Local Regulation of Cystic Fibrosis Transmembrane Regulator and Epithelial Sodium Channel in Airway Epithelium

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Regulation of cystic fibrosis transmembrane regulator (CFTR) and epithelial sodium channel (ENaC) in airway epithelia strongly influences the rate of mucociliary clearance (MCC) by determining the volume of airway surface liquid. MCC increases in response to stimuli originating on the airway surface, and CFTR and ENaC in airway epithelia appear to be regulated by local rather than systemic signaling. Although all signals that regulate CFTR and ENaC in airways have not been identified, the release of nucleotides from airway epithelial cells exposed to physical stimuli initiates a series of events that coordinately favor increased MCC. These events include activation of adenosine A_{2B} receptors that stimulate CFTR and P_{2Y2} receptors that inhibit ENaC. Together these actions result in an increased volume of airway surface liquid and increased MCC rates. Stimulation of CFTR by A_{2B}AR uses protein kinase (PK) A signaling elements that are localized within the apical/subapical compartment, including G proteins, adenylyl cyclase, PKA-II, A-kinase anchoring proteins, and phosphodiesterases. Inhibition of ENaC by P_{2Y2} receptors appears to be mediated by phospholipase C-β3, possibly through an effect on the levels of phosphatidylinositol 4,5-bisphosphonate in the apical membrane.

Keywords: ion channels; signaling complex; transduction

Protection of airways from inhaled particulates requires multiple lines of defense that cooperate to neutralize and remove inhaled particulates from the lung (1). The main process that removes foreign material from the lung is mucociliary clearance (MCC). In MCC, particulates stick to mucins secreted onto the airway surface and are then swept up and out of the lung by the synchronized beating of cilia that project from the luminal surface of most airway epithelial cells. Transport of mucus requires an aqueous nonviscous layer that allows cilia to beat effectively. The depth of this periciliary layer (PCL) is determined by solute and water transport by ciliated epithelia. Cystic fibrosis transmembrane regulator (CFTR) and epithelial sodium channel (ENaC) are the principal rate-limiting steps for Cl^- secretion and Na^+ absorption by ciliated airway epithelia (2, 3). These opposing processes are the main determinants of PCL depth, which must be maintained within a range that permits simultaneous MCC and unimpeded gas flow in the airway lumen. The importance of CFTR–ENaC regulation is documented in the disease cystic fibrosis (CF), whose patients suffer airway obstruction and chronic infection that results from decreased MCC secondary to missing CFTR and accelerated ENaC activity. Given the importance of CFTR and ENaC in lung defense mechanisms, much remains to be learned about their regulation in airway epithelia. One potential contributor to the slow pace of discovery in this area is the fact that PCL volume is controlled principally by stimuli originating at the airway surface. Thus, much of what has been learned about hormonal regulation of CFTR and especially of ENaC in other tissues does not appear to apply in airways. Outstanding questions in the regulation of CFTR and ENaC in the airways include what pathways link CFTR activity to local stimuli, what mechanisms normally restrain ENaC, and what is the molecular basis for CFTR influence on ENaC. This review considers these questions in the context of how nucleotide release in response to physical forces, such as shear stress, contributes to a signaling network that makes CFTR and ENaC responsive to local stimuli in airways.

CFTR REGULATION IS HIGHLY LOCALIZED

In characterizing the patterns and regulation of ion transport in models of airway epithelia, we found a substantial level of CFTR-mediated Cl^- conductance in the absence of intentional stimulation of cAMP production (4). This observation suggests both that very low levels of cAMP activate CFTR and that there exist signaling pathways, which produce small quantities of cAMP locally in response to tonic, low-level stimulation. We set out to identify the elements of cAMP signaling that were responsible for basal activity of CFTR in CalU3 cells. In early studies, we found CFTR to be selectively regulated by the membrane-associated isozyme of protein kinase A (PKA), PKA-II (5, 6). Building on this intact tissue observation, we asked whether PKA activity was closely physically associated with CFTR in CalU3 cell membranes (7). Using patch clamp techniques, membrane patches were excised in the inside-out configuration. CFTR channel opening is strongly stimulated by the degree of PKA-mediated phosphorylation. Therefore, we treated CFTR activity as a reporter for the activity of PKA that might be present in the patch. As seen in Figure 1, CFTR was activated by the cAMP analogue, 8-(4-chlorophenylthio)-CAMP (cpt-cAMP). If the patch was pretreated with PKI, a highly specific peptide inhibitor of PKA, NPo (n [number of channels] × Po [open probability]) was very low, and there was no stimulation by cpt-cAMP. These observations suggested that PKA remained associated with excised membranes that contained CFTR.

