Review

Rapid responses to steroid hormones: from frog skin to human colon. A homage to Hans Ussing

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Abstract

Fifty years ago, Hans Ussing described the mechanism by which ions are actively transported across frog skin. Since then, an enormous amount of effort has been invested in determining the cellular and molecular specifics of the transport mechanisms and their regulatory pathways. Ion transport in high-resistance epithelia is regulated by a variety of hormonal and non-hormonal factors. In vertebrates, steroid hormones such as mineralocorticoids, glucocorticoids and estrogens are major regulators of ion and water transport and hence are central to the control of extracellular fluid volume and blood pressure. Steroid hormones act through nuclear receptors to control the transcriptional activity of specific target genes, such as ion channels, ion transporters and ion pumps. These effects are observed after a latency of several hours and can last for days leading to cellular differentiation that allows a higher transport activity. This pathway is the so-called genomic phase. However, in the past 10 years, it has become apparent that steroid hormones can regulate electrolyte and water transport in tight epithelia independently of the transcription of these ion channels and transporters by regulating ion transporter activity in a non-genomic fashion via modulation of various signal transduction pathways. The molecular mechanisms underlying the steroid hormone-induced activation of signal transduction pathways such as protein kinase C (PKC), protein kinase A (PKA), intracellular calcium, intracellular pH and mitogen-activated protein kinases (MAPKs) and how non-genomic activation of these pathways influences epithelial ion transport will be discussed in this review.
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1. Introduction

Some of the first studies of rapid responses to steroid hormones in epithelia were performed on frog skin, which showed rapid (<1 min) activation of basolateral K^+ recycling and Na^+–H^+ exchange following exposure to aldosterone. These rapid 'non-genomic' effects are also observed in other Na^+- and Cl^--transporting epithelia such as human sweat gland and colon in response to physiological concentrations of aldosterone and estradiol. In this paper, we will describe the signal transduction pathways [Ca^{2+}, protein kinase C (PKC), protein kinase A (PKA), and mitogen-activated protein kinases (MAPKs)] involved in the rapid

responses to aldosterone and estradiol and discuss their physiological impact.

Understanding the relationship between rapid non-genomic effects of steroid hormones on intracellular second messengers and their subsequent cellular effects on ion transport targets requires an integrative approach in which both measurement of the cytosolic transduction processes and the macroscopic effects are determined simultaneously in whole tissue under physiological conditions. Aldosterone is the most important mineralocorticoid hormone released from the adrenal glands, regulating solute reabsorption in epithelia such as distal colon and kidney [1], acting in both a rapid and delayed manner to influence transport, that is, via both non-genomic and genomic pathways. Similarly, 17β-estradiol (E2), in addition to its primary functions to control the development of female reproductive organs, also regulates transepithelial transport by a rapid non-genomic mechanism [2,3]. Studies from our laboratory have demonstrated

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rapid (<1 min) non-genomic activation of PKC activity (PKCα for aldosterone, PKCα and PKCδ for E2), MAP kinase activity, a PKC-dependent Ca^{2+} entry and a PKCα-dependent effect on Na^{+}–H^{+} exchange and K^{+} recycling by mineralocorticoids and E2 in distal colon [2,4–8]. Rapid effects of E2 are female gender specific and insensitive to inhibitors of the classical estrogen receptor. Physiological concentrations of E2 reduced both basal and secretagogous-induced Cl^{-} secretion [3]. The antisecretory effect of E2 is sensitive to PKC inhibition, intracellular Ca^{2+} chelation, is female gender specific and insensitive to inhibitors of the classical ER. However, E2-induced activation of MAPK proceeds via an ER-dependent pathway. The primary ion transport target of the non-genomic signal transduction cascade elicited by aldosterone and E2 in epithelia is the Na^{+}–H^{+} exchanger. Both aldosterone and E2 produced a PKCα sensitive intracellular alkalinization within 1 min of hormone addition [8]. An increased intracellular pH up-regulates a basolateral ATP-dependent K^{+} channel, which is involved in K^{+} recycling to maintain an electrical driving force for Na^{+} absorption, while inhibiting a Ca^{2+}-dependent K^{+} channel, which generates the charge balance for Cl^{-} secretion. The non-genomic response to aldosterone and E2 in distal colon appears to enhance the capacity for absorption while down-regulating the potential for secretion [9]. These observations link rapid non-genomic activation of second messengers with a gender-specific physiological effect in the whole tissue. Aldosterone and E2 differ in their protein kinase signal transduction and both hormones stimulate specific PKC isoforms indicating both common and divergent signalling systems for salt-retaining steroid hormones [2,6,8]. The physiological function of non-genomic effects of aldosterone and E2 is to shift the balance from net secretion to net absorption in a pluripotential epithelium. In this review, we will focus initially on molecular mechanisms of steroid hormone-induced activation of PKC isoforms and MAPK before discussing the rapid effects of these hormones on ion transport in epithelial cells.

2. Rapid effects of steroid hormones on signal transduction pathways

2.1. Rapid action of steroid hormones on PKC

PKC comprises a large family of proteins with multiple isoforms—which can be divided into three subgroups, classical PKC (cPKC) isoforms (α, $\beta1$, $\beta2$, γ), novel PKC (nPKC) isoforms (δ, ε, η, θ, μ) and atypical PKC (aPKC) isoforms (ζ, λ), based on different activator/cofactor requirements. The various PKC isoforms exhibit subtly different kinetics, specific patterns of tissue distribution and intracellular localisation, differing modes of activation and distinct behaviour during the cellular response [10]. Many studies have been performed to identify specific roles for each PKC isoform. It has been proposed that some

isoforms may play a role in regulating membrane functions such as receptor down-regulation, modulation of ion channels and pumps and membrane phospholipid hydrolysis leading to the generation of lipid mediators [10].

Rapid non-genomic stimulation of PKC activity has been demonstrated by various steroid hormones and in numerous cell types—aldosterone [4,5,11], E2 [4,12], glucocorticoids [13], adrenal androgen—dehydroepiandrosterone [14], $1,25(OH)_2$ vitamin D_3 [15]. Similarly, many rapid steroid hormone effects are mediated by PKC, for example, aldosterone [4,5,7,16–18], estradiol E2 [3,6,12,19–21], $1,25(OH)_2$ vitamin D_3 [15].

Studies from various laboratories have also demonstrated direct binding and activation of different PKC isoforms by a variety of steroid hormones. Slater et al. [22] and Stubbs et al. [23] observed direct activation of rat brain PKC (α, β and γ) and PKC-α, -γ and -ε isoforms by $1,25(OH)_2$ vitamin D_3 at physiological concentrations. Results of these studies demonstrated that $1,25(OH)_2$ vitamin D_3 binds to PKC at the activator binding site and induces PKC activity in a manner resembling the effect of DAG and phorbol esters. The adrenal androgen, dehydroepiandrosterone, has also been shown to bind to purified PKC from rat brain, containing PKC α, β and γ isoforms and also to atypical PKCζ [14]. Similar results have been observed with glucocorticoids and in these studies, binding of the steroid to the regulatory subunit of PKC was demonstrated [24,25]. Doolan et al. [6] have also demonstrated direct and specific activation of human recombinant PKCα by physiological concentrations of aldosterone and E2 (Fig. 1). Estradiol also directly and specifically stimulated PKC-δ activity (Fig 1). Current evidence therefore, points to PKC isoforms as non-genomic receptors for rapid responses to steroids [6,22,23].

