Purinergic regulation of the epithelial Na\textsuperscript{+} channel

Lauren M. O’Mullane, David I. Cook and Anuwat Dinudom

The Discipline of Physiology, The Bosch Institute, Faculty of Medicine, The University of Sydney, NSW 2006, Australia

Summary

1. The epithelial Na\textsuperscript{+} channel (ENaC) is a major conductive pathway that transports Na\textsuperscript{+} across the apical membrane of the distal nephron, the respiratory tract, the distal colon and the ducts of exocrine glands. ENaC is regulated by hormonal and humoral factors, among which are extracellular nucleotides that can be available from the epithelial cells themselves.

2. Extracellular nucleotides, via the P2Y\textsubscript{2} receptors (P2Y\textsubscript{2}Rs) at the basolateral and apical membrane of epithelia, trigger signalling pathways that cause inhibition of the activity of ENaC and activation of Ca\textsuperscript{2+}-dependent Cl\textsuperscript{−} secretion.

3. Recent data from our laboratory suggest that stimulation of the P2Y\textsubscript{2}Rs at the basolateral membrane inhibits activity of ENaC by a signalling mechanism that involves G\textbeta\textgamma subunits freed from a PTX-sensitive G protein and phospholipase C-\beta. A similar signalling mechanism is also partially responsible for inhibition of ENaC during activation of apical P2Y\textsubscript{2}Rs.

4. Stimulation of apical P2Y\textsubscript{2}Rs also stimulates an additional signalling mechanism that inhibits ENaC, involving the activated G\alpha subunit of a PTX-insensitive G protein, and activation of an unidentified PLC. The effect of this PTX-insensitive system requires the activity of the basolateral Na\textsuperscript{+}/K\textsuperscript{+}/2Cl\textsuperscript{−} co-transporter.

Introduction

The epithelial Na\textsuperscript{+} channel (ENaC) mediates Na\textsuperscript{+} absorption across the apical membrane of polarised epithelia including the distal colon, lung, distal nephron, and excretory ducts of the sweat and salivary glands. Activity of ENaC creates an osmotic driving force for fluid reabsorption which, in turn, plays important roles in the regulation of blood pressure and the maintenance of the volume of alveolar fluid and mucociliary clearance. ENaC is comprised of 3 homologous subunits, \alpha-, \beta- and \gamma-ENaC, each of which shares 30-40% homology in their amino acid sequences. ENaC subunits contain short cytosolic NH\textsubscript{2}- and carboxy-termini, two membrane spanning domains, and a large amino acid loop that is exposed to the extracellular environment. Constitutively active ENaC at the cell membrane comprises all 3 subunits with a quaternary composition of 2\alpha, 1\beta and 1\gamma. Although, the presence of the transmembrane domains in all three ENaC subunits suggests that they may contribute to the formation of the ion conductive pore of the channel, the presence of the \alpha subunit is essential for generating the Na\textsuperscript{+} conductance of ENaC.

Electrogenic Na\textsuperscript{+} absorption is a passive process, driven by an electrochemical gradient across the apical membrane, which is generated by activity of the basolateral membrane Na\textsuperscript{+}/K\textsuperscript{+} ATPase. This electrochemical gradient favours influx of Na\textsuperscript{+} ions from the luminal solution into the cytoplasm. Activity of ENaC, therefore, causes development of a negative potential in the lumen, which in turn diminishes the driving force for Cl\textsuperscript{−} secretion. Hence, mechanisms that reduce activity of ENaC are likely to hyperpolarise the cells, which in turn promotes Cl\textsuperscript{−} secretion via Cl\textsuperscript{−} conductive pathways at the apical membrane of epithelial cells. Activity of ENaC is under tight regulation by a wide variety of hormonal and non-hormonal mechanisms. These regulators affect the expression, trafficking, or function of the channel.

