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G Protein-Coupled Receptors-Induced Activation of Extracellular Signal-Regulated Protein Kinase (ERK) and Sodium-Proton Exchanger Type 1 (NHE1)

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1. Introduction

G-protein-coupled receptors (GPCRs) comprise a large family of cell-surface molecules, involved in signal transmission, accounting for >2% of the total genes encoded by the human genome. GPCRs have been linked to key physiological functions, including immune responses, cardiac- and smooth-muscle contraction and blood pressure regulation, neurotransmission, hormone and enzyme release from endocrine and exocrine glands. Thus, GPCRs contribute to embryogenesis, tissue remodelling and repair, inflammation, angiogenesis and normal cell growth. Their dysfunction contributes to multiple human diseases, and GPCRs represent the target of over 50% of all current therapeutic agents (Reviewed by Pierce et al., 2002). In addition, recent studies indicate that many GPCRs are overexpressed in various cancer types, and contribute to tumor cell growth when activated by circulating or locally produced ligands, suggesting a crucial role of GPCRs in cancer progression and metastasis. For example, many potent mitogens such as thrombin, lysophosphatidic acid (LPA), endothelin and prostaglandins stimulate cell proliferation by acting on their cognate GPCRs in various cell types. (Reviewed by Dorsam & Gutkind, 2007). The mechanisms that control cellular proliferation are important in normal physiology and disease states. Multiple mitogens that activate GPCRs stimulate the extracellular signal-regulated protein kinase (ERK) and lead to proliferation of mammalian cells. Another extensively studied mitogenic effector pathway in addition to ERK that ultimately leads to cell proliferation, is the ubiquitous plasma membrane sodium-proton exchanger type 1 (NHE1). NHE1 and ERK have both been implicated as key mediators of growth signals (Noel & Pouyssegur, 1995; Rozengurt, 1986; Kapus et al, 1994; Krump et al, 1997), therefore the regulatory relationships between NHE1 and ERK have been the subject of a number of studies over the last decade. Because both proteins can serve mitogenic functions, and because both are activated by similar stimuli, it has been hypothesized that

one may be a regulator for the other. Indeed, in some cell types ERK plays a clear role in either the short or long term activation of NHE1 (Aharonovitz & Granot, 1996; Bianchini et al., 1997; Wang et al., 1997; Sabri et al., 1998; Bouaboula et al., 1999; Gekle et al., 2001). However, several groups were unable to demonstrate any role of ERK in regulation of NHE1 in a number of cell types (Gillis et al., 2001; Kang et al., 1998; Pederson et al., 2002; Garnovskaya et al., 1998; Di Sario et al., 2003). In addition, a number of recent studies suggested that certain stimuli such as mechanical stretch, hypertrophy and inflammatory mediators require NHE1 to regulate ERK (Takewaki et al., 1995; Nemeth et al., 2002; Yamazaki et al., 1998; Javadov et al., 2006; Chen et al., 2007). At present, very little is known about GPCR-induced NHE1-dependent ERK regulation. One report suggests that NHE1 is not a regulator for LPA-induced ERK activation in C6 glioma cells (Cechin et al., 2005) and another paper demonstrates the lack of role of NHE1 in angiotensin II (Ang II)- and endothelin 1-induced ERK activation in cultured neonatal rat cardiomyocytes (Chen et al., 2007). At the same time, our group showed that NHE1 activation plays a necessary role in activation of ERK by AII AT_1 and serotonin 5-HT_{2A} receptors in vascular smooth muscle cells (VSMC) (Mukhin et al., 2004), and in bradykinin B_2 receptor-induced ERK activation in renal carcinoma A498 cells (Garnovskaya et al., 2008) thus suggesting that the critical role of NHE1 in GPCR-induced ERK activation is not restricted to one specific cell type and receptor. Studies on the involvement of NHE1 in ERK regulation may also have pathophysiological relevance. NHE1 is usually referred to as a "housekeeping" protein and is normally inactive, but it gets activated in response to multiple specific stimuli, and maintains homeostatic cell volume and pH through Na^+/H^+ transport. The role of NHE1 has been well established in the myocardial remodeling and heart failure process (reviewed by Karmazyn et al., 2008). NHE1 may play a key role in the maintenance of blood pressure because increased activity of NHE1 has been observed in cells and tissues from hypertensive animals and humans (Rosskopf, et al., 1993; Lucchesi et al., 1994). Northern blot analysis showed that cultured VSMC from Sprague-Dawley and Wistar-Kyoto rats express only the NHE1 isoform, and that steady-state mRNA levels are similar for normal and spontaneously hypertensive animals (Lucchesi et al., 1994; LaPointe et al., 1995). Because no mutations in the NHE1 DNA sequence have been found in hypertensive animals, this suggests that increased activity of the antiporter is caused by an alteration in the regulation of NHE1 (Lucchesi et al., 1994). In addition, NHE1-mediated intracellular alkalinization has been proposed to play role in cancer cells growth, and over-expression of NHE1 contributes to the transformed phenotype of multiple cancer cells (Cardone et al., 2005). The role of NHE1 in renal diseases is less known. Mice with a spontaneous point mutation that results in truncation between the 11^{th} and 12^{th} NHE1 trans-membrane domains and causes loss of NHE1 function (Cox et al., 1997) do not present visible renal phenotype, consistent with the concept that NHE1 "housekeeping" activity under normal conditions is not required. However, NHE1 activity was increased in cell lines derived from patients with diabetic nephropathy (Ng et al., 1994), suggesting that NHE1 activity may be important in the context of cellular stress. Further, it has been shown that genetic or pharmacological loss of NHE1 function causes renal tubule epithelial cell apoptosis and renal dysfunction in several models of kidney disease (ureteral obstruction, adriamycin-induced podocyte toxicity, and streptozotocin-induced diabetes), suggesting that NHE1 activity may be beneficial for chronic kidney disease (Schelling & Abu, 2008). Moreover both, ERK and NHE1, have been

proposed as key therapeutic targets for vascular illnesses, such as congestive heart failure (Kusumoto et al., 2001), myocardial infraction and reperfusion injury (Avkiran & Marber, 2002), ventricular fibrillation (Gazmuri et al., 2001), and ventricular hypertrophy (Chen et al., 2001). Therefore, studies devoted to the regulatory relationships between NHE1 and ERK have a potential clinical relevance.