2.2. Rapid non-genomic effects of mineralocorticoids on PKC activity

Aldosterone has been shown to exert rapid non-genomic effects on PKC activity in vascular smooth muscle cells (VSMCs) [11], mammalian distal colonic epithelium [4,5] and a DAG-dependent PKC activation in cultured kidney cells [17]. In contrast, in neonatal rat cardiomyocytes, aldosterone rapidly represses PKC [26].

Various studies indicate that aldosterone non-genomically activates the Ca^{2+}-sensitive PKC isoform, PKCα. In VSMCs, aldosterone induced a rapid activation and translocation of PKCα from cytosolic to membrane fractions [11]. Doolan et al. [6] have demonstrated a direct and specific activation of human recombinant PKCα, by physiological concentrations of aldosterone, in a cell-free assay system (Fig. 1). However, no stimulatory effect of other PKC isoforms known to be present in distal colonic epithelium, PKCδ, PKCε and PKCζ [27], was observed in the presence of aldosterone (0.01–10 nM). The results of this study point to the PKCα isoform as a candidate non-genomic receptor for rapid responses to aldosterone.
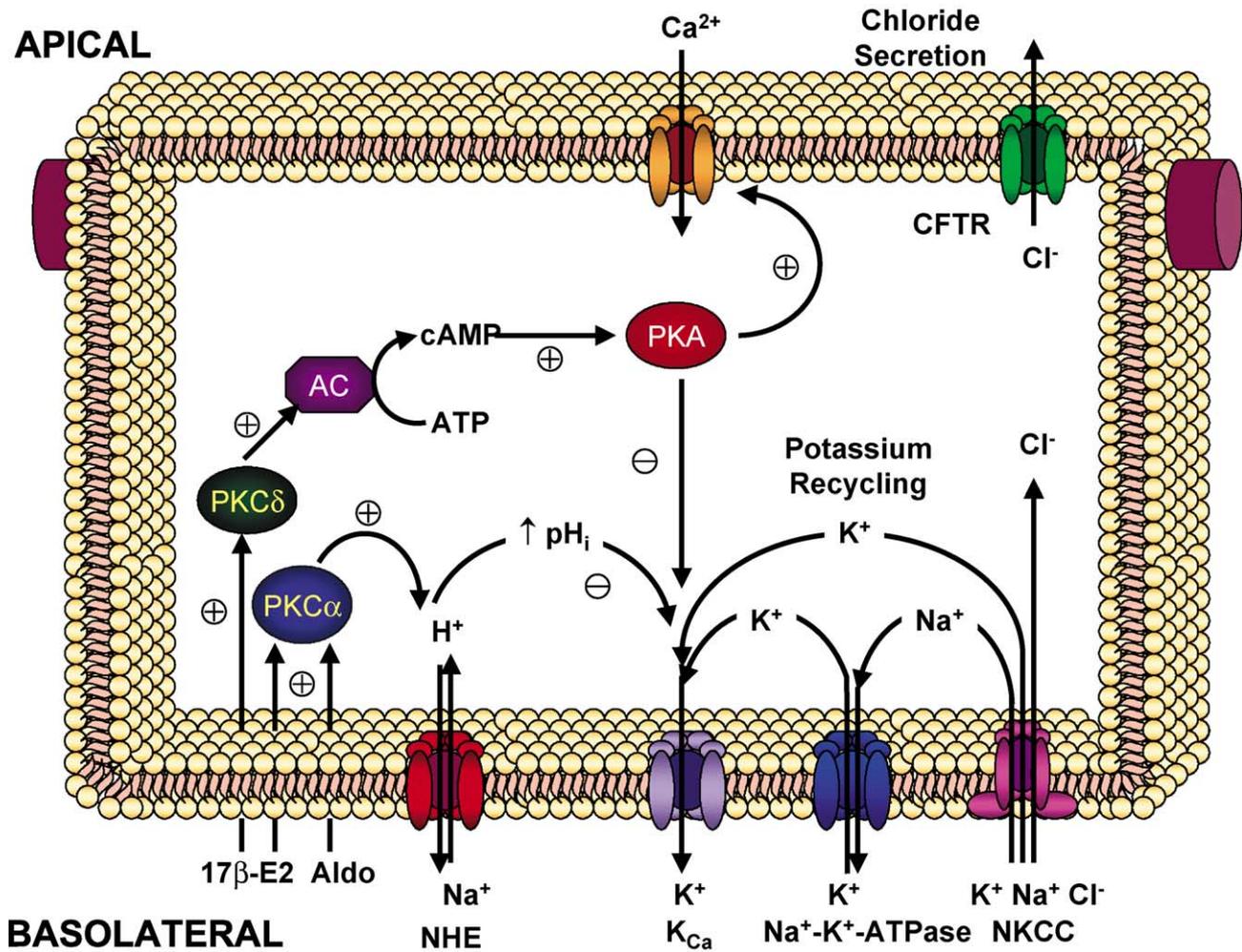
Fig. 1. Signal transduction pathways for non-genomic action of steroid hormones. Both aldosterone and estradiol directly stimulate PKCα activity resulting in activation of NHE and intracellular alkalinization followed by an inhibition of K_{Ca} channel activity. Estradiol directly and specifically stimulates PKCδ activity. PKCδ activates adenylyl cyclase activity resulting in an increase in PKA activity, followed by an inhibition of K_{Ca} channel activity. Both PKCδ and PKA are required for calcium entry. Inhibition of K_{Ca} channels reduces potassium recycling in the basolateral membrane reducing the driving force for chloride secretion.

Many rapid non-genomic aldosterone effects are mediated by PKC, for example, stimulation of human colonic K_{ATP} channel and Na^+–H^+ exchanger [7], activation of Na^+–H^+ exchanger in VSMCs [16], stimulation of H^+ conductive influx in renal epithelial cells [17] and increases in $[Ca^{2+}]_i$ in T84 colonic epithelial cells, mammalian distal colonic epithelium and M-1 cortical collecting duct cells [4,5,18,28] (Fig. 1).

Conflicting reports exist as to the rapid effects of aldosterone on intracellular cAMP levels and PKA activity. Christ et al. [29] have demonstrated a 2-fold increase in intracellular cAMP levels in VSMCs at physiological aldosterone concentrations. In contrast, however, no stimulation of the PKA pathway was observed by aldosterone in rat distal colonic epithelium [6].

Rapid effects of aldosterone on free intracellular calcium $[Ca^{2+}]_i$ have been demonstrated in VSMCs, porcine aortic endothelial cells [30,31], T84 colonic epithelial cells, human and rat distal colonic epithelium [4,5,18] and M-1 cortical collecting duct cells [28] with the Ca^{2+} source differing between various cell types. These rapid responses appear to be mineralocorticoid specific since glucocorticoid hormones (hydrocortisone, dexamethasone) either failed to produce a response or exhibited a very small response at supraphysiological doses.

