Phosphorylation of a new member of the bicarbonate cotransporter superfamily

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Phosphorylation of a new member of the bicarbonate cotransporter superfamily. Acid-base balance is regulated by cortical collecting duct cells in kidney, where the transporter functions of β-intercalated cells are controlled by such factors as isoproterenol, cyclic adenosine monophosphate (cAMP), and the protein kinase A (PKA) pathway. The apical anion exchanger (AE) in β-intercalated cells is thought to be involved in the secretion of bicarbonate into urine. In isolated cells, the Cl⁻ channel was shown to be activated by isoproterenol via the PKA pathway. The importance of the PKA pathway and phosphorylation in the regulation of its transporter activity is not yet known.

The kidneys play a pivotal role in acid-base homeostasis, and acid-secreting (α type) and bicarbonate-secreting (β type) intercalated cells in the collecting ducts are major sites for the final modulation of urinary acid secretion [1]. These intercalated cells are regulated by several humoral factors and in vivo and in vitro acid-base balance. It has been shown that isoproterenol, cyclic adenosine monophosphate (cAMP), and the protein kinase A-dependent pathway regulate transporter functions of β-intercalated cells, such as activities of basolateral Cl⁻ channel and apical anion exchanger (AE).

ISOPROTERENOL AND cAMP INCREASES TRANSPORTER ACTIVITIES OF β-INTERCALATED CELLS

In β-intercalated cells, which are present in the cortical collecting ducts of the kidney, three transporters are mainly related to their cell function [1]. Vacuolar-type H⁺-ATPase is located in the basolateral membrane and transports H⁺ to basolateral side [2]. Chloride channel is also present in the basolateral membrane [3]. On the other hand, AE on the apical membrane is thought to play a major role in bicarbonate secretion into urine. Early work by Schuster, Bonsiv, and Jennings showed that peanut lectin binds exclusively to β-intercalated cells, and currently peanut lectin is widely used as a marker of these cells [4]. It has been reported that the transporters in β-intercalated cells, which are often defined by peanut lectin binding, are regulated by cAMP and isoproterenol. The first evidence of isoproterenol-dependent activation of β-intercalated cells was presented by Schuster [5], who showed that isoproterenol and cAMP stimulate HCO₃⁻ secretion by the rabbit cortical collecting ducts perfused in vitro. It has been reported that the lumen-to-bath Cl⁻ flux, which presumably indicates apical AE and basolateral Cl⁻ channel activities, was increased by these factors, too [6]. In our previous article, we reported that the function of β-intercalated cells is regulated by the cAMP-dependent pathway [7]. In that study, we measured intracellular pH (pHi) with 2',7'-biscarboxyethyl-5(and -6)carboxyfluorescein-acetoxyethyl ester (BCECF-AM) in the in vitro microperfused tubules. The β-intercalated cells were identified by FITC-peanut lectin. Isoproterenol or 8-bromine (Br)-cAMP was added to the bath, and the changes in the pH, were measured. The pH of β-intercalated cells was acidified by these factors, indicating that either the apical AE or the basolateral Cl⁻ channel was stimulated. In the absence of luminal Cl⁻, however, this regulation by isoproterenol and cAMP was abolished, suggesting that apical AE could be activated by these factors (Fig. 1).

In other cell types, regulation of cell function by isoproterenol, cAMP, and the protein kinase A (PKA)-dependent pathway also have been reported. In kidney, the Cl⁻ channel in α-intercalated cells is known to be activated by isoproterenol and the cAMP pathway. In RCC-28A cells, an α-intercalated cell line derived from rabbit cortical collecting duct (CCD) [8], and cultured medullary collecting duct cells (MDCD) [9], patch clamp studies clearly indicated that the Cl⁻ current was induced by isoproterenol and cAMP. To clarify whether the Cl⁻ channel in the β-intercalated cells is also regulated by these pathways, we examined the effect of isoproterenol on the Cl⁻ channel in purified β-intercalated cells [10]. The β-intercalated cells obtained by using a fluo-
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Fig. 1. Ion transporters in α- and β-intercalated cells. Basolateral Cl⁻ channel and apical anion exchanger (AE) in β-intercalated cells are considered to be regulated by isoproterenol via the protein kinase A (PKA)-dependent pathway. Basolateral AE in α-intercalated cells, the truncated form of AE1, is inhibited by diisothiocyanostilbene disulfonic acid (DIDS), while apical AE of β-intercalated cells is resistant to DIDS.

rescence-activated cell sorter with fluorescein isothiocyanate (FITC)-peanut lectin as a marker of these cells, and the sorted cells were cultured for two to four days. These β-intercalated cells were primary cultured, and the effects of isoproterenol and cAMP on chloride current were examined. Isoproterenol activated channel activity, and this effect was abolished by the β1 sympathetic receptor blocker atenolol [10]. In addition, the effect of isoproterenol was not additive to the effect of cAMP, indicating that isoproterenol exerts its effect via the PKA pathway. Furthermore, our previous study showed that this response of cultured β-intercalated cells was abolished by a PKA inhibitor. These series of experiments show that a apical AE and basolateral Cl⁻ channel are stimulated by isoproterenol via a PKA-dependent pathway. It is suggested that phosphorylation of this channel may be involved in this action of isoproterenol.