The purpose of this review is to describe the relationship between NHE1 and ERK when both pathways are activated by GPCRs, with a particular emphasis on the situations when NHE1 is regulating ERK activity leading to cell proliferation.

2. Mitogen-activated protein kinases and Sodium-Hydrogen Exchanger-1 as mediators of growth signals

2.1 Mitogen-activated protein kinases

Mitogen-activated protein kinases (MAPKs) are a family of highly conserved proline-directed serine/threonine kinases that are activated by a large variety of extracellular stimuli and play integral roles in controlling many cellular processes, from the cell surface to the nucleus (Widmann et al, 1999). The MAPK family in mammals includes four distinctly regulated groups of MAPKs: extracellular signal-regulated kinase 1/2 (ERK), p38, c-Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPKs), and ERK5/Big MAPK (BMK1) (Chang & Karin, 2001; Bogoyevitch & Court, 2004; Johnson & Lapadat, 2002). MAPK cascades typically consist of three levels of protein kinases that are consecutively activated by phosphorylation events: MAPK kinase kinase (MAPKKK or MAP3K or MEKK) activates MAPK kinase (MAPKK (MKK or MEK) or MAP2K), which in turn activates MAPK (Figure 1). Even so, the different tiers are composed of many similar isoforms that can be activated by more than one MAPK, increasing the complexity and diversity of MAPK signaling. Regulation and function of different MAPKs as well as complexity of MAPK signaling have been recently discribed in several review articles (Pearson et al., 2001; Chang & Karin 2001; Shaul & Seger, 2007; Bodart; 2010). MAPKs are involved in transmitting signals from a wide variety of extracellular stimuli including those of growth factor receptors and GPCRs, as well as physical or mechanical stimuli. In fact, MAPKs are major components of signaling pathways regulating a large array of intracellular events, such as proliferation, differentiation, acute signaling in response to hormones, stress response, programmed cell death, and gene expression (Pearson et al., 2001; Chang & Karin 2001; Kim & Choi, 2010). ERK is one member of a family of kinases that participate in mitogenic signaling through complex phosphorylation cascades that convert cell surface signals into nuclear transcription programs. In the typical scenario, GTP-bound Ras, a small G protein, activates Raf-1 kinase. In an alternative scenario, protein kinase C (PKC) or other signaling molecules activate Raf-1. In either case, Raf-1 phosphorylates and activates mitogen and extracellular signal-regulated kinases kinase (MEK), which in turn phosphorylates and activates ERK (Cobb & Goldsmith, 1995). Activated ERK translocates to the nucleus, where it activates a number of transcription factors such as Elk-1.

Recently a number of scaffolding proteins that play important role in ERK regulation have been described. Examples of such proteins include the Kinase Suppressor of Ras (KSR), β-arrestins1/2, PEA15 (phosphoprotein enriched in astrocytes), paxillin, and Raf-1 (Kolch,

2005). Paxillin, a multi adaptor protein in focal adhesion assembly, serves as a connector between ERK and Focal Adhesion Kinase (FAK) signaling pathways binding Raf-1 and ERK in response to hepatocyte growth factor in epithelial cells (Ishibe et al., 2004).

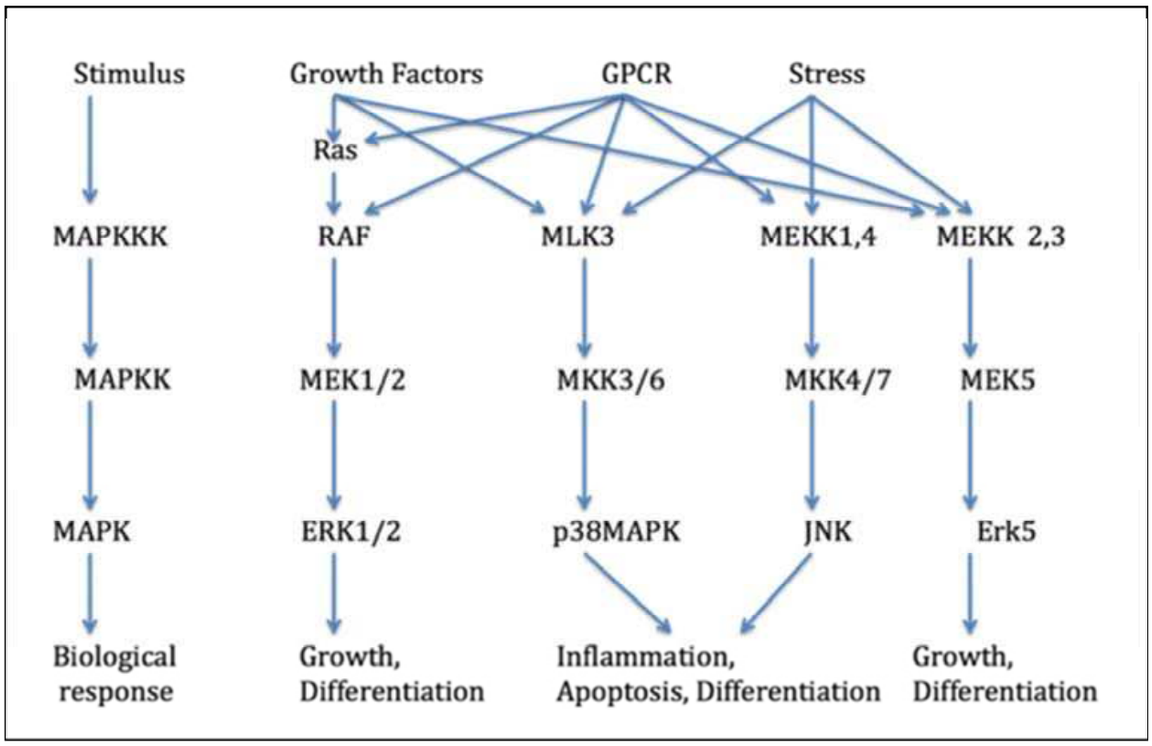

Fig. 1. Mitogen-Activated Protein Kinase Cascades.

