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Short communication

Molecular mechanism of arachidonic acid inhibition of the CFTR chloride channel

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Abstract

Arachidonic acid inhibits the activity of a number of different Cl^- channels, however its molecular mechanism of action is not known. Here we show that inhibition of cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels by arachidonic acid is weakened following mutagenesis of two positively charged pore-lining amino acids. Charge-neutralizing mutants K95Q and R303Q both increased the K_d for inhibition from ~3.5 μM in wild type to ~17 μM. At both sites, the effects of mutagenesis were dependent of the charge of the substituted side chain. We suggest that arachidonic acid interacts electrostatically with positively charged amino acid side chains in the cytoplasmic vestibule of the CFTR channel pore to block Cl^- permeation.

Keywords: Arachidonic acid; Blocker binding site; Chloride channel; Open channel block; Site directed mutagenesis

1. Introduction

Arachidonic acid affects the activity of many different classes of ion channels (Ordway et al., 1991; Meves, 1994; Besana et al., 2005) and in some cases specific regions and/or amino acid residues in cation-selective channel proteins have been implicated in arachidonic acid-mediated modulation (Macica et al., 1997; Kim et al., 2001; Honoré et al., 2002; Hamilton et al., 2003). Many different classes of Cl^- channels have been shown to be inhibited by arachidonic acid (e.g. Anderson and Welsh, 1990; Hwang et al., 1990; Kubo and Okada, 1992; Gosling et al., 1996; Xu et al., 1997; Riquelme and Parra, 1999; Linsdell, 2000; Dutta et al., 2002). However, the molecular basis of these inhibitory effects on anion-selective channels has not been reported.

One example of a Cl^- channel that is potently inhibited by arachidonic acid is the cystic fibrosis transmembrane conductance regulator (CFTR) (Linsdell, 2000), an epithelial cell cAMP-regulated channel that is mutated in cystic fibrosis (Sheppard and Welsh, 1999). In this channel, arachidonic acid applied to the cytoplasmic face of the membrane appears directly to inhibit chloride current (Linsdell, 2000). Chloride permeation through the CFTR channel is subject to inhibition by a broad range of organic anions that enter the pore from its cytoplasmic end and physically occlude it (Cai et al., 2004; Linsdell, 2006), a mechanism that apparently involves an electrostatic interaction with a positively charged amino acid side chain that lines the pore, K95 (Linsdell, 2005). This residue, along with another positive charged side chain (R303), also plays important roles in attracting intracellular Cl^- ions into the pore (Linsdell, 2005; St. Aubin and Linsdell, 2006). In the present study we show that mutagenesis of these two pore-lining positively charged residues weakens the inhibitory effect of arachidonic acid, in a manner that depends on the charge of the substituted amino acid side chain. These results indicate that arachidonic acid inhibition of CFTR Cl^- currents results from an electrostatic interaction between arachidonic acid and the cytoplasmic part of the CFTR channel pore.

2. Materials and methods

Experiments were carried out on baby hamster kidney (BHK) cells transiently transfected with wild type or mutant

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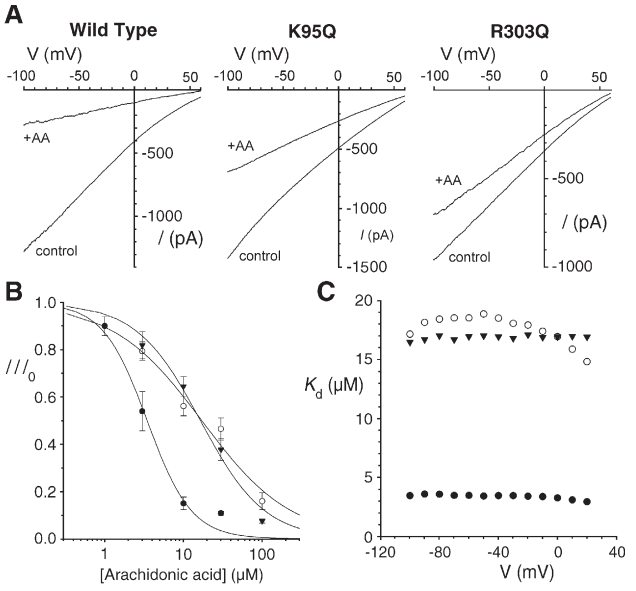