2.3. Interaction between PKC and estrogens

The first direct evidence that E2 modulates PKC was obtained by Drouva et al. [32]. This study demonstrated that estradiol implants in ovariectomised rats significantly increased Ca^{2+} and phospholipid-dependent PKC in both soluble and particulate fractions of anterior pituitary. The stereoisomer 17α-estradiol was without effect. Recent stud-

ies have demonstrated an up-regulation of PKCδ expression in the rat and rabbit corpus luteum by estrogen [33–35]. In contrast, however, E2 treatment of the MCF-7 breast cancer cell line resulted in a significant decrease in PKCδ protein and mRNA expression in a time- and dose-dependent manner [36]. These authors suggest that the differential ability of E2 to regulate PKCδ expression could be a result of differential signalling through either ERα or ERβ. Lahooti et al. [37] report that PKCδ participates in the signalling pathways that lead to ER phosphorylation. These studies therefore indicate a role for PKC in the biological response to estrogen.

One of the earliest reports of rapid non-genomic effects of estradiol was in 1969 by Szego and Davis [38]. In this study, estrogen treatment of rats in vitro resulted in an acute, very rapid elevation of uterine cAMP. Estradiol has also been shown to exhibit rapid non-genomic effects in various cell types on (i) adenylyl cyclase activity and cAMP production in rat pulmonary VSMCs, human coronary artery endothelium and rat uterine cells and MCF-7 cells [39–41], (ii) PKA activity in muscle and mammalian distal colon [6,42], (iii) PKC activity in mammalian distal colon and chondrocytes [4,5,12,43], (iv) Ca^{2+} (with the Ca^{2+} sources differing in various cell types) in female rat osteoblasts, chicken and pig granulosa cells, single rat hepatocytes, mammalian distal colonic crypts and rat duodenal enterocytes, [6,20,44–47] and in human monocytes via the activation of an estrogen surface receptor [48], and (v) targeting ion transport pathways such as pumps and channels in mammalian distal colonic epithelium [3,9,49]. Both PKC and PKA have been shown to play a role in E2-dependent Ca^{2+} responses in many tissue types such as duodenal enterocytes, mammalian distal colon and primary rabbit proximal tubule cells [6,20,21,47] indicating multiple signalling pathways which influence E2-dependent changes in $[Ca^{2+}]_i$.

E2 has been shown to activate specific PKC isoforms in a non-genomic manner. In both growth zone and resting zone chondrocytes, E2 rapidly activated and translocated PKCα from cytosolic to membrane fractions. The stereoisomer, 17α-estradiol, was without effect [43]. E2 has also been shown to directly and specifically stimulate PKCδ activity in a cell-free assay system (Fig. 1). Other steroid hormones such as aldosterone and 17α-estradiol were without effect on PKCδ activity. E2 also rapidly and directly activated PKCα activity in a concentration-dependent manner. However, E2 exhibited no stimulatory effect on other PKC isoforms known to be present in distal colonic epithelium, PKCε and PKCζ [2,6]. Studies from our laboratory have also demonstrated an E2-induced PKCδ-specific activation of PKA activity in rat distal colonic epithelium (Fig. 1). Therefore, the cytosolic protein responsible for rapid non-genomic E2-PKA activation is the Ca^{2+}-independent, phospholipid-dependent PKCδ isoform [2,6]. The results of this study point to the PKCδ isoform as a candidate non-genomic receptor for rapid responses to E2.

2.4. Gender-specific rapid non-genomic E2 effects

Studies from many laboratories have demonstrated gender-specific non-genomic responses to E2. Sylvia et al. [43] have shown that E2 stimulated PKC activity in a sex-dependent manner in rat costochondral chondrocyte cultures. Studies from our laboratory have shown E2-stimulated, PKC-sensitive female gender-specific effects on PKA activity, intracellular Ca^{2+} concentration, intracellular pH and forskolin-induced chloride secretion in rat distal colonic epithelium [3,6,8]. The reason for this gender specificity is as yet unknown. One possible explanation is that there may be different PKC isoform distribution between male and female tissue. Studies performed in rat liver have in fact shown both gender specific and developmental differences in PKC isoform expression with levels of both PKCα and PKCδ being higher in females vs. males [50].

2.5. Common and divergent signalling pathways for rapid effects of aldosterone and estradiol

Aldosterone and E2 differ in their protein kinase signal transduction and both hormones stimulate specific PKC isoforms. Isozyme-specific properties such as substrate specificities, subcellular localisation, activation requirements and rates of down-modulation suggest that different PKC isoforms may perform unique cellular functions. Initially, there appeared to be a common stimulatory signalling pathway for rapid non-genomic responses to aldosterone and estradiol, that is, PKC. Our recent results [6] now suggest that these steroid hormones stimulate different PKC isozymes with aldosterone stimulating PKCα selectively and estradiol stimulating both PKCα and PKCδ. These properties confer both common and divergent signalling pathways for salt-retaining steroid hormones [2,6,8] (Fig. 1).

2.6. Rapid non-genomic activation of extracellular-regulated kinase (Erk) 1/2 by 17β-estradiol

Mitogen-activated protein (MAP) kinases are serine/threonine kinases that become activated in response to a diverse array of extracellular stimuli including growth factors, cytokines, UV irradiation and shear stress [51]. They exert their effects by phosphorylating a wide variety of cytoplasmic targets and transcription factors. MAPKs can be subdivided into about five families, three of which, Erk, jun N-terminal kinase (Jnk) and p38 kinase, have been very well characterised. In general, mitogenic stimuli such as growth factors activate the Erk family of MAPKs (composed of two closely related isoforms Erk1 and Erk2) while stressful stimuli usually activate Jnk and p38 MAPKs. All MAPKs are the final component of a highly conserved module of three kinases, known as a MAPK pathway, which are sequentially activated by phosphorylation [51]. There exists a degree of cross-talk between the previously mentioned PKC family of signalling proteins and the MAPK

pathway. Specifically, it has been shown that the PKCα isoform phosphorylates and activates Raf-1, which in turn leads to downstream activation of Erk1/2 [51]. Similarly, a recent study has demonstrated that activation of PKCδ leads to phosphorylation/activation of Erk1/2 in intestinal epithelial cells [52].

It has been well established that 17β-estradiol triggers a rapid (1–10 min) but transient activation of Erk1/2 in a large number of different cell types and tissues [53–57]. In our laboratory, we have shown that in vitro treatment of rat colonic epithelia with physiological concentrations of the hormone can stimulate a transient activation of Erk1/2 within 1 min (unpublished data). It has also been shown previously that E2 rapidly activates Erk1/2 in Caco-2 cells, a human colon carcinoma cell line [58]. Therefore, it is clear that Erk1/2 in intestinal epithelial cells is responsive to treatment with estrogen. An exception to the observation that E2 induces activation of Erk1/2 is VSMCs. In these cells, hormone treatment actually causes a reduction in basal levels of Erk1/2 and E2 also acts to inhibit Erk1/2 activation induced by serum treatment [59]. A possible reason for these variable effects of E2 on Erk1/2 is discussed below.