We next examined the molecular basis of PKA association with the cell membrane (7). The PKA-II isoform is known to associate with subcellular surfaces by binding proteins called A-kinase anchoring proteins (AKAPs) (8). The nature of this interaction is well understood. A region in the early amino terminus of RII binds with α-helical regions found in AKAPs, a class of otherwise diverse proteins (9, 10). When membrane patches were exposed to HT31, an α-helical peptide that disrupts the anchoring of PKA-II to AKAPs, we found that CFTR was no longer activated by cpt-cAMP (Figure 2). Importantly, a control peptide that contained prolines to disrupt the α-helical conformation of HT31 was completely ineffective. The stimulation of CFTR in isolated membrane patches by a cAMP analogue and the inhibition of this effect by a peptide capable of dislodging

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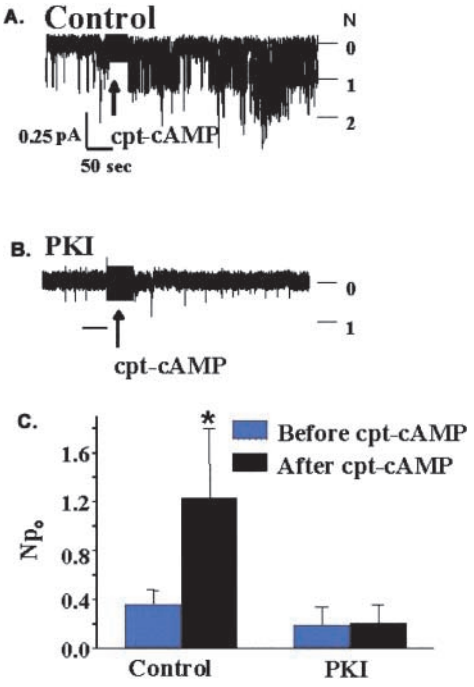

Figure 1. cAMP analogue activates cystic fibrosis transmembrane regulator (CFTR) in excised membrane patches (7). (*A*) Control. Excised inside-out patch containing CFTR exposed to chlorophenylthio-cAMP. (*B*) Same experiment as in *A* but in the presence of PKI, a specific inhibitor of protein kinase (PK) A. (*C*) Summary of all experiments like *A* and *B*.

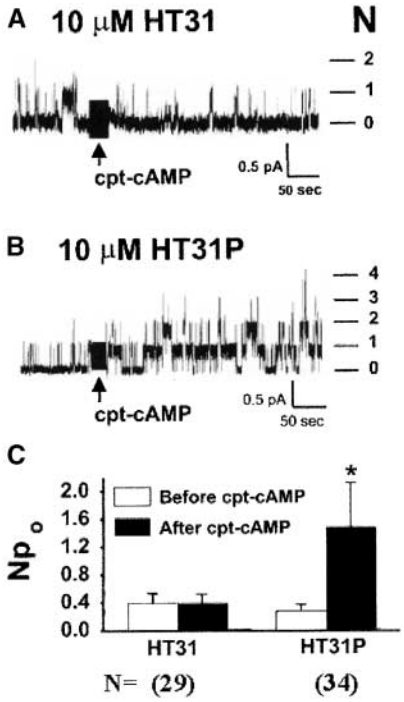

Figure 2. A-kinase anchoring proteins (AKAPs) localize PKA to membrane patches containing CFTR (7). (*A*) Membrane patch containing CFTR in the presence of HT31 peptide to compete with PKA-II binding to AKAPs. cpt-cAMP added at *arrow* to activate PKA had no effect. (*B*) Control experiment with HT31P, a peptide that does not dislodge PKA-II from AKAPs. (*C*) Summary data for the experiments illustrated by *A* and *B*.

PKA-II from preferred binding sites provide a clear indication that organized pathways for PKA-mediated regulation of CFTR exist. Such pathways could help explain how CFTR could be controlled by local stimuli.