2.1.1 Regulation of ERK by GPCRs

The ability of G-protein-coupled receptors (GPCRs) to generate signals that control cellular proliferation via activation of ERK pathways has been demonstrated in several studies (reviewed by Kranenburg & Moolenaar, 2001; and Luttrell, 2002). Although the mechanisms by which GPCRs control the activity of ERK vary between receptor and cell type, typically there are three categories of mechanisms: (1) signals that initiate classical G protein effectors, e.g., protein kinase A or protein kinase C causing the production of second messengers, (2) mechanisms that involve cross-talk between GPCRs and classical receptor tyrosine kinases, e.g., "transactivation" of epidermal growth factor (EGF) receptors, and (3) signals initiated by direct interaction between beta-arrestins and components of the MAP kinase cascade, e.g., beta-arrestin "scaffolds". Mitogenic pathways activated by different Gα families including activation of adenylyl cyclase/cAMP and phospholipase Cβ/protein kinase C second messenger pathways have been described in detail (reviewed by New & Wong, 2007). Angiotensin II promotes DNA synthesis and proliferation in many cell types by activating the G_q-coupled AT_1 receptor. AT_1 receptor activity in human adrenal cells induces Ras-dependent ERK activity, leading to increased levels of c-Fos and c-Jun transcription factors and to proliferation of the cells (Watanabe et al., 1996). Other mitogenic

GPCRs, including M_1 muscarinic and α_{1B}-adrenergic and purinergic receptors, induce ERK activity via the Ras-independent protein kinase C phosphorylation and activation of Raf-1 (Luttrell, 2002). G_s-coupled GPCRs utilize the adenylyl cyclase/ cAMP /Epac/Rap-1/B-Raf pathway to activate MAPK cascades and proliferation. In bone cells parathyroid hormone receptor promotes cAMP accumulation, which binds directly to the Rap-1 guanine nucleotide exchange factor Epac. Epac in turn activates Rap-1, a Ras family GTPase, which activates the kinase B-Raf, triggering ERK cascades (Fujita et al., 2002). Alternatively, PKA may directly activate Rap-1 (Luttrell, 2002).

However, activation of classical second messenger cascades cannot fully explain roles of GPCRs in stimulation of MAPK cascades. Additional signaling mechanisms including transactivation of the Receptor Tyrosine Kinases (RTKs) via the autocrine/paracrine release of epidermal growth factor (EGF)-like ligands at the cell surface and scaffolding of MAPK cascades, appear to contribute to GPCR-mediated MAPK activation. GPCR-mediated proliferation via the Gα or Gβγ subunit transactivation of RTKs has been described in several cell types (Ohtsu et al., 2006; Schafer et al., 2004). Thus, ligands for the LPA, endothelin-1 and thrombin receptors all stimulate cell proliferation in Rat-1 fibroblasts by transactivation of the epidermal growth factor receptor (EGFR, an RTK). Such transactivation requires the activation of matrix metalloproteases (MMPs) to release EGF from its membrane bound form, which then stimulates the EGFR and downstream ERK pathways (Schafer et al., 2004). Studies from our group demonstrated that bradykinin B_2 receptor activates ERK via EGFR transactivation in kidney cells (Mukhin et al., 2003; Mukhin et al., 2006; Kramarenko et al., 2010). The similar MMP/EGFR/ERK pathway have been also demonstrated in kidney cancer cells stimulated by LPA and angiotensin II (Schafer et al., 2004). A significant advance in the understanding of how GPCRs activate MAPK cascades is the discovery that beta-arrestin, a protein well known for its roles in both receptor desensitization and internalization, serves as a scaffolding protein for the GPCR-stimulated the extracellular signal regulated kinase ERK cascade. For example, agonist stimulation of the proteinase-activated receptor-2 (PAR2) leads to the formation of a large complex, which includes the receptor and beta-arrestin, MAPKKK, Raf-1, and activated ERK. Similarly, activation of neurokinin-1 receptor with the substance P, results in the formation of a complex, which includes the receptor, and beta-arrestin, c-Src and ERK. (Reviewed by Pierce et al., 2001).

ERK activation occurring via EGF receptor transactivation or via pathways employing second messengers (PKA- or PKC-dependent pathways) typically leads to sustained ERK activity and nuclear translocation of the kinase, thus contributing to regulation of cell cycle progression (Kranenburg & Moolenaar, 2001; Luttrell, 2002). In contrast, beta-arrestin/endocytotic pathway usually results in the retention of ERK in the cytoplasm and transient ERK activity, which is probably not sufficient to stimulate cell proliferation (Luttrell, 2002).

The intracellular pathways that mediate GPCR -induced ERK activation and regulation of cellular proliferation were recently reviewed by New & Wong (New & Wong, 2007).