2.7. Molecular mechanisms underlying the modulation of Erk1/2 activity by 17β-estradiol

By employing ER antagonists such as ICI 182,780, the majority of studies have found that the classical ER is necessary for hormone-induced activation [53–57]. In our experiments on isolated rat colonic epithelia (unpublished results) and on Caco-2 cells [58], ICI 182,780 inhibits estrogen-dependent activation of Erk1/2, suggesting a key role for ER. There is evidence in the literature demonstrating the expression of functional estrogen receptors in normal intestinal epithelia. Thomas et al. [60] were the first to conclusively show that epithelial mucosa from male and female rat duodenum, jejunum, mid-ileum and colon all express ERα. While they were unable to identify if crypt or villar epithelial cells from these tissue samples both express ERα, they demonstrated that IEC-6 cells, which are derived from small intestinal crypts cells, express ERα and that 17β-estradiol induces rapid up-regulation of c-fos in IEC-6 cells [60]. This strongly suggests that, in vivo, pluripotential crypt cells, which undergo replication and differentiation into secretory and absorptive epithelial cells in the villus, are responsive to E2. In a more recent study of normal human colonic mucosa, expression of ERα and ERβ was observed, with ERβ levels in greater abundance than ERα [61]. Immunolocalization experiments also showed ERβ staining in superficial epithelial cells, whereas crypt cells were negative [61].

Because E2 triggers such a rapid activation of Erk1/2 in so-varied cell types, many researchers have postulated the existence of a membrane receptor to which the hormone binds resulting in a signal transduction process that causes the activation of the MAPK pathway. In support of this

hypothesis, several groups have shown that the membrane-impermeable conjugate, E2-BSA, is capable of rapidly activating signalling pathways [62–64]. On a cautionary note, however, it has been reported that some preparations of the conjugate may be contaminated with free hormone and in addition, experiments have shown that purified E2-BSA does not bind ER in vitro. This would suggest that the conjugate does not mimic the biological hormone [65].

The requirement for ER participation in Erk1/2 activation has led to the hypothesis that the ER itself may be localised at or near the plasma membrane and be involved in the signal transduction process. In support of this, Razandi et al. [66] have shown that overexpression of both ERα and ERβ in CHO cells enables E2 to activate a number of signal transduction pathways in these cells, including the MAPK pathway. Moreover, highly purified preparations of plasma membrane from the transfected cells contain significant quantities of ER as determined by Western blotting [66]. In addition, immunocytochemical staining of cells with anti-ER antibodies shows strong binding to plasma membranes [67]. In a recent experiment on MCF-7 cells, a breast cancer cell line, treatment with E2 increased anti-ER staining at the plasma membrane suggesting that hormone treatment may cause a translocation of ER to the cell membrane [68].

Because neither ERα nor ERβ possesses hydrophobic transmembrane helices necessary for insertion in the lipid bilayer of the plasma membrane, the receptor may actually associate with integral plasma membrane proteins. Caveolae are specialized cholesterol-rich plasma membrane microdomains that compartmentalize signal transduction molecules such as membrane receptors and signalling proteins. They also contain scaffolding proteins that belong to a family of proteins known as caveolins that associate with these signal-modulating proteins [69]. Caveolin-1 and -2 are ubiquitous while caveolin-3 is restricted to muscle cells. It has recently been established that ER can associate with either caveolin-1 or -2 in a number of cell types, thereby localising ER to the caveolar microdomains in close proximity with signalling molecules [70,71]. The effect of E2 on the association between ER and caveolins is cell-type dependent, however. In VSMCs, there is little association between ER and caveolins in the absence of estrogen but hormone treatment triggers a 3-fold increase in association. The situation in MCF-7 cells is reversed, with hormone treatment reducing the strong interaction between ER and caveolins present in unstimulated cells [71]. These opposing effects of 17β-estradiol on caveolin/ER association may explain why the hormone activates Erk1/2 in MCF-7 cells and inhibits it in VSMCs. Caveolins, while crucial for assembling signalling complexes, also act to inhibit signal activation and their dissociation from the signalling complex, as occurs in MCF-7 cells upon E2 treatment, is necessary for activation of Erk1/2 to occur. Because the hormone increases the association of caveolin and ER in VSMCs, signal transduction to Erk1/2 is suppressed [71]. Supporting the proposed role of caveolins in assembling

signalling complexes, overexpression of caveolin-1 in MCF-7 cells induces an increased translocation of ER to the cell surface [71].

Normal intestinal epithelial cells and Caco-2 cells express either very little or no caveolin-1 and caveolin-2 and rarely exhibit classic plasmalemmal caveolae [72,73]. However, a recent study using immunocytochemical analysis has determined that in normal intestinal epithelia cells and in T84 cells, an intestinal carcinoma cell line, caveolin-1 staining was most likely localised mainly to the basolateral plasma membrane [73], the region of the cell that steroid hormones would encounter in vivo. While it remains to be investigated, it seems likely that ER may associate with caveolins at the basolateral membrane of intestinal cells. While caveolins associate with ER and modulate non-genomic actions of E2 on Erk1/2, they are not signalling molecules. However, the localisation of ER in caveolar structures would permit its association with signalling proteins situated here. A small number of candidate proteins have been investigated to date. Src kinase, a tyrosine kinase normally tethered to the cytoplasmic side of the plasma membrane, is a key signalling molecule which upon activation leads to the stimulation of MAPK pathways in many cell types [74]. Migliaccio et al. [53] have published a number of key papers investigating the role of Src kinase in E2-induced activation of Erk1/2. First, they showed that E2 treatment of MCF-7 cells triggers a very rapid activation of Src and that pharmacological inhibition of Src activity blocks the activation of Erk1/2 suggesting that Src kinase lies upstream of Erk1/2. The same group also demonstrated that Erk1/2 activation by estrogen in Caco-2 cells also requires prior activation of Src [58]. Second, they demonstrated that E2 treatment promotes a physical interaction between Src and both ERα and ERβ in MCF-7 cells resulting in the activation of Src kinase [75]. Mutagenesis studies revealed that phosphotyrosine 537 in the ligand binding domain of ER is crucial for ER association with Src and Src activation by E2 [75]. This implies that the SH2 domain of Src binds to this phosphotyrosine residue on ER perhaps as a result of a conformational change in the ER triggered by hormone binding. More recently, another group has confirmed these findings and has further shown that the ligand binding domain of ER alone is sufficient to confer responsiveness to E2 [76]. Src kinase can activate the MAPK pathway via the adaptor protein Shc. Shc binds to active Src and acts as a docking site for Grb2 and Sos which in turn trigger Ras activation leading to the activation of the MAPK pathway [51]. In a recent paper, it was claimed that E2 rapidly induces the physical association between ERα and Shc in MCF-7 cells [68]. This would suggest the ER can physically interact with both Src and Shc when these signalling molecules form a complex.

Another putative ER binding protein is the p85 subunit of phosphatidylinositol 3-OH kinase (PI3K). This signalling molecule is classically activated by insulin growth factor-1 (IGF-1) binding to its receptor and has numerous downstream targets involved in cell proliferation including protein kinase B (or Akt) and in some cell types, the MAPK pathway [77]. Recently, it has been shown that E2 rapidly activates Akt in VSMCs in a PI3K-dependent manner [78]. Moreover, hormone treatment triggers an association between the catalytic subunit (p85) of PI3K and ERα but not ERβ [78]. Another report also describes the E2-dependent association between ERα (but not ERβ) and the IGF-1 receptor which results in the activation of the IGF-1 receptor [79]. The Castoria et al. laboratory has very recently clarified the association of ER with both Src and PI3K in MCF-7 cells [80]. The Src inhibitor, PP1, blocks 17β-estradiol-induced activation of PI3K, while in turn the PI3K inhibitor, LY294002, blocks the Src activation. Moreover, they show that, while ER can form complexes with both signalling molecules in response to hormone treatment, these binary complexes are weak and easily dissociate. However, in vitro, a ternary complex of ER, Src and PI3K induced by E2 is much stronger suggesting that in vivo, the hormone will trigger a rapid association of all three leading to a strong activation of both Src and PI3K (Fig. 2) [80].