It has become apparent that purinergic regulation is one important factor that controls Na\textsuperscript{+} transport via ENaC. Extracellular nucleotides such as ATP and UTP can become available from many cell types including epithelial cells themselves. Epithelial cells release nucleotides in response to physiological signals, such as mechanical stimulation, changes in ion concentration, rate of flow of fluid bathing the apical cell surface, and pathological states and infections. In addition, nucleotides can become available from pathogens that infect epithelial cells. Extracellular nucleotides, in turn, regulate a range of physiological mechanisms by activating nucleotide-sensitive P2Y receptors at the plasma membrane. These effects include modulating Na\textsuperscript{+} and water transport in the renal and gastrointestinal epithelia, increasing mucociliary clearance of the respiratory epithelium and regulating blood pressure. Moreover, extracellular ATP and UTP can be further metabolised by ecto-nucleotidases and other hydrolytic activities into nucleotide-diphosphates and nucleosides, allowing stimulation of other classes of purinergic receptor.
Purinergic regulation of ENaC

Figure 1: Extracellular nucleotides mediate inhibition of ENaC via P2Y2 receptor. A (left panel): representative tracings of transepithelial potential measurement in FRT cell monolayers transfected with ENaC obtained under open-circuit conditions (see Lee et al. for methods). Cells were co-transfected with plasmids containing α-, β- and γ-ENaC subunits in pcDNA3.1 (0.7 µg/ml each). UTP (100 µM) was added to the solution bathing the apical or basolateral side of the monolayers. Activity of ENaC was determined by adding amiloride (10 µM) to the apical bath solution. B (upper panel): RT-PCR analysis of the effect of the siRNAs on expression of P2Y2, P2Y4, P2Y6 receptors. Cells were transfected with ENaC alone or co-transfected with ENaC and scrambled siRNA or siRNA directed against P2Y2, P2Y4 or P2Y6 (50 pmol each). Expression of β-actin was used as a control. B (lower panel): effect of apical or basolateral UTP on the relative I_{amil} in FRT cells. * and ** indicates p < 0.05 and p < 0.01, respectively.

largely attributable to activation of P2 purinoceptors of the P2Y2 class.

P2Y2 receptors (P2Y2Rs) are members of the purine or pyrimidine nucleotide-sensitive G protein-coupled receptor (GPCR) family, which are known to couple with Gq/11, Go or Gi2. Stimulation of the receptors by nucleotides leads to activation and dissociation of α and βγ subunits of heterotrimeric G proteins and subsequent stimulation of phospholipase C-β (PLC-β) and its downstream signalling cascades. So far, the exact identity of the G proteins responsible for mediating the P2Y2, R signalling pathways that inhibit the activity ENaC in epithelia remains elusive. The first description of the G protein involved in the purinergic regulation of ENaC was provided by a study by Kunzelmann et al., where the effect of UTP on the activity of ENaC in mouse trachea was investigated. The inhibitory effect of UTP, applied to the apical cell surface, on the activity of ENaC in the tracheal epithelium was attenuated by pertussis toxin (PTX). This finding suggested that the mechanism by which nucleotides regulate the activity of ENaC may involve a G protein of either the Gi or Go class. Our own studies in Fisher Rat Thyroid (FRT) cells expressing ENaC confirm with this previous report. FRT cells do not express ENaC endogenously, however, when transfected with ENaC subunits, FRT cell monolayers grown on permeable supports exhibit an amiloride-sensitive current that is sensitive to ENaC regulators, including insulin, aldosterone, SGK1, AKT1 and PI3K. The presence of UTP (100 µM) in the solution bathing the apical membrane of FRT cell monolayers inhibited activity of ENaC by 45%, whereas only 15% inhibition was observed when UTP was added into the solution bathing the basolateral cell surface (Figure 1A). This differential effect of apical and basolateral nucleotides on the activity of ENaC observed in this cell type is in agreement with reports in porcine tracheal epithelium and mouse collecting duct cells.