2.2 Sodium-Hydrogen Exchanger-1 (NHE1)

The Na^+/H^+ exchange system was described in 1977 by Aickin and Thomas (Aickin & Thomas, et al., 1977), and the first Na^+/H^+ exchanger (NHE) gene was cloned in 1989 (Sarget et al., 1989). To date nine mammalian isoforms (NHE 1-9) have been identified in the family of Na^+/H^+ exchangers (Kemp et al, 2008). In this review we will focus only on the

ubiquitously expressed, amiloride-sensitive integral plasma membrane protein NHE1 known as the "housekeeping enzyme", which is activated by various stimuli including growth factors, mitogens, and hyperosmolarity (Orlowski & Grinshtein, 2004; Wakabayashi et al., 1997). NHE1 is highly conserved across vertebrate species and is a major membrane transport mechanism, which plays an essential role in pH regulation, volume homeostasis, cell growth and differentiation (Bertrand et al., 1994). NHE1 is a phosphoglycoprotein of 815 amino acids that contains two functional domains: an NH2-terminal transmembrane ion translocation region with a proposed topology of 12 transmembrane domains, and a COOH-terminal cytoplasmic regulatory domain (Figure 2). The ion translocation domain catalyzes electroneutral exchange of extracellular sodium ion for intracellular hydrogen. Regulation of NHE1 activity in response to multiple stimuli including growth factors, hormones, and osmotic stress is mediated by a COOH-terminal cytoplasmic regulatory domain. The regulatory domain controls transport activity probably by altering affinity of a proton site in the transmembrane domain (Takahashi et al., 1999). This cytoplasmic domain includes a number of distinct subdomains modified either by phosphorylation or by the binding of regulatory proteins. The cytoplasmic domain contains high and low affinity Ca^{2+}/calmodulin-binding sites and several potential phosphorylation sites (Bertrand et al., 1994; Yan et al., 2001). Bertrand et al. first identified two calmodulin-binding sites on the

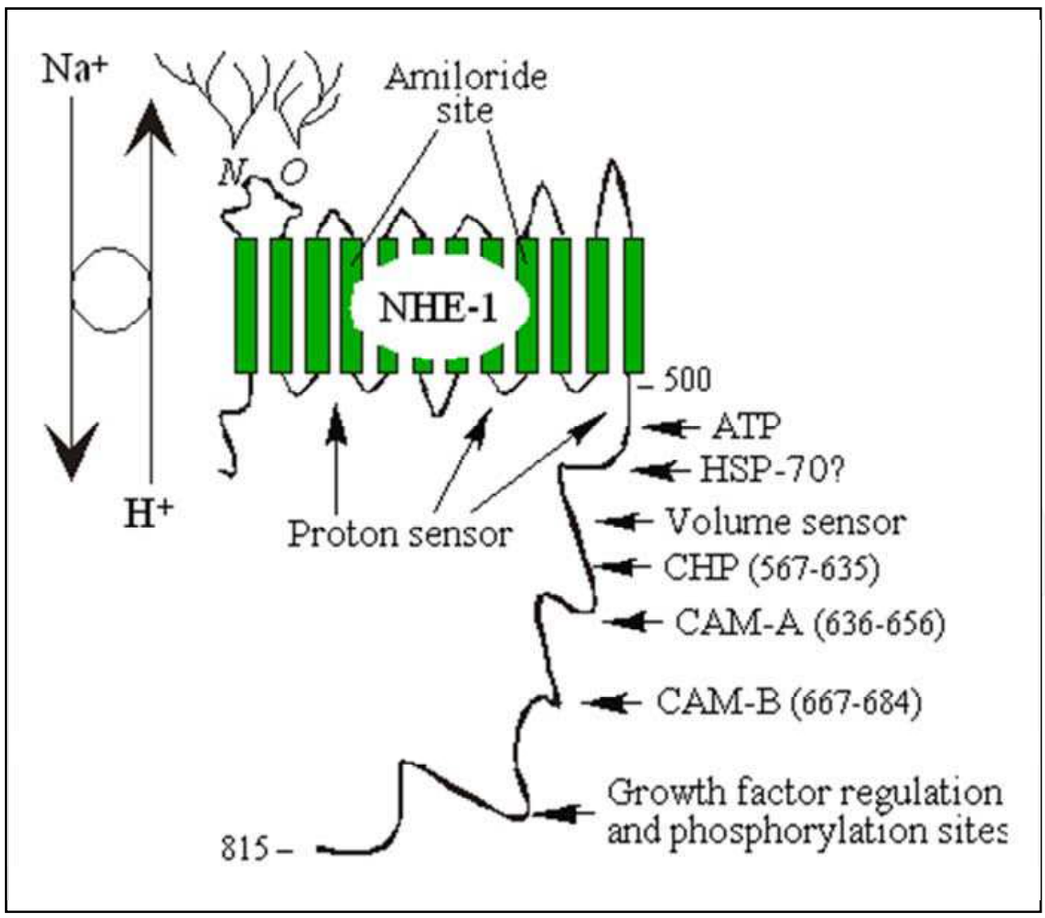

Fig. 2. Topographical model of NHE1.