2.8. Biological consequences of 17β-estradiol-induced activation of Erk1/2

As mentioned previously, Erk1/2 has a wide variety of cytoplasmic and nuclear targets, including a number of transcription factors which Erk1/2 can activate by phosphorylation. To date, it has been shown that E2 is capable of activating the transcription factors Elk-1, and as a result c-*fos*, in MCF-7 cells, Egr-1 in cardiomyocytes and CREB in adipocytes [57,81,82]. In each of these cases, Erk1/2 stimulation is required for hormone-induced activation of the transcription factors. Once activated, these factors can regulate the transcription of other genes; this allows estrogen to modulate the expression of genes that do not contain classical estrogen response elements (ERE), thus increasing the repertoire of biological effects of the sex hormone (Fig. 2).

A second biological consequence of rapid activation of Erk1/2 by E2 may be a modulation of the classical genomic action of the hormone. Both ERs are also phosphoproteins and to date, five phosphorylation sites, Ser104, Ser106, Ser118 Ser167 and Tyr537 have been located on human ERα (Ref. [83] and references therein). It has been suggested that the phosphorylation of these residues promotes the recruitment of co-activator proteins stimulating increased transcription [84]. Growth factor (EGF and IGF-1)-induced stimulation of the MAPK pathway triggers phosphorylation of Ser118 and Ser167 on ERα leading to stimulation of transcriptional activation [85,86]. Recently, it has been shown that growth factor activation of MAPK pathway also results in the phosphorylation of SRC-1, AIB1 and GRIP1, members of the SRC/p160 family of steroid co-activators resulting in an increased rate of ER transcriptional activity [87–89]. Because E2, itself, is capable of activating
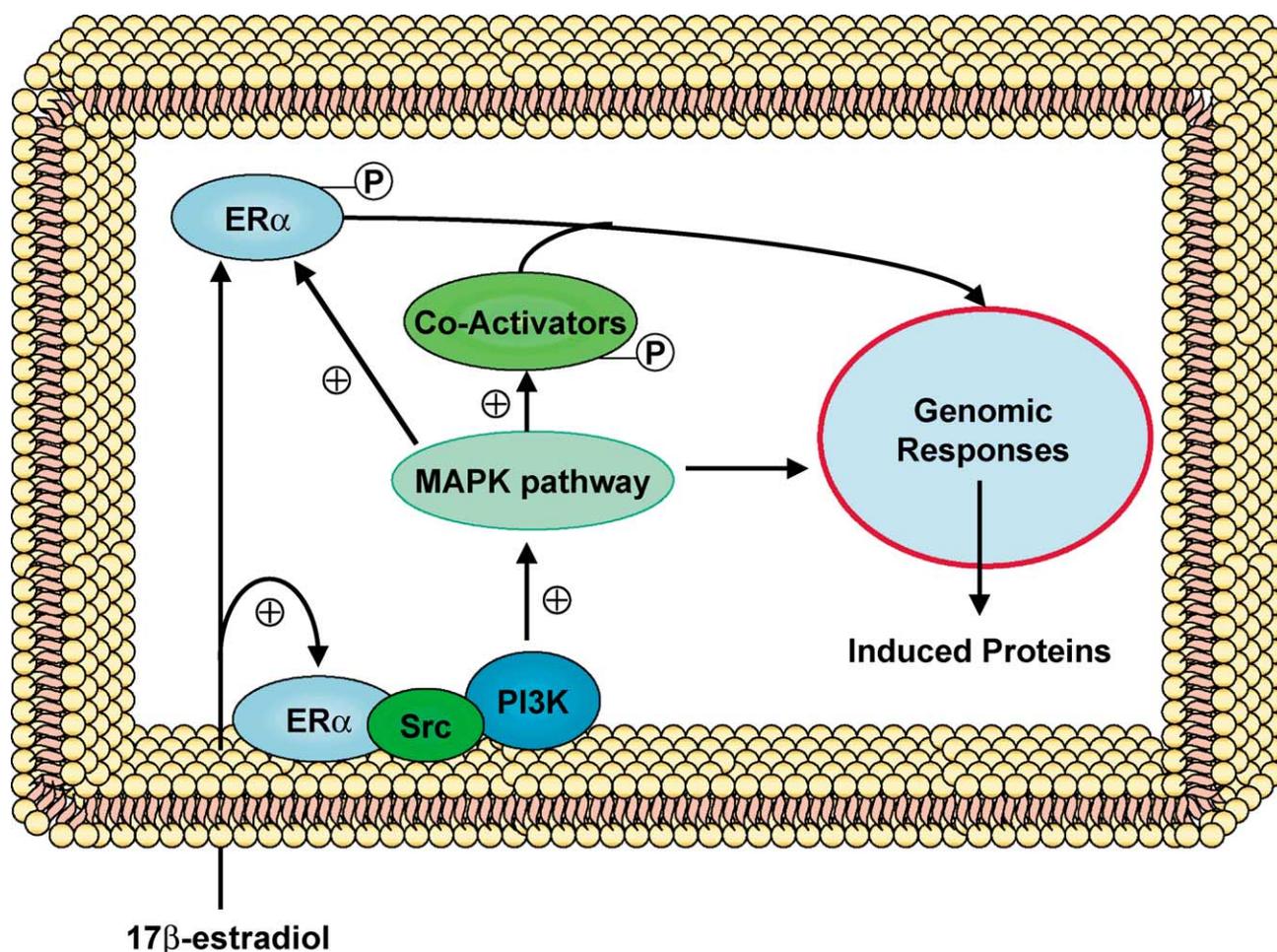
Fig. 2. Localisation of estrogen receptor (ERα) at the plasma membrane is required for rapid non-genomic activation of MAPK pathway. 17β-Estradiol binding to ERα triggers an association with the signalling proteins Src kinase and PI3K at the plasma membrane. The formation of this complex has been shown to activate signal transduction leading to stimulation of MAPK activity. A number of transcription factors are phosphorylated and activated by MAPK resulting in the modulation of transcriptional events. While it has not yet been shown conclusively, hormone-induced activation of MAPK may also lead to phosphorylation of ERα and a number of steroid co-activator proteins. The effect of this should be to enhance the genomic effects of ERα.

the MAPK pathway, it might be expected that hormone induction of Erk1/2 may also result in similar phosphorylation events. There have in fact been some early reports of rapid increases in phosphorylation of the ER in some cell types following treatment with estrogen [90]. This rapid phosphorylation should have the effect of priming the genomic response by increasing the transcriptional activity of the receptor. However, a detailed investigation into estrogen-induced phosphorylation of both ER and the family of steroid co-activators and its effect on rates of transcription has not yet been performed (Fig. 2).