MULTIPLE AKAPS ARE EXPRESSED IN AIRWAY EPITHELIA CELLS

We have begun to identify and characterize AKAPs expressed in airway epithelial cells to discover the AKAP or AKAPs important for regulation of CFTR by PKA (11, 12). These studies have led to the discovery of novel AKAPs and verification of the expression of a number of known AKAPs. In one study, we screened a lung cDNA expression library using biotinylated RII as probe and identified a novel cDNA that shares sequence homology with AKAP18, a previously identified membrane-associated AKAP associated with the regulation of calcium channels (13, 14). However, the AKAP18 cDNA we cloned encodes

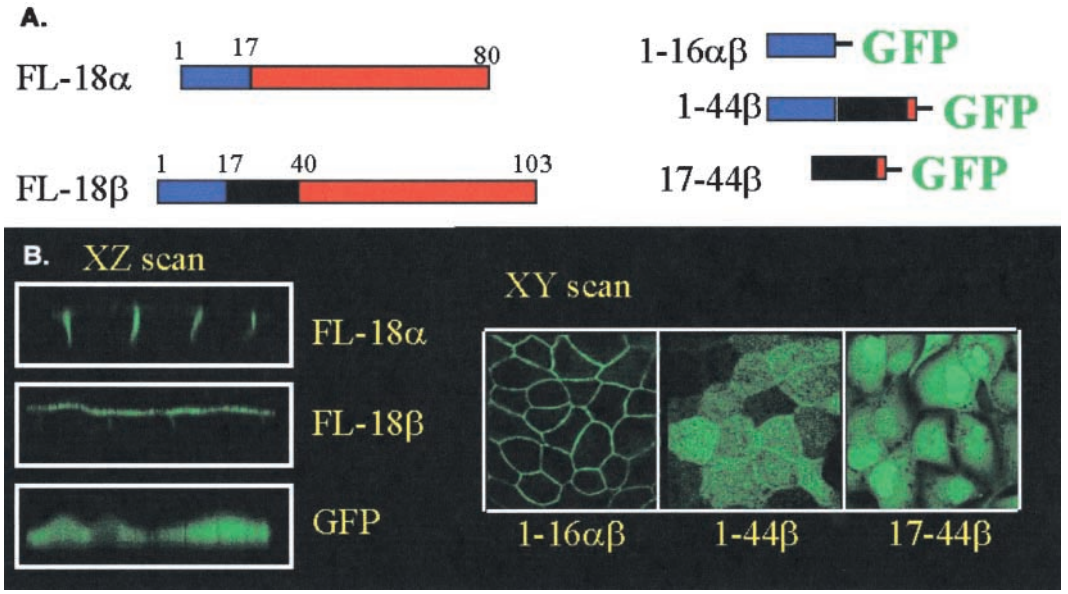

Figure 3. AKAP18β insert targets the apical membrane in polarized epithelial cells (11). (*A*) Fusion proteins made to test cellular distribution of isoforms of AKAP18. The key difference is the 23 amino acids that occupy positions 17–40 in AKAP18β but are absent in AKAP18α. (*B*) Confocal microscopy of proteins described in *A*.

a protein of 326 amino acids, the first 262 amino acids of which are unique with no significant homology with any known proteins (11). This finding led to the recognition of two novel isoforms of AKAP18, which arise from alternative splicing. We showed both AKAP18α (original AKAP18) and AKAP18β to be strongly membrane associated. Interestingly, AKAP18α distributes preferentially to lateral cell membranes in polarized Madin Darby Canine Kidney (MDCK) epithelial cells, whereas AKAP18β is exclusively apical in its distribution (Figure 3). Studies are underway to test the possibility that AKAP18β colocalizes with and regulates CFTR in the apical membrane of airway epithelial cells. Using a similar cloning strategy and highly purified ciliary axonemes, we identified a novel AKAP expressed in ciliated airway epithelia, named AKAP28 (12). Immunolocalization of endogenous AKAP28 revealed that it is located exclusively in the cilial shaft. Thus, it is probable that multiple AKAPs serve to position PKA-II at specific locations within the apical pole of airway epithelial cells. We hypothesize that this positioning of PKA is required for efficient regulation of CFTR and other functions, such as ciliary beating, by stimuli present in airway surface liquid. Such stimuli include adenosine (discussed later here), which is known to regulate PKA (15). Other kinases that regulate CFTR activity, such as PKC epsilon, are also optimally localized with respect to CFTR in the membrane (16). These and other findings, such as differential regulation of CFTR channel gating by specific phosphatase isozymes, suggest how CFTR activity could represent a balance between signaling pathways whose elements are strategically localized to access CFTR regulatory domains (17).