cytoplasmic domain of NHE1 at amino acids 636-656 and 664-684, with high (Kd ~ 20 nM) and low (Kd~ 350 nM) affinities, respectively (Bertrand et al., 1994). In quiescent cells, the high-affinity calmodulin-binding domain may act as an autoinhibitory domain by interacting with the transmembrane domain, thus inhibiting ion translocation. Upon activation, NHE1 undergoes a conformational change that allows the Ca^{2+} -dependent binding of calmodulin (Wakabayashi et al., 1997). A phosphorylation domain at the distal COOH-terminus (amino acids 656-815 of human NHE1) contains a number of serine residues constitutively phosphorylated in quiescent cells that have increased phosphorylation levels in response to growth factors (Sarget et al., 1989). COOH-terminal serine residues on NHE1 molecule can be phosphorylated by the ERK-regulated kinase p90RSK (Tominaga et al., 1998) and by the Ste20-like Nck-interacting kinase (NIK) in response to growth factor receptors (Putney et al., 2002) and by Rho kinase 1 (ROCK1) in response to activation by GPCRs for thrombin and lysophosphatidic acid (Tominaga et al., 1998; Putney et al., 2002) and by integrin-induced cell adhesion (Tominaga & Barber, 1998). Because the cytoplasmic regulatory domain associates with multiple binding partners including the cytoskeleton-plasma membrane linker protein ezrin of the ezrin, moesin, radixin, (EMR) family (Denker et al., 2000), the calcineurin homolog protein CHP1 (Pang et al., 2001), calmodulin (CaM) (Yan et al., 2001), carbonic anhydrase II (Li et al., 2002), heat shock protein (Silva et al., 1995), and 14-3-3 protein (Lehoux et al., 2001), a novel function of NHE1 as a plasma membrane scaffold in the assembly of signaling complexes has been suggested (Baumgartner et al., 2004).

2.2.1 Regulation of NHE1 by GPCRs

While the activation of NHE1 and the kinetic alterations to the exchanger have been widely studied (Noel & Pouyssegur, 1995; Wakabayashi et al., 1997; Orlowski & Grinshtein, 1997) the signaling pathways that regulate NHE1 have not been fully elucidated. Because G protein-coupled receptors (GPCRs)-mediated regulation of sarcolemmal NHE activity is likely to play significant roles in modulating myocardial function in both physiological and pathophysiological conditions, most of the studies devoted to GPCR-induced NHE1 regulation were performed in cardiac myocytes (reviewed by Avkiran & Haworth, 2003). Sarcolemmal NHE activity is subject to exquisite regulation by a variety of extracellular stimuli, most of which act through GPCRs. Intriguingly, although the majority of the GPCR systems that have been studied to date have been shown to *stimulate* sarcolemmal NHE activity, there is also evidence that some may *inhibit* NHE activity or its stimulation through other pathways. A number of GPCRs, such as α_1-adrenergic receptors , angiotensin II AT_1 receptors, endothelin ET_A receptor, thrombin receptor, muscarinic receptors have been shown to increase sarcolemmal NHE activity through a change in the pH_i sensitivity of the exchanger. Interestingly, in contrast to the evidence that various G_q –coupled receptors (e.g. α_{1A}-ARs and angiotensin AT_1) mediate an increase in sarcolemmal NHE activity, GPCRs that signal through other G protein families (G_s and G_i) may attenuate NHE activity or its stimulation. Thus, β_1-AR stimulation *inhibits* sarcolemmal NHE activity, while adenosine A_1 and angiotensin AT_2 receptors attenuate stimulation of NHE1 by other ligands (Avkiran& Haworth, 2003). The mechanisms of GPCR-induced NHE1 activation are not fully understood. To date, several mechanisms of activation of NHE1 by G protein-coupled receptors have been proposed although not fully characterized : α_{1A}-adrenoceptor activates NHE1 through protein kinase C (Snabaitis et al., 2000; Avkiran & Haworth, 2003); lysophosphatidic acid stimulates NHE1 through RhoA and its effector ROCK (Tominaga et

al., 1998), and angiotensin II AT_1 receptor regulates NHE1 activity through RSK (Takahashi et al., 1999). In addition, Wallert et al provided evidence that the specific α_1-adrenergic agonist, phenylephrine and the lysophosphatidic acid (LPA) activate NHE1 in CCL39 cells, and demonstrated a direct involvement of ERK in the α_1-adrenergic activation of NHE1 and a significant role for both ERK and RhoA in LPA stimulation of NHE1 in CCL39 fibroblasts (Wallert et al., 2004). Our group reported that a fibroblast NHE1 can be rapidly stimulated through the transfected human serotonin 5-HT_{1A} receptor via pertussis toxin-sensitive G protein α-subunits $G_{i\alpha2}$ and $G_{i\alpha3}$, in CHO cells (Garnovskaya et al., 1997), by endogenously expressed G_q-coupled bradykinin B_2 receptor in kidney cells (Mukhin et al., 2001), and by endogenously expressed G_q-coupled angiotensin II AT_1 and serotonin 5-HT_{2A} receptors in vascular smooth muscle cells (Garnovskaya et al., 2003). While studying the signaling pathway of bradykinin B_2 receptor-induced NHE1 activation in mIMCD-3 kidney cells, we found a new mechanism for the GPCR-induced regulation of Na^+/H^+ exchange (Mukhin et al., 2001). This novel pathway involved activation of phospholipase C, elevation of intracellular Ca^{2+}, activation of the non receptor tyrosine kinase, Janus kinase 2 (Jak2), tyrosine phosphorylation of Ca^{2+}/calmodulin (CaM), and binding of CaM to NHE1. Bradykinin rapidly stimulated the assembly of a signal transduction complex that includes CaM, Jak2, and NHE1. We suggested that Janus kinase 2 is involved in the activation of NHE1 by increasing the tyrosine phosphorylation of calmodulin, which appears to be a direct substrate for phosphorylation by Janus kinase 2. Further the same pathway has been demonstrated for the bradykinin B_2 receptor-mediated activation of Na^+/H^+ exchange in KNRK and CHO cells (Lefler et al., 2003), and for the G_q-coupled angiotensin II AT_1 and serotonin 5-HT_{2A} receptors, which stimulated NHE1 activation in vascular smooth muscle cells (Garnovskaya et al., 2003), suggesting that this pathway represent a fundamental mechanism for the rapid regulation of NHE1 by G_q-coupled receptors in multiple cell types. Further we have shown that the G_i – coupled serotonin 5-HT_{1A} receptor also rapidly stimulates NHE1 through a pathway that involves 1) activation of Janus kinase 2 downstream of the 5-HT_{1A} receptor; 2) formation of a complex that includes NHE1, Jak2, and CaM; 3) tyrosine phosphorylation of CaM through Jak2; and 4) increased binding of CaM to the carboxyl terminus of NHE1 (Turner et al., 2007).