3. Na⁺–H⁺ exchanger: a target for rapid responses to steroid hormones

3.1. Structure and function of Na⁺–H⁺ exchangers

The process of $Na^+–H^+$ exchange was first demonstrated in intestinal and renal brush border vesicles in 1976 [91].

However, it was not until 1989 that the first gene encoding a $Na^+–H^+$ exchanger (NHE) was identified and cloned [92]. To date, six mammalian isoforms of the $Na^+–H^+$ exchanger (NHE1 to NHE6) have been identified. The isoforms share a similar hydropathy profile which indicates the existence of two major structural domains: a hydrophobic transmembranous N terminal responsible for catalysing Na^+ and H^+ exchange and a more variable hydrophilic C terminal involved in regulation of the activity and subcellular distributions of the exchangers [93]. NHE1 is ubiquitously expressed at the basolateral membrane and plays a housekeeping role in intracellular pH (pHi) and cell volume regulation. In contrast, NHE2 to NHE6 have more limited tissue distribution and specialized functions. NHE2 to NHE4 are abundant in epithelial cells of kidney, intestine and stomach, whereas NHE2 and NHE3 are apically localised and NHE4 is mainly basolateral. NHE5 is primarily expressed in brain, spleen and testis. The most recently identified isoform, NHE6, is only localised in mitochondria. The antiporter, under normal conditions, catalyses the elec-

troneutral exchange of one extracellular Na^+ for one intracellular H^+. Na^+–H^+ exchange activity is essential for pH homeostasis, cell volume regulation, transepithelial ion and water transport, and plays a role in cell proliferation and adhesion (see Ref. [94] for review).

3.2. Activation of NHE by steroid hormones

Steroid hormones have been shown to activate the Na^+–H^+ exchanger in a wide variety of tissues including epithelia, vascular smooth muscle and lymphocytes. Stimulation of the Na^+–H^+ exchanger can influence the activity of other ionic transporters, which in turn determine cell volume, secretion and absorption. This section of the review will focus on the effects of aldosterone and estrogen on the activity of the Na^+–H^+ exchanger.

It has long been known that hyperaldosteronism, resulting in salt deprivation, stimulates Na^+ absorption as well as K^+ secretion, particularly in the late segments of the kidney tubules and intestine [1]. These actions of aldosterone are mediated by genomic—as well as non-genomic—pathways and in distal nephron and intestine affect the electrogenic transport pathways for Na^+ absorption and K^+ secretion. Turnamian and Binder [95] have also demonstrated an increase in electroneutral NaCl absorption, 1 day after aldosterone administration, in rat proximal and distal colon. The same effects of aldosterone have been observed in rat proximal colon [96]. Using specific inhibitors of the different Na^+–H^+ exchanger isoforms, these authors have shown that aldosterone stimulates Na^+–H^+ exchange by increasing expression of NHE3 specifically. As NHE3 is known to be the isoform involved in Na^+ absorption, they concluded that aldosterone regulates Na^+ transepithelial movement in rat proximal colon via activation of NHE3. It has been shown, in VSMCs, that the genomic activation of the Na^+–H^+ antiporter was preceded by a rapid non-genomic activation. This rapid activation was not blocked by inhibitors of de novo protein synthesis, whereas the genomic effects, occurring after 24 h, were suppressed [16]. These and other studies have linked the nongenomic and genomic actions of aldosterone and have suggested a two-step model for aldosterone effects.

Moura and Worcel [97] were the first to suggest a nongenomic mineralocorticoid action by demonstrating an increase of transmembrane Na^+ flux in tail artery of adrenalectomized rats 15 min after injection of aldosterone. This response was not suppressed by inhibitors of transcription and translation. Since this study, rapid actions of aldosterone on Na^+–H^+ exchange have been described in a wide variety of cell types. Aldosterone activates the Na^+–H^+ exchanger resulting in an increase in intracellular pHi in frog kidney diluting segment [98]. As this stimulation was prevented by the mineralocorticoid receptor antagonist, spirolactone, and inhibitors of transcription and translation, the authors concluded that this action was mediated by the genomic pathway. Recent studies from our laboratory have demonstrated rapid (<1 min) nongenomic activation of Na^+–H^+ ex-

changer in human and rat distal colon and sweat gland, which was not affected by spirolactone [99,100]. Similar results were obtained in both VSMCs and human mononuclear leukocytes [101,102]. Rapid effects of aldosterone on the Na^+–H^+ exchanger have been widely characterised in Madin–Darby canine kidney cells (MDCK). Gekle et al. [103] have shown that aldosterone initially activates a plasma membrane proton (H^+) conductance, which in turn activates the Na^+–H^+ exchanger. These authors have proposed that the aldosterone-sensitive H^+ conductance maintains Na^+–H^+ exchange activity by providing an acidic environment in the vicinity of the exchanger.

The most widely studied nongenomic effects of estradiol are its action on intracellular Ca^{2+} and cAMP levels and on PKC and MAPK activation with non-genomic effects of estradiol on the Na^+–H^+ exchanger not being extensively documented. E2 is essential for male fertility by enhancing fluid reabsorption for concentrating sperm in the head of the epididymis. Studies have shown that this action is due to the regulation by E2 of the Na^+–H^+ exchanger isoform 3 (NHE3) and the rate of Na^+ transport was sensitive to an NHE3 inhibitor [104]. These effects are non-genomic as they could not be inhibited by the classical estrogen receptor antagonist, tamoxifen, or by inhibitors of transcription and translation. In MCF7 cells, E2 up-regulates expression of the Na^+–H^+ exchanger regulatory factor NHE-RF. NHE-RF is a protein co-factor essential for PKA-mediated inhibition of NHE3 and links NHE3 to the actin cytoskeleton. As NHE-RF plays a critical role in endocytosis, internal trafficking and targeting membrane proteins to the apical membrane, estradiol regulates NHE3 activity by controlling the number of exchangers at the plasma membrane [105]. An early report demonstrated a rapid activation of Na^+–H^+ exchange by E2 in rat pituitary cells with the responses being insensitive to classical ER antagonists and inhibitors of transcription and translation [106]. Recent studies from our laboratory have demonstrated an increase in intracellular pHi within 1 min after aldosterone and estradiol addition in rat female distal colonic crypts. Inhibition of the alkalinization by inhibitors of the Na^+–H^+ exchanger, amiloride and EIPA, demonstrated that these steroids act on the Na^+–H^+ exchanger [8,107].

In our laboratory, the mechanisms of activation of the Na^+–H^+ exchanger by aldosterone and E2 have been investigated. Our studies have shown that the effects of these steroid hormones on the antiporter are both mineralocorticoid and sex steroid hormone specific. The mineralocorticoid, fludrocortisone, and the glucocorticoids, dexamethasone and hydrocortisone, did not activate the Na^+–H^+ exchanger. Similarly, the sex-steroid hormones, 17α-estradiol, progesterone and testosterone did not induce an alkalinization. Moreover, the stimulation of the antiporter by E2 is gender specific. We have shown that the effects of estradiol could only be observed in female rat distal colon. In contrast, aldosterone activates the exchanger in both sexes.