ELEMENTS FOR AUTOCRINE REGULATION OF CFTR ARE CONTAINED IN MEMBRANE PATCHES

We began investigating the importance of autocrine/paracrine regulation of CFTR by examining the effect of extracellular adenosine (ADO) on CFTR-mediated Cl^- secretion and CFTR channel activity. In Ussing chambers, we found that ADO stimulated Cl^- secretion, and the rank order potency of well-studied ADO analogues identified the responsible receptor as $A_{2B}AR$ (18). A striking finding in these studies was that maximal stimulation of CFTR-mediated Cl^- secretion by luminal ADO was accompanied by no measurable change in total cellular cAMP. In parallel patch clamp studies, we made a second surprising but informative observation. Our objective was to measure the effect of increasing concentrations of ADO in the pipette on CFTR single-channel gating in cell-attached recordings. In these studies, we encountered a substantial basal activity of CFTR with no ADO added to the pipette. We speculated that this activity could arise from the presence of endogenous ADO at the face of the membrane patch isolated by the pipette tip. This supposition was verified by including the nonselective ADO receptor antagonist 8-sulfophenyltheophylline in the pipette, which resulted in nearly complete inhibition of CFTR gating. We further speculated that ADO was being generated on the extracellular face of the patched membrane by the ectometabolism of ATP. This speculation was also confirmed when we observed almost no CFTR activity with β-methylene-adenosine-5′-diphosphate (AMPCP) in the pipette solution. This poorly hydrolyzed ADP analogue strongly inhibits 5′-nucleotidase, which generates ADO from AMP. These simple observations have two important implications for the notion of local regulation of CFTR. The first is that an $A_{2B}AR$ in the apical membrane can signal to CFTR Cl^- channels contained within the same patch. This implies, and we subsequently demonstrated, that the molecular components of G protein–coupled receptor (GPRC) signaling, including G proteins and adenylyl cyclase, are also accessible within the patched membrane. The second implication is that cellular ATP moves across the cell-attached membrane patch and is broken down on the extracellular surface of the plasma membrane. Given that the formation of gigaseals to attain the cell-attached configuration is a highly localized physical stimulus, these results are consistent with induced ATP release. The effectiveness of AMPCP in decreasing the apparent basal activity of CFTR places the enzymes necessary to generate ADO from ATP on the cell surface (19).

REGULATION OF CFTR BY $A_{2B}AR$ AT THE APICAL MEMBRANE IS LATERALLY COMPARTMENTALIZED

Once we understood that extracellular ADO regulated CFTR through $A_{2B}AR$ and downstream signaling elements in a tiny area of membrane confined within a cell-attached patch pipette, we turned to the question of lateral spread of such signaling. We made cell-attached patches with AMPCP in the pipette to prevent generation of ADO (Figure 4). We then recorded CFTR gating for a control period and after the addition of ADO to the solution, bathing the entire apical membrane surface outside the cell-attached patch. We observed that ADO, even at high concentrations, had little effect on CFTR. However, the patched membrane contained CFTR Cl^- channels and PKA sufficient to activate them because forskolin caused a marked stimulation of CFTR channel gating (18). These results are not surprising given the relative effects of ADO and forskolin on cellular cAMP levels (discussed previously here) and support the idea that receptor-mediated cAMP production efficiently activates PKA in close proximity but does not spread very far. As receptor-mediated regulation of CFTR is expected to reflect local stimuli on the airway surface, a picture of highly localized acute regulation of CFTR-mediated Cl^- conductance emerges. The question of what limits the lateral spread of apical membrane signaling is currently under investigation, with a strong focus on the role of phosphodiesterases (20).