2.3 Relationships between NHE1 and ERK

2.3.1 MAPK regulates NHE1

Whereas it has been known for some time that mitogens typically activate both NHE1 and ERK in concert (Noel & Pouyssegur, 1995; Rozengurt, 1986; Kapus et al., 1994; Krump et al., 1997) the exact relationships between NHE1 and ERK have only recently been explored in any great detail. Recent studies have shown that multiple stimuli that rapidly activate ERK pathways also rapidly increase NHE activity in many cell types, particularly in fibroblasts. Those stimuli include, but are not limited to: growth factors that modulate tyrosine phosphorylation cycles, integrins, hyperosmotic stress or cell shrinkage, protein kinase C (PKC), tyrosine phosphorylation cascades and heterotrimeric G proteins (Clark & Limbird, 1991; Barber, 1991; Rozengurt, 1986; Lowe et al., 1990). Those similarities provide evidence to suggest that ERK may be a direct proximal component of an NHE regulatory pathway (Noel & Pouyssegur, 1995; Aharonovitz & Granot, 1996). There is a growing awareness that tyrosine phosphorylation cycles are critical in regulating NHE activities in a number of cell types (Donowitz et al., 1994; Yamaji et al., 1995; Good, 1995; Fukushima et al., 1996) as has also been shown for ERK (Blumer & Johnson, 1994). Other studies have demonstrated that

NHE and ERK activities are modulated by overlapping upstream enzymes, including phosphoinositide 3´-kinase (PI-3K), phospholipase C, and PKC (Levine et al., 1993; Kapus et al., 1994; Voyno-Yasenetskaya et al., 1994; Bertrand et al., 1994; Ma et al., 1994; Dhanasekaran et al., 1994; Inglese et al., 1995). In aggregate, those studies implicate G proteins, lipid-recognizing enzymes, tyrosine kinases, and NHEs as playing interrelated roles along with ERK in cell growth (Barber, 1991; Noel & Pouyssegur, 1995; Aharonovitz & Granot, 1996; Blumer & Johnson, 1994; Lin et al., 1996). Relevant to the hypothesis that ERK regulates NHE1, are studies showing that microinjection of activated Ras (Hagag et al., 1987) or transfection of the Ha-Ras oncogene (Doppler et al., 1987; Maly et al., 1989; Kaplan & Boron, 1994) stimulates NHE activity in fibroblasts. The classical effect of GTP-bound Ras is the activation of the ERK1 and ERK2 (Blumer & Johnson, 1994). This is thought to occur primarily through a linear signalling pathway that flows as follows: Ras-GTP → Raf-1 kinase → MEK (MAPK/ERK kinase) → ERK. Thus, because Ras functions upstream of both NHE and ERK activities, ERK has been proposed as a logical funnel for signals from extracellular stimuli to the effector NHE. The effect of NHE activation due to the sustained activation of ERK is most likely secondary to the activation of transcription cascades that upregulate the NHE message/protein or modulate expression of key regulators of NHE activity. However, several studies suggest that ERK might regulate NHE activity in the short term, as well. The possibility that ERK rapidly regulates NHE activity was tested in platelets by Aharonovitz and Granot (Aharonovitz & Granot, 1996) who showed that arginine vasopressin (AVP) and PMA rapidly activated NHE by a pathway which was sensitive to PD98059, a specific inhibitor of MEK1. Moreover, the signal initiated by AVP was sensitive to genistein, a broad-spectrum inhibitor of tyrosine kinases (Aharonovitz & Granot, 1996). Bianchini et al. (Bianchini et al., 1997) went further to characterize the role of ERK in regulating NHE when cells were stimulated by combinations of growth factors or serum. Specifically, they showed that expression of a dominant negative p44 ERK or of the MAPK phosphatase MKP-1, or treatment with the MEK1 inhibitor PD98059 reduced activation of NHE-1 by mixtures of growth factors by about 50%. Further, it has been shown that short-term activation of ERK leads to rapid stimulation of NHE1 in multiple cell types (erythrocytes, fibroblasts, MDCK-11 cells, rabbit skeletal muscle, and cultured rat neonatal and adult ventricular cardiomyocytes) when activated by diverse stimuli including growth factors, angiotensin II, and aldosterone (Wang et al., 1997; Sabri et al., 1998; Bouboula et al., 1999; Gekle et al., 2001; Wei et al., 2001; Moor et al., 2001; Snabaitis et al., 2002). At least in some cases, the short term-stimulation of NHE1 by ERK is mediated by phosphorylation of NHE1 either by ERK itself, or by p90RSK, an ERK-regulated kinase (Takahashi et al., 1999). Cuello et al. demonstrated that ERK- dependent 90kDa ribosomal S6 kinase (RSK) is the principal regulator of cardiac sarcolemmal NHE1 phosphorylation and NHE activity after α_1-adrenergic stimulation in adult myocardium (Cuello et al., 2007). Thus, there is clear evidence that ERK can increase the activity of NHE1 by increasing its expression and/or by stimulating the activity of existing NHE1 molecules.