Aldosterone and E2 require PKCα to activate the Na^+-H^+ exchanger [8] (Fig. 1). We have shown that pre-incubation with the PKCα inhibitor, HBDDE blocks the hormone-induced activation of the antiporter. Gekle et al. [108] have shown that non-genomic activation of the Na^+-H^+ exchanger by aldosterone involves the MAPK pathway in MDCK cells. The aldosterone-induced stimulation of the Na^+-H^+ exchanger activity was prevented by the two specific inhibitors of Erk1/2 activation, PD98059 and U0126. Mechanisms, which link the stimulation of Erk1/2 to Na^+-H^+ exchanger activation in MDCK cells, are not yet known.

In pluripotential epithelia, such as intestinal tract, kidney and sweat gland, intracellular alkalinization, following activation of the Na^+-H^+ exchanger, affects transepithelial ion and water transport (Fig. 1). The Na^+-H^+ exchanger is therefore a primary ion transport target of the non-genomic signal transduction cascade induced by aldosterone and E2 (see Ref. [2] for review). The consequences of its activation on transepithelial ion and water transport are discussed in the subsequent section.

4. Non-genomic effects of steroid hormones on ion transport in epithelia

The mineralocorticoid hormone aldosterone has been identified as one of the major regulators in body fluid, electrolyte, and pH homeostasis [109,110]. Classically, aldosterone exerts its action on high-resistance epithelia, such as kidney, distal colon, sweat and salivary glands, by increasing the expression of epithelial sodium ion channels (ENaC), potassium ion channels and Na^+-K^+-ATPases. Sodium ion transport across the tight epithelia of reabsorbing tissues is the major factor determining total-body Na^+ and fluid levels, and thus, long-term control of blood pressure [111]. However, upon treating target epithelia with aldosterone, the beginning of the physiological response precedes the accumulation of induced proteins, indicating that there must be an earlier "regulatory" phase of aldosterone action that precedes the genomic phase.

4.1. Kidney

Regulation of sodium re-absorption in the kidney occurs mostly in the distal nephron where re-absorption is a two-step process as originally described by Ussing for frog skin. Sodium enters renal cells from the luminal compartment through ENaC in the apical membrane before it is actively transported out of the cell by the basolateral Na^+-K^+-ATPase. ENaC is usually the rate-limiting step for Na^+ transport and, therefore, a key target in aldosterone action. Classically, because of the observation that mRNA transcription and protein synthesis are required for aldosterone to increase sodium transport, it was hypothesized that aldosterone induces ENaC synthesis and insertion in the apical membrane. While the evidence from electrophysio-

logical measurements remains somewhat controversial [112–114], most of the available biochemical evidence suggests that the number of sodium channel proteins in the apical membrane does not change after aldosterone treatment, at least in the first 2–4 h when the increase in sodium transport is most dramatic [115–118]. This suggests that aldosterone can also regulate sodium transport by a rapid mechanism in the nephron. The post-translational modification of the channels that increases their open probability, offers an alternative pathway for the rapid aldosterone action. Zhou and Bubien [119] demonstrated rapid (<2 min) aldosterone-induced activation of ENaC in principal cells from the renal collecting ducts, this effect is sensitive to inhibitors of methyl esterification suggesting methylation of one of the ENaC subunits by methylases. Previous reports by Sariban-Sohraby et al. [120] showed that application of aldosterone also leads to the methylation of membrane proteins. However, neither of the ENaC subunits contains methylation consensus sequences. Thus, another protein must be methylated which in turn activates ENaC. The link between methylation and ENaC activation was found recently when Al Baldawi et al. [121] and Stockand et al. [122] demonstrated that K-Ras2A protein is a target for methylation and that its methylation is induced by aldosterone in A6 distal nephron cells. These studies do not clearly show the mechanism(s) by which K-Ras activates ENaC. The simplest mechanism would be direct ENaC/K-Ras interaction. However, it is likely that there are other signalling elements that lie between Ras and ENaC. The traditional effector of Ras is Raf kinase, Raf activation leads to activation of the MAPK pathway. However, activation of MAPK pathway appears to inhibit ENaC activity rather than stimulate it. Nevertheless, rapid aldosterone-induced activation of Na^+-H^+ exchanger (NHE) in MDCK cells [108] and M1 cortical collecting duct cells (unpublished data) are a MAPK-dependent process (Fig. 2).

Activation of ENaC is not the only event elicited by aldosterone to increase sodium absorption. Active transport of sodium in the basolateral membrane not only requires the activity of Na^+-K^+-ATPase but also potassium recycling through basolateral potassium channels. It has been demonstrated in principal frog skin cells that aldosterone induces rapid activation of ATP-sensitive potassium channels (K_{ATP}) [123]. The time course for the rapid activation of K_{ATP} channels is similar to the rapid ENaC activation time (<2 min). This effect is dependent on previous activation of NHE and intracellular pH alkalinization; K_{ATP} channels are highly sensitive to variations in intracellular pH within the physiological range (7.0–7.5). The role of aldosterone-induced activation of K_{ATP} channels is to preserve the driving force for Na^+ absorption by acting as a "physiological voltage-clamp" which compensates membrane depolarisation producing by Na^+ entry into the cell [123]. Fig. 3 illustrates the pro-absorptive mechanism of aldosterone in kidney.
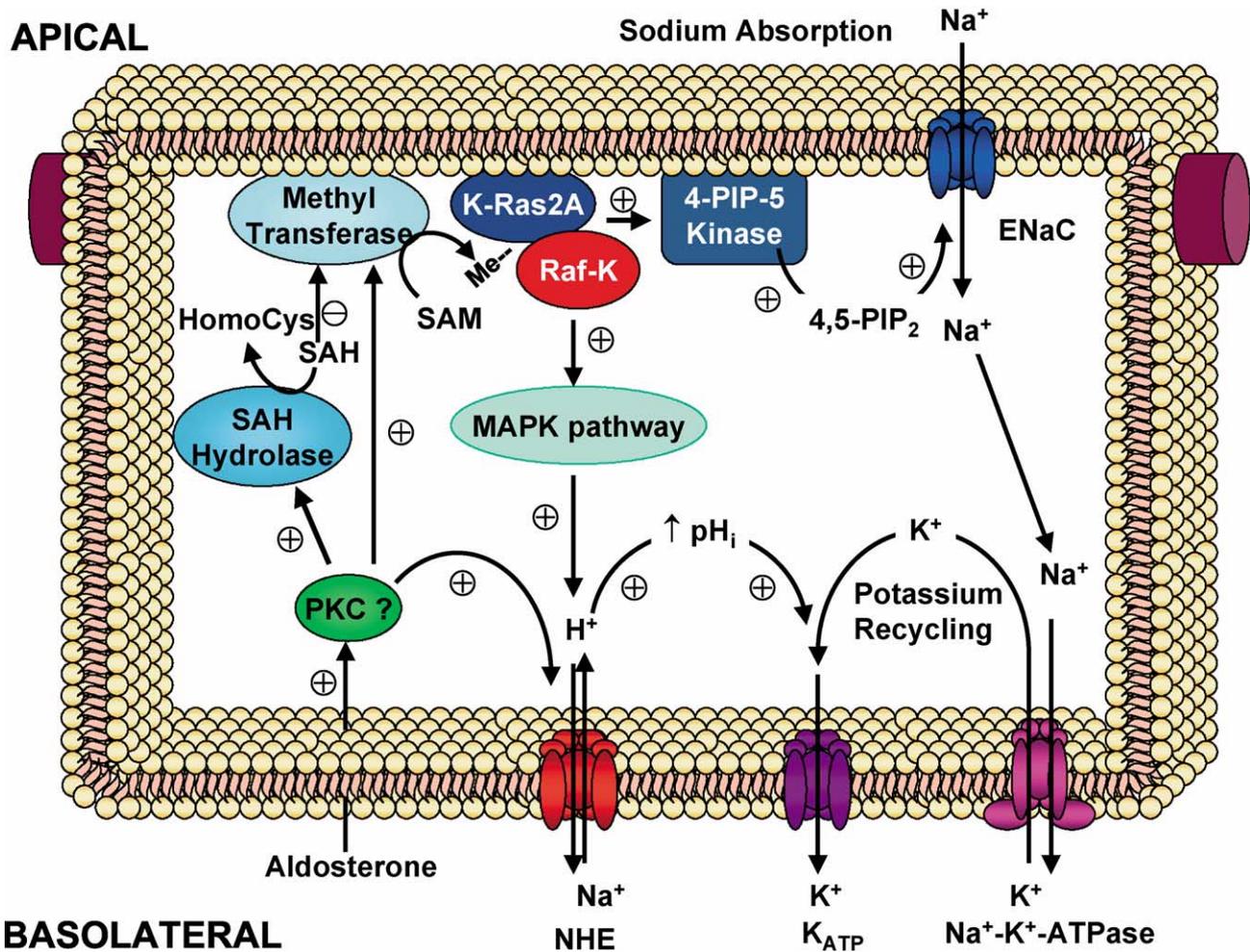
Fig. 3. Schematic diagram of the non-genomic aldosterone signalling pathway in kidney. The schematic is based on the observations that aldosterone-induced activation of sodium transport requires activation of methyl transferase to methylate K-Ras2A, and stimulation of SAH-hydrolase to eliminate inhibition of methyl transferase by SAH. Possible effectors for K-Ras2A are Raf-Kinase (Raf-K), which in turn can activate MAPK pathway, and 4-PIP-5 that mediates 4,5-PIP$_2$, a strong activator of ENaC. MAPK activation stimulates NHE activity.