To elucidate the purinergic receptor responsible for mediating the effect of extracellular nucleotides on ENaC in this cell type, we used gene interference techniques to specifically knockdown expression of P2Y2, P2Y4 and P2Y6 receptors, all of which are sensitive to UTP. We found that the inhibitory effect of UTP on the activity of ENaC was completely abolished in cells in which expression of the P2Y2R was inhibited (Figure 1B). Knocking down expression of P2Y2 or P2Y4 receptors, however, has no effect on the inhibition of ENaC by UTP. The effect of nucleotides on ENaC in FRT cells is, therefore, mediated via the P2Y2R. The contribution of PTX-sensitive G proteins was subsequently determined by incubating FRT cell monolayers in PTX (200 ng/ml) for 16 hours before the effect of UTP on ENaC was determined. We found, in agreement with a previous report, that the effect of UTP on the activity of ENaC in FRT cells is inhibited by PTX (Figure 2A). It is notable that PTX completely inhibits the effect of apical UTP on ENaC. Taken together, these data support the existence of a PTX-sensitive G protein pathway linked to the P2Y2Rs in both the basolateral and apical membranes. Given that the effect of apical P2Y2, R stimulation on ENaC activity is only partially inhibited by PTX, it seems likely that the effect of apical P2Y2, R stimulation on ENaC is mediated by both PTX-sensitive and PTX-insensitive mechanisms.
activate distinct cellular signalling pathways and regulatory proteins. For instance, the free Gβγ released during P2Y₆,R stimulation is involved in activation of PLCb₄ and G protein-activated inwardly rectifying K⁺ channels. Our own studies in human colon epithelial (HT29) cells suggested that free Gβγ, released during M₃ muscarinic receptor stimulation, mediates mobilization of Ca²⁺ from intracellular stores, and that free Gβγ, released during P2Y₁₆,R stimulation, regulates membrane Ca²⁺ influx (unpublished data). One of our most surprising findings is the discovery that the mechanisms by which P2Y₆,R signalling regulates the activity of ENaC involves the βγ subunits of G proteins. We found that over-expression of the c-terminal of β-adrenergic receptor kinase (βARK) that acts as a scavenger of Gβγ, completely abolishes the effect of basolateral UTP on the activity of ENaC (Figure 3A). βARK, however, only partially inhibits the effect of the apical application of UTP on the channel. These findings suggest that the PTX-sensitive component of the P2Y₆,R signalling may be mediated by the Gβγ subunits. The inhibitory effect of βARK on the inhibition of ENaC by apical UTP further suggests a degree of similarity between signalling mechanisms utilized by the apical and basolateral P2Y₆,R. Conversely, the inability of βARK to completely abolish the effect of apical UTP (Figure 3A) suggests that free Gα may be involved, at least in part, in mediating the effect of apical nucleotides on ENaC.

To further investigate the characteristics of the P2Y₆,R-mediated signalling system that regulates the activity of ENaC, we used siRNAs to specifically knockdown expression of PLC-β isoforms in FRT cells. siRNA-mediated knockdown of PLC-β4 expression totally abolished the effect of basolateral UTP on ENaC, whereas siRNAs directed against PLC-β1, PLC-β2 or PLC-β3 were without any effect (Figure 3B). Thus, PTX-sensitive P2Y₆,R signalling in the basolateral membrane is coupled with PLC-β4. Knocking down expression of PLC-β4 partially inhibits the effect of apical UTP on ENaC (Figure 3B). This finding is consistent with a notion that a similar signalling mechanism to that generated by the basolateral P2Y₆,R is partially involved in the apical P2Y₆,R signalling. Interestingly, the effect of apical UTP, but not that of basolateral UTP, on the activity of ENaC is sensitive to a blocker of phospholipase C, U73122 (Figure 3C). Given that the effect of basolateral UTP on ENaC is insensitive to this blocker, we conclude that the P2Y₆,R-activated PTX-sensitive signalling pathway is coupled with PLCb-4. The PTX-insensitive component of the P2Y₆,R signal, activated by apical nucleotides, however, is coupled with a different PLC isoform that is sensitive to this blocker. Other phosphoinositol-specific PLC isoforms such as PLC-γ₂, δ and ε isoforms that are sensitive to U73122, might be considered as candidates of the mediator of the PTX-insensitive pathway.