2.3.2 MAPK and NHE1 do not regulate each other

On the other hand, several groups have been unable to show any role for ERK in activating NHE1 in multiple cell types, including Xenopus oocytes (Kang et al., 1998), Ehrlich Ascites cells (Pederson et al., 2002), CHO cells (Garnovskaya et al., 1998), or hepatic stellate cells (Di Sario et al., 2003). Moreover, there is one report in which ERK was shown to mediate inhibition of NHE1 activity in MTAL cells (Watts & Good, 2002). Our group tested the hypothesis that ERK could mediate rapid, short-term activation of NHE activity in

fibroblasts when both signals were initiated by a single G protein-coupled serotonin 5-HT_{1A} receptor (Garnovskaya et al., 1998). These studies revealed a number of similarities between the regulation of ERK and NHE. Activation of the two processes shared similar concentration–response and time-course characteristics. Receptor-activated NHE and ERK also shared an overlapping sensitivity to some pharmacological inhibitors of tyrosine kinases (staurosporine and genistein), PI-3K (wortmannin and LY294002), and PC-PLC (D609), and neither pathway was sensitive inhibition of PKC. However, definitive studies designed to block signaling molecules possessing well-defined roles in activating ERK through the 5-HT_{1A} receptor by transfecting cDNA constructs encoding inactive mutant PI-3K, Grb2, Sos, Ras, and Raf molecules were successful in attenuating ERK, but had essentially no effect upon NHE activation. Thus, our data do not support the hypothesis that ERK is a proximal short-term regulator of NHE in CHO cells when the signal is initiated by the $G_{i/o/z}$ protein-coupled 5-HT_{1A} receptor. Therefore, the ability of ERK to stimulate NHE1 activity has not been a universal finding.

2.3.3 NHE1 as a regulator of MAPK

Despite the increasing interest in potential roles for ERK in the activation of NHE1, much less is known regarding the role of NHE1 in regulating ERK. There have been several reports that suggest that NHE1 might play a role in regulating ERK activation (reviewed by Pedersen et al., 2007). Mitsuka et al. had shown that specific inhibitors of NHE1 could reduce neointimal proliferation in a rat model of carotid artery injury (Mitsuka et al., 1993). However, in C6 glioma cells although lysophosphatidic acid (LPA) - increased proliferation was sensitive to NHE1 inhibitors, LPA-induced ERK activation was unaffected (Cechin et al., 2005). Takewaki et al. presented some evidence that a potent antagonist of NHE1 could partially inhibit stretch-induced activation of ERK in the cultured cardiomyocytes (Takewaki et al., 1995). Later the same group reported that in cultured neonatal rat cardiomyocytes NHE1 inhibition blocked the stretch-induced activation of Raf-1 and ERK, while angiotensin II (Ang II)- and endothelin 1-induced ERK activation remained unaffected (Yamazaki et al., 1998). On the other hand, our work in vascular smooth muscle cells (VSMC) demonstrated that activation of ERK by AII and serotonin was strongly dependent of NHE1 activity, and the effect of NHE1 occurs at or above the level of Ras (Mukhin et al., 2004). In human colon cancer epithelial cells, NHE1 inhibition suppressed activation of ERK and NF-κB and led to decreased production of interleukin-8 in response to inflammatory signals (Nemeth et al., 2002). Recently it has been also demonstrated that NHE1 inhibition prevented ERK activation during phenylephrine-induced hypertrophy in neonatal rat cardiomyocytes (Javadov et al., 2006), and prevented glucose-induced ERK activation in a high glucose model of cardiomyocyte hypertrophy (Chen et al., 2007). In Ehrlich Lettre Ascites cells under osmotic cell shrinkage NHE1 regulates ERK acting at or above the level of MEK (Pederson et al., 2002). Therefore, NHE1-dependent regulation of ERK in most cases has been described in cells stimulated by mechanical stretch, osmotic shrinkage, hypertrophy and inflammatory mediators (Takewaki et al., 1995; Nemeth et al., 2002; Yamazaki et al., 1998; Javadov et al., 2006; Chen et al., 2007; Mitsuka et al., 1993; Pederson et al., 2007). Very little is known about GPCR-induced NHE1-dependent ERK regulation. One report suggests that NHE1 is not a regulator for LPA-induced ERK activation in C6 glioma cells (Cechin et al., 2005) and another paper demonstrates the lack of role of NHE1 in AII- and endothelin 1-induced ERK activation in cultured neonatal rat cardiomyocytes (Chen et al., 2007).

2.3.3.1 NHE1 regulates ERK activity in GPCR-activated VSMC

Angiotensin II (Ang II), a potent hypertrophic factor for vascular smooth muscle cells, mediates its effects via specific plasma membrane AT_1 receptors that belong to GPCR family. Ang II stimulates multiple signaling pathways (reviewed by Touyz & Schiffrin, 2000) including MAPKs, Src family kinases, phospholipase D, and Janus kinase (Jak2). Ang II also has been shown to stimulate NHE1 activity in VSMC (Berk et al., 1987) but does not appear to increase the steady state levels of NHE1 mRNA. There also are reports on relationship between Ang II-induced NHE1 and ERK activities in VSMC suggesting that activation of the AT_1 receptor first leads to activation of the MEK-ERK-p90RSK pathway, and that activated p90RSK in turn directly phosphorylates and activates NHE1 in VSMC (Takewaki et al., 1995). However, this suggestion was based mainly on *in vitro* experiments in which p90RSK immunoprecipitated from Ang II-stimulated VSMC was able to phosphorylate recombinant NHE1, and it is still not clear whether Ang II-induced phosphorylation of NHE1 takes place in VSMC *in vivo* and if this phosphorylation is physiologically significant. Our group has described a novel pathway of the regulation of NHE1 activity in VSMC by two mitogens, Ang II and serotonin (5-HT) that involves the activation of Jak2, tyrosine phosphorylation of Ca^{2+}/calmodulin, and binding of calmodulin (CaM) to NHE-1 (Garnovskaya et al., 2003). In the same study we were not able to support any role for ERK in Ang II-induced NHE1 activation in VSMC (Garnovskaya et al., 2003). Further, we specifically investigated the roles of NHE and ERK (as stimulated by either 5-HT or Ang II) in the activation of each other in VSMC (Mukhin et al., 2004), and we have found evidence to support a novel role for NHE in the activation of ERK in VSMC. This evidence includes 1) dual stimulation of NHE and ERK by Ang II and 5-HT, with the activation of NHE preceding that of ERK, 2) similar concentration-response relationships for the stimulation of NHE and the phosphorylation of ERK by 5-HT and Ang-II, 3) blockade of the activation of ERK induced by 5-HT and Ang II by chemical inhibition of NHE, 4) blockade of the activation of ERK induced by 5-HT and Ang II by removal of sodium from incubation buffers, and 5) phosphorylation of ERK during recovery from an imposed acid load, a maneuver that induces receptor-independent activation of NHE.