4.2. Distal colon

The mammalian colon is a major target for aldosterone, and the level of mineralocorticoid receptor gene expression is higher in the distal colon than in any other target tissue including the kidney [124]. In the distal colon, aldosterone causes a switch from electroneutral NaCl absorption to stimulated electrogenic Na^+ absorption by inducing expression of apical ENaC and basolateral Na^+–K^+-ATPase [125]. In parallel, net K^+ absorption is converted to net K^+ secretion by induction of apical K^+ channels. This process has been well characterized; however, several studies have demonstrated that changes in electrolyte transport precedes the increase in induction of these proteins. Early studies in the 1970s reported rapid effects of aldosterone on ion transport in rabbit distal colon [126]. Aldosterone induced a rapid (<30 min) increase in the short-circuit current (I_{sc}), an equivalent increase in the rate of active Na^+ absorption ($J_{Na\ net}$) and a decline in tissue resistance (R_t). Aldosterone produced no change in Na^+–

K^+-ATPase protein number or activity within the time frame of these studies. Also, aldosterone had no effect on the bi-directional or net transepithelial movements of K^+ under short-circuit conditions, suggesting that the enhanced secretion of K^+ observed in vivo is the result of increased diffusion of K^+ from plasma to lumen via paracellular pathways in response to an increased transepithelial electrical potential difference (lumen negative). More recently, it has been found that aldosterone also regulates basolateral potassium channels in rat distal colon [7]. These channels are responsible for potassium recycling in the basolateral membrane and play an essential role in sodium absorption and chloride secretion [125]. Basolateral potassium channels in colonic epithelia are very sensitive to changes in intracellular pH within the physiological range [7]. In the rat distal colon, aldosterone induces rapid activation of NHE that leads to intracellular alkalinization. This change in intracellular pH has a significant impact on basolateral potassium conductances producing activation of K_{ATP} channels and inhibition of a calcium-dependent

potassium channel (K_{Ca}) [7,123]. Previous studies have demonstrated that K_{ATP} channels are involved in electrogenic Na^+ absorption in distal colon [7,107,127]. This result suggests that aldosterone's non-genomic action will lead to a rapid increase in Na^+ absorption. In contrast to rabbit tissue, there are no reports of rapid activation of ENaC in rat distal colon; this is consistent with previous studies that reported lack of rapid ENaC activation in mice and rat kidney cells [119]. Therefore, it appears that the activation of K_{ATP} channels will not increase Na^+ absorption by itself but will counteract membrane depolarisation produced by Na^+ entry into the cell during the genomic phase of aldosterone action.

On the other hand, K_{Ca} channels are involved in electrogenic chloride secretion. The molecular identity of K_{Ca} channel remains unknown but they have been characterized pharmacologically. This channel is sensitive to quaternary ammonium salts (tetrapentyl and tetraethyl ammonium) and partially blocked by barium [7]. Inhibition of this channel leads to a decrease in basolateral potassium recycling, thus producing reduction in chloride secretion. Similar effects on basolateral potassium conductance were found following 17β-estradiol application [127]. 17β-Estradiol was shown to have anti-secretory effects on basal and stimulated cAMP-dependent (forskolin) or Ca^{2+}-dependent (carbachol) chloride secretion [3]. Also, pretreatment of the tissues with 17β-estradiol for 10 min prevented the effect of forskolin and carbachol [3]. 17β-Estradiol is PKC dependent and sensitive to calcium chelation. Also, this effect is gender specific since it was not observed in male rats. It is possible that this gender-specific mechanism contributes to the salt and water retention associated with high estrogen states. Therefore, the non-genomic action of aldosterone and 17β-estradiol (in female) in the distal colon leads to an up-regulation of sodium absorption while down-regulating the capacity for chloride secretion (Fig. 1).

4.3. Airway epithelium

In airway epithelium glucocorticoid, non-genomic effects on intracellular calcium have been characterized [128]. As in the kidney and colon, the mechanism of chloride secretion requires a basolateral potassium conductance to provide the driving force (ref). However, further experiments are needed to analyse the role of glucocorticoid non-genomic action on ion transport in the lung.

5. Conclusions

Our current knowledge of non-genomic action of steroid hormones in epithelia points to a role for rapid regulation of ion transport. The physiological function of non-genomic effects is to shift the balance from net secretion to net absorption in pluripotential epithelia. To achieve this effect,

several pathways are activated, such as PKC, PKA, MAPK, intracellular calcium and pH. Initially, PKC appeared to be a common pathway for rapid responses to steroid hormones. However, now it is clear that activation of specific PKC isoforms confers selectivity to steroid hormone action. Non-genomic and genomic actions may even synergize, leading to the appearance of biphasic effects that have both a rapid onset and a long-lasting persistence. A better knowledge of non-genomic actions of steroids is likely to open new perspectives in the understanding of animal and human physiology, as well as in the pharmacological treatment of some pathological conditions.

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