The P2Y₆,R inhibition of ENaC: role of intracellular Cl⁻

As mentioned earlier, inhibition of ENaC activity hyperpolarises the epithelial cells, which, in turn, increases

It is known that P2Y₆,R may functionally couple with more than one G protein. For example, the effect of P2Y₂ receptor stimulation on the activation of phospholipase C (PLC) in human erythroleukemia cells was mediated by two distinct G proteins i.e., a PTX-sensitive G protein and the Gα₁₆. It is, therefore, possible that the signalling mechanism generated by the apical P2Y₆,R of FRT cells may be associated with more than one class of G protein. Interestingly, siRNA-mediated knockdown of expression of Gαq, Gα₁₁, Gα₁₄ and Gα₁₆ (Figure 2C) has no effect on the inhibitory effect of apical UTP on the activity of ENaC (Figure 2B). Thus, the PTX-insensitive component of the apical P2Y₆,R signalling system is not associated with Gq/11 or 16. Whether the PTX-insensitive G12 or G13 are involved in these mechanisms remains to be investigated.

G-protein βγ subunits play a role in the P2Y₆,R inhibition of ENaC

The free Gβγ subunits released from heterotrimeric G proteins subsequent to ligand stimulation of GPCRs can
Purinergic regulation of ENaC

Figure 3: Gβγ and PLCβ-4 are involved in mediating the effect of P2Y,R stimulation on ENaC. A (left panel): Relative I_{Na} of FRT monolayers in response to apical or basolateral UTP treatment. Cells were co-transfected with ENaC and an empty pSE vector (3 μg/ml) or c-myc tagged βARK in pSE vector (3 μg/ml). A (right panel): Immunoblot analysis showing expression of βARK detected by an antibody directed against c-myc. Expression of β-actin was used as a control. B (left panel): The effect of apical or basolateral UTP on the relative I_{Na} in FRT cells co-transfected with ENaC and scrambled siRNA or siRNA directed against PLC-β1, PLC-β2, PLC-β3 or PLC-β4. B (right panel): Immunoblot analysis of the effect of siRNAs on the expression of PLC-β isozymes. Expression of β-actin was used as a control. C: Effect of apical or basolateral UTP on the relative I_{Na} in ENaC transfected FRT cell monolayers treated with 1 μM PLC inhibitor U73122 or its inactive analogue U73343. * indicates p < 0.01.

the driving force for Cl− efflux across the apical membrane. Cl− secretion, however, cannot proceed effectively unless there is a pathway or pathways in the basolateral membrane that allows an influx of Cl− from the interstitium into the cell to supply necessary Cl− ions for secretion. Recent evidence indicates that the basolateral Cl− transport mechanism itself may influence the P2Y,R signalling mechanism that regulates activity of ENaC. The Na+/K+/2Cl− co-transporter is one of the basolateral Cl− transport mechanisms that play an important role in Cl− secretion. Inhibition of this co-transporter significantly attenuated nucleotide-induced Cl− secretion in the respiratory epithelium. An insight into the role of basolateral Cl− transport on purinergic regulation of ENaC came from a previous report that showed that the inhibitory effect of apical UTP on the activity of ENaC in mouse trachea was abolished if the concentration of Cl− in the extracellular fluid bathing both sides of the membrane is low. In our own studies, replacing all but 5 mM Cl− in the solution bathing the basolateral surface of monolayers of FRT cells expressing ENaC with gluconate inhibits the effect of apical and basolateral UTP on ENaC (Figure 4A). Conversely, depletion of Cl− from the apical bathing solution is without any effect on inhibition of ENaC by UTP. Moreover, the presence of bumetanide in the basolateral, but not in the apical, bathing solution inhibited the effect of apical UTP (Figure 4B). However, the effect of basolateral UTP on the activity of ENaC is not influenced by bumetanide (Figure 4B). Taken together, these data suggest that Cl− transport via the basolateral Na+/K+/2Cl− co-transporter is required for the apical P2Y,R to generate its inhibitory signal that regulates activity of ENaC.

Evidence has emerged from recent studies that, indeed, intracellular Cl− concentration ([Cl−]i) plays an important role in the regulation of the activity of ENaC in epithelia. The effect of [Cl−]i on ENaC was first described in our studies in isolated mouse mandibular duct cells. Subsequent studies reported a similar negative relationship between Cl− transport and [Cl−]i. The activity of ENaC in a variety of cell systems. This inhibitory effect of [Cl−]i may be of physiological important in the regulatory mechanism by which cystic fibrosis transmembrane conductance regulator (CFTR) downregulates activity of ENaC in epithelia. Using chloride-sensitive enhanced yellow fluorescent protein YFP on ENaC was first described in our studies in isolated mouse mandibular duct cells.