MOLECULAR CLONING OF APICAL AE IN β-INTERCALATED CELLS

The α-intercalated cells possess H⁺-ATPase and an AE on the apical membrane and the basolateral membrane, respectively [1]. This basolateral AE in the α-intercalated cells is a truncated form of band 3 protein [11], AE1 [12], and its localization has been revealed by immunohistochemical studies [13]. On the other hand, apical AE in the β-intercalated cells exhibits several differences in its transporter characteristics, such as its relative resistance to 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) [14], which inhibits AE1. Furthermore, since immunohistological studies with a specific antibody directed against the band 3 protein have not shown positive staining on the apical membranes of β-intercalated cells [4, 13], it has been suggested that this apical AE is a transporter that is distinct from AE1. For a long time, the molecular nature of this apical AE had not been elucidated. To clarify a role of the PKA-dependent pathway on the regulation of apical AE activity at a molecular level, we succeeded in molecularly cloning this AE [15]. Sorted β-intercalated cells were used as a source of mRNA for reverse transcription-polymerase chain reaction (RT-PCR) cloning with degenerate primers [15]. Since the details of our cloning strategy have been reported in our previous article, the detailed procedure for molecular cloning is not described here. The nucleotide sequences obtained from mRNA of sorted β-intercalated cells were
3164 and 3116 bp in length and contained open-reading frames of 2865 and 2817 bp, respectively. The shorter form (AE4b) of the cDNA lacked 48 bp of the longer form (AE4a), which was considered to be derived from alternative splicing. The proteins predicted for AE4a and AE4b were composed of 955 amino acids and 939 amino acids, respectively, and AE4a showed 48 and 34% sequence identities with human sodium bicarbonate co-transporter (NBC) 1 and rabbit AE2, respectively. The topology showed a marked similarity to that of the AE and NBC superfamily [16–18]. In the region homologous to the AE-consensus DIDS-binding motif [19], KLXX (where X is I, V, or Y), AE4 has the sequence KMLN (amino acids 517 to 520), and there are a few PKA and protein kinase C sites in the cytoplasmic domain. The difference in DIDS-binding motif may explain the relative resistance of AE4 to DIDS. Northern blots of rabbit liver RNA hybridized with an AE4a cDNA probe showed that AE4 is exclusively present in the kidney cortex. The expressed AE4 in COS-7 cells showed AE activity despite its closest homology to NBC family, and this AE activity was sodium independent and DIDS insensitive.

The immunohistological study with specific antibody raised against c-terminus of AE4 revealed that its immunostaining was detected only on apical cell membranes of a certain cell type of collecting ducts. Double staining with FITC-labeled peanut lectin and rhodamine-labeled anti-rat IgG was performed to characterize the AE4-positive cells. Double-staining studies showed that all of the AE4-positive cells were also peanut lectin-positive, indicating that AE4 is only expressed in β-intercalated cells. There were also a few cells that were peanut lectin-positive and AE4-negative. These cells may represent variants of the intercalated cells [13]. It is also possible that there may be different forms of apical AE in β-intercalated cell, since all of the peanut lectin-positive cells showed apical AE activity in the previous in vitro microperfusion studies.

**EFFECTS OF cAMP ON AE4 ACTIVITY**

To clarify whether apical AE is also regulated by isoproterenol and the cAMP pathway, AE activities of superfused COS-7 cells were measured with AE4 transfection. Preincubation of COS-7 cells with 8-Br-cAMP enhanced intracellular alkalization in response to bath Cl− removal. This effect of 8-Br-cAMP was mimicked by preincubation with forskolin. Since AE4 possesses two PKA sites in its intracellular domain, the activities of mutated AE4, which lacks each PKA site by site-directed mutagenesis, will reveal the importance of these sites in its regulation. The success in molecularly cloning AE4 should shed light upon the importance of the PKA-dependent pathway and phosphorylation in the regulation of its transporter activity.

**SUMMARY**

In β-intercalated cells, bicarbonate-secreting cells in the collecting ducts, isoproterenol, and cAMP regulate transporter activities. In isolated cells, the Cl− channel is activated by isoproterenol via the PKA-dependent pathway. Newly cloned AE4, which is presumably an apical AE of β-intercalated cells, has a PKA consensus motif, and its AE activity is increased by cAMP. The importance of the PKA-dependent pathway and phosphorylation in the regulation of its transporter activity should be revealed in the near future.

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