [3,18].

Northern blotting of rat parotid RNA using a rtNKCC1 probe (Fig. 2) revealed an abundant mRNA transcript at 6.3 kb, a less abundant transcript at 3.8 kb and a weaker still signal at 4.5 kb. Transcripts of similar sizes were found for the mouse NKCC1 (10; 6.5, 3.9 and 4.7 kb, respectively) and multiple transcripts for the human NKCC1 have also been observed [11]. The cDNA isolated by Delpire et al. for the mouse NKCC1 was 4.7 kb in length with a 964 bp 3′-UTR [10]. Using RNA mapping experiments these authors were able to demonstrate that this clone corresponds to the intermediate (4.7 kb) mouse NKCC1 transcript and that the longer (6.5 kb) dominant transcript apparently represents an extension of this 3′-UTR with an alternative polyadenylation site. In fact, the dominant transcripts of all previously cloned NKCC1s (6.5–7.5 kb) are significantly longer than the corresponding cDNAs isolated (4.1–5.2 kb) suggesting that a full length 3′-UTR sequence may not yet have been obtained [9–12].

As indicated above (see Section 2), in our initial experiments we determined that our rat parotid cDNA library contained two groups of clones that corresponded to rtNKCC1 cDNAs with 3′-UTRs of different lengths. One clone from each of these groups, nkcc1/S and nkcc1/L with 3′-UTRs of length 294 bp and 2685 bp, respectively, were chosen for

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3'-terminal sequence of nkcc1/S:

    3601  TTCTACTCTT AATTGTCCTG CCCCATGGAC TCCCTCTAAG AATGGTACTT
    3651  CAGTGCCTAG TCAGAGTGAC TGGATCGTCA GCAACACCGC AGCATCACAG
    3701  TGGCGAACTG GGTCTTTGTT TCACTACTTC ATTCTTTGAA AGCACATGGA
    3751  CACGTTGCTC CACTGATAGA TCTGTGGAGA CTTCGGTTTT AGTCTACTCC
    3801  AGATCTCAGT CTTATGACTG CTGATTCTTC CTTGTTGGAC TGAAGTTCTT
    3851  GAGAGTAAAG TTTTCCTTTG CTACTTGACA GCAAAAAAAA AAAAAAAAAA
    3901  AAA


3'-terminal sequence of nkcc1/L:

    3851  GAGAGTAAAG TTTTCCTTTG CTACTTGACA GCAATAAGAG TGAGTTATTT
    3901  TTGATTGCTG AAAGGAGTAC ATAAGCCTTT AGCCTTGAGG TGCCTTCTGA
    3951  AATTAACCAA ATTTCATCCA TAGATCTATC GTCTTTTGTA CATTTATAGA
      :
    6201  TTTAACCCCG AGTATTGCAG TTGCTGCTCC GTACAGAGGT TACTGCAATA
    6251  AAGGAAGTGG AATCATTAAA ACTTTAAAAA AAAAAAAAAA AAAA
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Fig. 3. 3′-UTRs of rtNKCC1 clones. See text for details. The stop codon (TAA) of clone nkcc1/S is underlined. Nucleotides are numbered relative to the beginning of the coding region of rtNKCC1.

detailed study. The sum of the length of the 3′-UTR of clone nkcc1/L and the coding region of rtNKCC1 is 6294 bp, corresponding to the length of the longest and most dominant rtNKCC1 transcript (Fig. 2) and strongly suggesting that this clone contains the complete 3′-UTR of rtNKCC1. The corresponding sum for nkcc1/S is 3903 bp consistent with the length of the shortest rtNKCC1 transcript (Fig. 2). In our experiments no rtNKCC1 clone with a 3′-UTR intermediate between those of nkcc1/S and nkcc1/L was ever observed. The sequence of the 3′-UTR of nkcc1/S is given in the upper panel of Fig. 3. The