Conclusion

Our current data suggest that there are two distinct signalling pathways activated during P2Y,R stimulation which can inhibit activity of ENaC in epithelia (Figure 5). The first signalling pathway is associated with P2Y2,Rs in
L.M. O’Mullane, D.I. Cook & A. Dinudom

Figure 4. Extracellular Cl⁻ and bumetanide inhibits the effect of UTP on the activity of ENaC. Effect of apical and basolateral UTP on the relative Iamil in FRT cell monolayers transfected with ENaC. A: The apical or basolateral surface of FRT cell monolayers were perfused with a modified physiological solution containing 5 mM Cl⁻ for 10 minutes prior and during the presence of UTP and amiloride. B: Bumetanide (1 mM) was present in the apical or basolateral bathing solution 10 minutes prior to and in the presence of UTP and amiloride.* and ** indicates p < 0.05 and p < 0.01, respectively.

both the apical and basolateral membranes. This pathway involves the Gβγ subunits of the PTX-sensitive G protein, which are coupled with PLC-β4. The second signalling pathway is exclusive to apical P2Y₂R signalling and mediates approximately half of the inhibitory effect of P2Y₂R activation on ENaC. This pathway involves activation of the α-subunit of a PTX-insensitive G protein that is not Gq,11,14 or 16 and an unidentified PLC that is inhibited by U73122. The sensitivity of this second signalling mechanism to the presence of Cl⁻ in the extracellular fluid bathing the basolateral cell surface and to bumetanide suggest that the inhibitory effect of this PTX-insensitive signalling pathway on the activity of ENaC may depend on its ability to increase [Cl⁻] i concentration, which is known to be elevated by extracellular nucleotides. It has been previously suggested that attenuation of the activity of ENaC during P2Y₂R activation is caused by PLCβ-dependent reduction of membrane phosphatidylinositol 4,5-bisphosphate, a phospholipid that is essential for stabilising and maintaining activity of ENaC. An increase of [Cl⁻] i mediated by the PTX-insensitive signalling system, which is specific to apical P2Y₂R stimulation, may provide an additional inhibitory effect on the activity of ENaC allowing for extracellular nucleotides present in the luminal fluid to have a more potent effect on Na⁺ reabsorption.

Figure 5. Schematic diagram of the P2Y₂R signalling pathways that regulate the activity of ENaC. This model predicts the presence of a PTX-sensitive signalling system that is activated by apical and basolateral P2Y₂Rs and a PTX-insensitive signalling system that is specific to the apical P2Y₂R. The PTX-sensitive pathway involves the activation of Gβγ and PLCβ4. The PTX-insensitive pathway involves activation of Ga and a PLC that is sensitive to U73122, and there is also a role for Cl⁻ absorption via NKCC1. GPTX = pertussis toxin-sensitive G protein, Ga = α-subunit of G protein, Gβγ = Gβγ subunits of G protein, PLC = phospholipase C, NKCC1 = Na⁺/K⁺/2Cl⁻ co-transporter.

Acknowledgements

This work is supported by National Health and Medical Research Council of Australia grant 508086 and Australian Research Council grant DP0774320. Anuwat Dinudom is a National Health and Medical Research Council Senior Research Fellow. We appreciate comments on improving this manuscript from Craig Campbell.

References


36. Kolachala VL, Bajaj R, Chalasani M, Sitaraman SV.


Purinergic regulation of ENaC


Received 1 June 2009, in revised form 8 June 2009. Accepted 8 June 2009. © A. Dinudom, 2009

Author for correspondence:

Anuwat Dinudom,
Medical Foundation Building,
92-94 Parramatta Road,
Camperdown, NSW 2050,
Australia.
Tel: +61 2 9036 3314
Fax: +61 2 9036 3316
E-mail: anuwat@physiol.usyd.edu.au