Fig. 1. Inhibition of wild type and mutant CFTR Cl^- currents by intracellular arachidonic acid. (A) Example leak-subtracted current–voltage relationships recorded from inside-out membrane patches for wild type, K95Q, and R303Q-CFTR. Currents were recorded following maximal current stimulation with ATP, PKA and PPi, before (control) and after (+AA) addition of 10 μM arachidonic acid to the intracellular solution. (B) Mean fraction of control current remaining (I/I_0) after the addition of different concentrations of arachidonic acid at a membrane potential of −100 mV, for wild type (●), K95Q (○) and R303Q (▼). Mean of data from 3 to 7 patches. In each case the fitted lines are to Eq. (1), giving a K_d of 3.5 μM and n_H of 1.54 for wild type, K_d of 17.2 μM and n_H of 0.77 for K95Q, and K_d of 16.5 μM and n_H of 1.06 for R303Q. (C) Relationship between K_d (calculated as described in (B)) and membrane potential for these three channel variants.

forms of CFTR (Gong et al., 2002). Macroscopic currents were recorded from inside-out membrane patches excised from these cells using the patch clamp technique, as described in detail previously (Gong et al., 2002; Gong and Linsdell, 2003). After patch excision and recording of background currents, CFTR channels were activated by exposure to protein kinase A catalytic subunit (PKA; 20 nM) plus MgATP (1 mM) in the cytoplasmic solution, followed by treatment with sodium pyrophosphate (PPi; 2 mM) to "lock" channels in the open state. In all experiments the intracellular (bath) solution contained (mM): 150 NaCl, 2 $MgCl_2$, 10 *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonate (TES); the extracellular (pipette) solution was identical except 150 mM Na gluconate was substituted for NaCl. These solutions were adjusted to pH 7.4 using NaOH. All chemicals were from Sigma-Aldrich (Oakville, ON, Canada) except PKA (Promega, Madison, WI). Arachidonic acid was prepared as described previously (Linsdell, 2000).

Current traces were filtered at 100 Hz using an 8-pole Bessel filter, digitized at 250 Hz, and analyzed using pCLAMP software (Molecular Devices, Sunnyvale, CA, USA). Macroscopic current–voltage relationships were constructed using depolarizing voltage ramp protocols (Linsdell and Hanrahan, 1996, 1998). Background (leak) currents recorded before addition of PKA have been subtracted digitally, leaving uncontaminated CFTR currents (Linsdell and Hanrahan, 1998; Gong and Linsdell, 2003). Given voltages have been corrected for liquid junction potentials calculated using pCLAMP software.

Arachidonic acid concentration–inhibition relationships were fitted by the equation:

$$\text{Fractional unblocked current} = 1/(1 + ([\text{arachidonic acid}]/K_d)^{n_H}) \quad (1)$$

3. Results

Application of arachidonic acid to the intracellular face of membrane patches inhibits CFTR Cl^- currents (Linsdell, 2000), a finding that is illustrated in Fig. 1A. Inhibition is concentration-dependent, with an apparent K_d of around 3.5 μM (Fig. 1B), and is independent of membrane potential (Fig. 1C). As also shown in Fig. 1, inhibition by arachidonic acid is dramatically weakened by point mutations which remove important positive charges from the pore inner vestibule, K95Q and R303Q, leading to approximately a 5-fold increase in K_d in both cases.

In order to understand the interaction between arachidonic acid and these pore-forming residues, we investigated the effects of additional substitutions of these two amino acids. As shown in Fig. 2, inhibition by arachidonic acid was strongly dependent on the charge of the amino acid side chain present at each of these two sites. Thus, while the charge conservative K95R and R303K mutations were not associated with a significant increase in K_d ($P > 0.3$, two-tailed *t*-test) all mutations which neutralized or reversed either of these two positive charges were associated with a significant increase in K_d (Fig. 2). For both positions, the potency of arachidonic acid was dependent on the amino acid side chain present, with K_ds following the rank order: (arginine, lysine < alanine < glutamine < glutamic acid). This order strongly suggests a side chain charge-dependent effect. This indicates that the interaction between the negatively charged arachidonic acid molecule and the CFTR pore is likely to be electrostatic in nature.