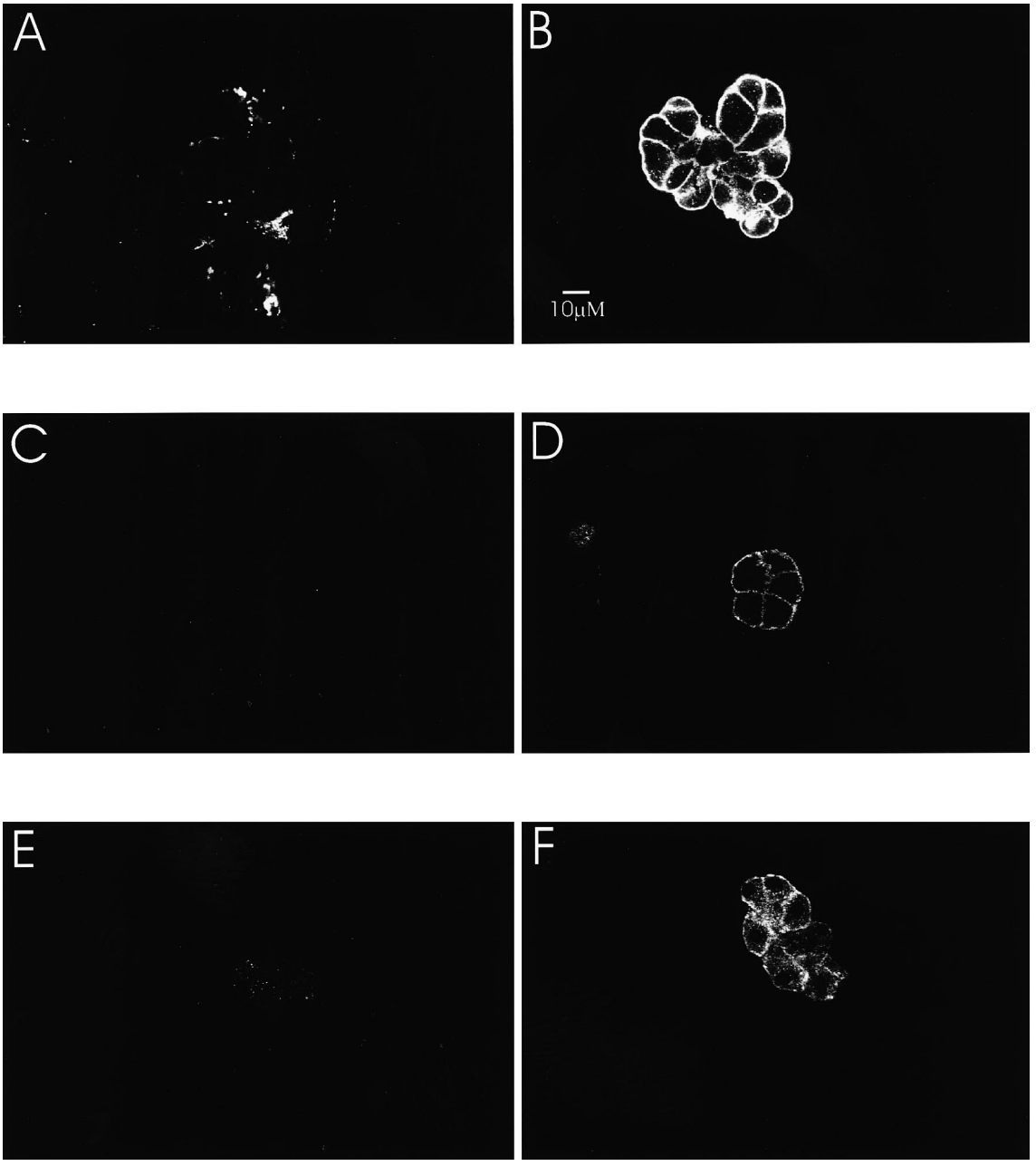

Fig. 4. Immunolocalization of N- and C-terminal rtNKCC1 epitopes. Experimental procedures were as described in Section 2. Immunofluorescent confocal micrographs of acini permeabilized with methanol are shown on the right and control acini (no methanol treatment) are shown on the left. The primary antibodies were α-wCT (A,B; diluted 1/2500), LL232 (C,D; diluted 1/100) and α-NT (E,F; diluted 1/100). Acini labeled with α-NT were also washed with 0.01% SDS (see Section 2).

corresponding sequence of the 3′-UTR of clone nkcc1/L (lower panel of Fig. 3) aligns exactly with that of nkcc1/S in the region preceding the poly(A) tail of the latter (i.e. to nucleotide number 3884 in both clones), indicating that the 3′-UTR of nkcc1/S is simply a truncation of that of nkcc1/L. The significance of the multiplicity of NKCC1 transcripts remains to be determined; however, elements in the 3′-UTR have been found to affect a variety of mRNA properties such as stability, localization, and translation efficiency. In this regard we point out that we have recently demonstrated that NKCC1 mRNA and protein levels are in fact upregulated during differentiation of the human intestinal cell line HT29-18 [19].

As already mentioned, Forbush and collaborators have previously demonstrated that the conserved threonines, Thr-208 and Thr-1126 (Fig. 1), are phosphoreceptors in the shark NKCC1 [3,18]. The location of these residues in the predicted hydrophilic N and C termini of the protein is consistent with these termini being inside the cell. In order to confirm this suggestion for rtNKCC1 we utilized immunofluorescent confocal microscopy and antibodies directed against specific regions in the N and C termini of rtNKCC1 to determine whether these epitopes were found on the extracellular or intracellular surface of rat parotid acinar plasma membranes. The antibodies used in our experiments were α-NT, raised against a 24 amino acid peptide found in the N terminus of rtNKCC1, LL232, raised against a 22 amino acid peptide amino found in the C terminus of rtNKCC1, and α-wCT, raised in rabbit against the terminal 454 amino acids of rtNKCC1 (see Section 2). In Western blots of rat parotid basolateral membranes (not shown) all three of these antibodies recognize a single 170 kDa protein, the molecular weight we have previously established as that of the Na^+-K^+-$2Cl^-$ (fully glycosylated) cotransporter in this tissue [7].

Representative confocal micrographs of permeabilized (panels B, D and F) and unpermeabilized (panels A, C and E) acini immunolabeled with each of the three antibodies are shown in Fig. 4. In immunolabeled unpermeabilized cells we typically observed a dim diffuse punctate pattern of fluorescence consistent with background labeling (cf. Fig. 4A,C,E). In addition, we occasionally observed larger irregularly shaped bright spots of fluorescence (cf. Fig. 4A) presumably due to trapping of the antibodies in the three-dimensional lobular acinar structures. In permeabilized acini, on the other hand, all three antibodies showed a distinctive web-like pattern consistent with the labeling of acinar cell membranes (cf. Fig. 4B,D,F). Some additional diffuse labeling exceeding that typically found in unpermeabilized cells was also frequently observed. This additional labeling of permeabilized acini was also seen when preimmune serum was used as the primary antibody (not shown) indicating that this effect was likely due to intracellular trapping. Taken together, the results illustrated in Fig. 4 provide strong evidence that the epitopes recognized by the antibodies αwCT, LL232 and α-NT are exposed in permeabilized but not in unpermeabilized acini and thus that the predicted large hydrophilic N and C termini of rtNKCC1 are located on the intracellular side of the plasma membrane.

Acknowledgements

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