REGULATION OF ENaC IN AIRWAY EPITHELIA

The epithelia of all adult mammalian airways that have been characterized exhibit net Na^+ absorption (2, 3). This Na^+ absorption has been inferred to be required to reduce PCL/airway surface liquid volume as MCC "funnels" liquid onto proximal airways of ever-decreasing net surface area. In this role, the appropriate rate of Na^+ absorption reduces the volume of airway surface liquid/PCL to prevent airway occlusion but maintains sufficient depth for adequate hydration of mucus (21). Against a background of constitutive Na^+ absorption, it is important to note that acute inhibition of ENaC has the effect of increasing airway surface liquid/PCL depth. Thus, it is not surprising that stimuli that increase the rate of MCC can have as a component of their actions ENaC inhibition. This contrasts to the major actions in hormonal regulation of ENaC observed in kidney and colon, where changes in ENaC expression or trafficking mediate increased Na^+ conductance for the purpose of salt conservation (22). It is increasingly clear that physiologic regulation of ENaC in airways is mediated by local stimuli that decrease Na^+ absorption (23). Furthermore, although changes in the expression or trafficking of ENaC are clearly associated with hormonal regulation of Na^+ absorption in kidney and colon (24), local stimuli are thought more likely to regulate ENaC open probability (22, 25).

GPCR-MEDIATED INHIBITION OF ENaC

Based on the previously mentioned reasoning, we have examined regulation of ENaC by pathways that are capable of sensing local conditions on the airway surface, focusing on GPCR and on downstream signaling components that may be common to

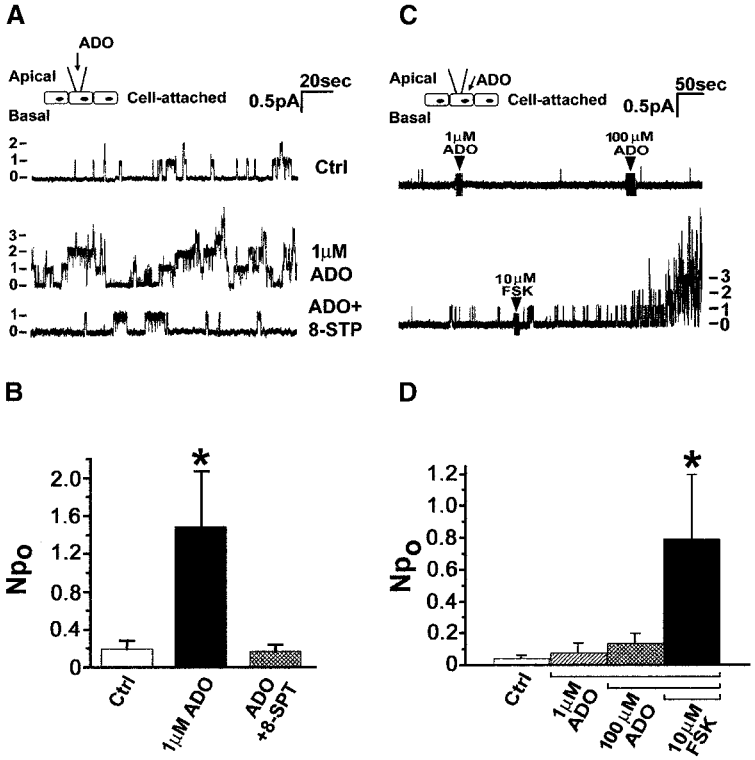

Figure 4. Adenosine signaling in the apical membrane is laterally compartmentalized (18). (*A*) Cell-attached patches under control conditions (β-methylene-adenosine-5′-diphosphate in pipette), with 1-μM adenosine and 1-μM adenosine + the adenosine receptor blocker 8-sulfophenyltheophylline (8-SPT). (*B*) Summary data of experiments illustrated in *A*. (*C*) Cell attached recording during sequential additions of adenosine and forskolin to the bath. (*D*) Summary of experiments illustrated by *C*.

specific families of receptors. Thus far, we have examined regulation of ENaC by endothelin ET_B receptors and by P_{2Y2} receptors. Each of these receptors is known to couple through Gq to phospholipase C-β (PLCβ), although cross-talk with other signaling pathways has been reported (26, 27).