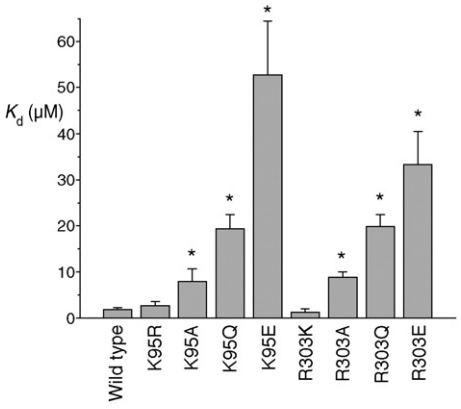

Fig. 2. Amino acid side chain charge-dependence of arachidonic acid inhibition. For each channel variant, K_d at −100 mV was estimated as described in Fig. 1. Mean of data from 3 to 11 patches. * indicates a significant difference from wild type, $P < 0.0005$ (two-tailed *t*-test).

4. Discussion

Many different classes of Cl^- channels are inhibited by arachidonic acid (see Introduction), however, the molecular mechanism by which arachidonic acid affects these channels has not previously been described. Our present results define a molecular mechanism of action for arachidonic acid inhibition of the CFTR Cl^- channel. Arachidonic acid appears to interact with positively charged amino acid side chains located within the Cl^- permeation pathway, K95 and R303, since mutations which eliminate the positive charge at these positions lead to significant weakening of the inhibitory effects of arachidonic acid. K95 is located in the first transmembrane region of the CFTR protein, and is thought to form part of the wide inner vestibule of the channel pore (Linsdell, 2005). Mutations which remove this positive charge reduce Cl^- entry into the pore from the cytoplasmic solution, as well as dramatically weakening the inhibitory effects of a broad range of negatively charged open channel blocking molecules (Linsdell, 2005). R303, which is situated at the very intracellular end of the fifth transmembrane region, appears to be located at the cytoplasmic mouth of the pore where it attracts Cl^- ions into the pore by a surface charge mechanism (St. Aubin and Linsdell, 2006). The amino acid side chain-dependent effects of mutations at both of these two positions on the apparent affinity of arachidonic acid inhibition (Fig. 2) suggest that the negatively charged arachidonic acid molecule interacts electrostatically with both of these positive charges within the CFTR channel pore. Thus it appears that arachidonic acid enters into the cytoplasmic end of the channel pore to inhibit CFTR Cl^- currents, most likely by physically occluding the Cl^- permeation pathway.

As noted above, many other negatively charged substances – such as sulfonylureas, disulfonic stilbenes, indazoles, arylaminobenzoates, and conjugated bile salts – inhibit CFTR Cl^- currents by interacting electrostatically with K95 and blocking the open channel (Linsdell, 2005). The present results therefore suggest that the molecular mechanism of inhibition by arachidonic acid is similar to these open channel blocker substances. In spite of this, several properties of arachidonic acid inhibition appear different from those of other open channel blockers that interact with K95. The inhibitory effects of the other classes of open channel blockers listed above are weakened by both depolarization of the membrane potential and by increasing extracellular Cl^- concentration (Cai et al., 2004; Linsdell, 2005) — indeed, these properties have become diagnostic of an open channel block mechanism of action in CFTR (Cai et al., 2004). In contrast, arachidonic acid inhibition is practically independent of both membrane potential (Linsdell, 2000; Fig. 1C) and Cl^- concentration (Linsdell, 2000). While the reasons for these differences are unclear, they presumably reflect some difference in the interaction between blocking anions, the CFTR pore and permeant Cl^- ions inside the pore.

Identification of key molecular determinants of arachidonic acid inhibition of CFTR points to a simple molecular mechanism — negatively charged arachidonic acid molecules are drawn into the cytoplasmic mouth of the pore by electrostatic attraction, where they impede Cl^- permeation. Since inhibition by arachidonic acid is common to so many different types of Cl^- channels (see Introduction), we speculate that a similar mechanism of action may also result in arachidonic acid inhibition of other Cl^- channel types.

Acknowledgement

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