ET_B RECEPTORS INHIBIT ENaC THROUGH AN Src FAMILY KINASE

We initially observed in airway epithelia that luminal application of endothelin inhibited amiloride-sensitive short circuit current (I_{SC}) within 5–10 minutes. Whereas this has turned out to be an inconsistent effect in our airway epithelial models, we proceeded to examine its molecular basis in 3T3 cells expressing α-, β-, and γ-rENaC subunits (28). First, we determined in whole-cell studies that endothelin inhibited amiloride-sensitive whole-cell current. Next, we used specific inhibitors of endothelin receptor subtypes ET_A and ET_B to show that this regulation of ENaC was mediated in fibroblasts by ET_B receptors. A logical downstream effector of PLCβ is PKC, which is activated by endothelin in many cell types and has been reported to inhibit ENaC. After we found little effect of PKC inhibition on endothelin's action on ENaC, we tested for a role of Src family kinases, which are also reported to be activated by ET_B receptors in some cell types. In one series of studies, we looked at the effect of P2, a potent and somewhat selective inhibitor of Src family kinases. Inhibition of amiloride-sensitive whole-cell current was completely blocked by a concentration of P2 that inhibited Src kinase activity of fibroblasts in a biochemical assay. Interestingly, P2 itself, but not a chemically similar control compound P3, stimulated amiloride-sensitive whole-cell currents. These results suggest that Src family kinases were tonically active in fibroblasts and serve to restrain ENaC-mediated current under "basal" conditions. Although this is a very interesting mechanism for potentially restraining ENaC activity in airway epithelia, so far it does not appear to be a dominant mechanism in airways and may turn out to be more important in other tissues.

P_{2Y2} RECEPTOR ACTIVATION INHIBITS ENaC

Like others, we found that stimulation of P_{2Y2} receptors in airway epithelia inhibits Na^+ absorption (29). We view identification of the pathway(s) that links P_{2Y2} receptors to regulation of Na^+ absorption as a strategy to identify mechanisms that inhibit ENaC. P_{2Y2} receptors couple via Gαq to activation of PLCβ and stimulate the rapid hydrolysis of phosphatidyl inositol 4,5-bisphosphonated (PIP2) to generate diaglycerol and inositol 1,4,5-trisphosphate. PIP2 may also function as a signaling molecule that affects the activity of multiple membrane transporters and ion channels, including ENaC. Therefore, activation of PLCβ may regulate ENaC function via second-messenger pathways, such as PKC, or by depleting the amount of PIP2 at the apical membrane. The four PLCβ isozymes (β1–β4) share a common domain organization but differ in their primary sequence and ability to be activated by G proteins (30). Because PLCβ isozymes display different relative sensitivities to stimulation by G protein subunits and are differentially regulated by phosphorylation, it is important to identify the specific PLCβ protein(s) involved. Therefore, we plan to identify PLCβ isozymes expressed in airway and to determine which are compartmentalized together with GPCRs that regulate apical ion transport. We also plan to explore whether PLCβ activity regulates ENaC by reducing PIP2, which has been recently reported to increase ENaC open probability (31, 32), or by activating downstream effectors such as PKC, a pathway also implicated in the regulation of ENaC (33).

CFTR REGULATION OF ENaC

CF airway epithelia are virtually devoid of CFTR-mediated Cl^- conductance but absorb Na^+ at two to three times the normal rate (34). In normal airway epithelia, stimuli that raise intracellular cAMP have no effect on the rate of Na^+ absorption, but in CF, airway epithelia cAMP further stimulates the already elevated rate of Na^+ absorption (35). These and secondary support-

ing observations, such as increased ouabain-sensitive oxygen consumption in CF airways, suggest that the expression of CFTR in airway epithelia restrains the activity of ENaC (36). Although there have been many reports consistent with this notion, the molecular basis for a functional connection between CFTR and ENaC has remained elusive. Over the years, it has emerged that ENaC in airways is constitutively active and subject to regulation by acute inhibition, although the responsible signaling pathways are not yet identified. In this context, we speculate that CF mutations that effectively prevent CFTR functional expression indirectly decrease the expression of a signaling pathway that inhibits ENaC or, conversely, permit the expression of a stimulatory pathway that is normally repressed in airway epithelial cells. A better understanding of the pathways that inhibit ENaC in airway epithelial cells, coupled with genomic and proteomic analyses to identify differentially expressed genes in normal and CF airways, offers the best approach to solving this riddle.

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