Moreover, in the case of receptor-induced activation of ERK, NHE appears to be located upstream of MEK and ERK, and downstream of Ang II and 5-HT-mediated transactivation of the EGF receptor. NHE intersects the classical pathway of activation of ERK at or above the level of Ras. Figure 3 depicts one possible scheme that can account for our findings. Because it has been described that G_q-coupled receptors such as Ang II AT_1 and serotonin $5\text{-}HT_{2A}$ receptors activate ERK in VSMC through transactivation and phosphorylation of the epidermal growth factor (EGF) receptor (Eguchi et al., 1999), we wanted to establish whether NHE regulates ERK activation upstream of the EGF receptor. It appeared that inhibition of NHE activity by depriving the exchanger of extracellular sodium, or by blockade with the specific inhibitors, amiloride analog, methylisobutylamiloride (MIA), prevents activation of ERK by two GPCR ligands, Ang II and 5-HT. Those same maneuvers have no effect on EGF-stimulated ERK, suggesting that there are some differences in the pathways used by Ang II and 5-HT to activate ERK when compared with that used by EGF. Interestingly, the close connection between NHE and ERK activation is further underscored by the observation that receptor-independent activation of NHE also results in ERK phosphorylation only when the exchanger is allowed to mediate recovery from an imposed

intracellular acid load. Thus, NHE activation is *necessary* for Ang II and 5-HT-induced activation of ERK, and is *sufficient* to activate ERK under conditions of an imposed acid load. In contrast, NHE activation is not *necessary* for EGF-mediated activation of ERK. The most likely explanation is that 5-HT or Ang II requires the parallel activation of NHE and the EGFR to activate ERK in VSMC. The two pathways intersect downstream of trans-phosphorylation of the EGFR, and upstream of ERK and MEK, most likely at or upstream of Ras (Figure 3). The precise mechanisms of NHE-dependent ERK activation by Ang II and 5-HT remain to be defined. One possibility is that NHE plays an accessory role in Ang II and 5-HT induced activation of ERK by facilitating cytoskeletal reorganization or by altering Na^+ or H^+ concentrations in cellular microdomains, thereby affecting enzyme activity or protein-protein interactions.

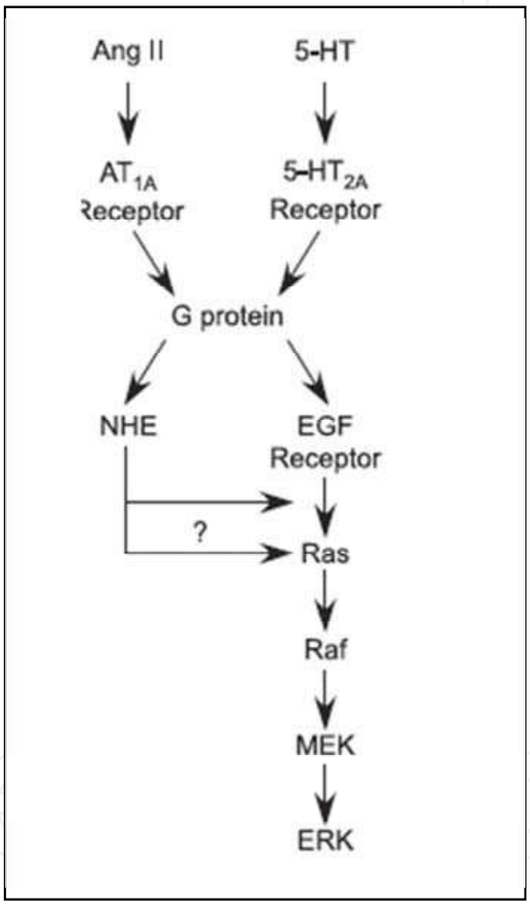

Fig. 3. Hypothetical scheme of NHE1-dependent ERK activation by GPCRs in VSMC.

The speculation regarding the cytoskeletal effects of NHE is particularly intriguing in light of work by Barber's group showing important functional links between NHE activity and the cytoskeleton (Denker et al., 2000). These findings have potential implications for the regulation of vascular tone, as well as for vascular pathobiology because Ang II and 5-HT are both potent vasoconstrictors, and Ang II has been shown to play major roles in various cardiovascular diseases including left ventricular hypertrophy and hypertension (Mitsuka et al., 1993).

2.3.3.2 NHE1 regulates ERK activity in Bradykinin-activated renal carcinoma cells

Because we have been able to detect the critical role of NHE in GPCR-mediated activation of ERK in cells of contractile phenotype, we thought that this relationship might be restricted to specific cell types and receptors. The cellular specificity of the relationship between NHE1 and ERK could be mediated by alternate accessory components of each signaling pathway, or by cell-specific compartmentalization of scaffolded signal transduction platforms (Luttrell & Lutrell, 2003). However, we have collected data that support a role of NHE1 in bradykinin B_2 receptor-induced ERK activation in renal carcinoma A498 cells, thus suggesting that the critical role of NHE1 in GPCR-induced ERK activation is not restricted to one specific cell type and receptor (Garnovskaya et al., 2008). In this study we investigated the endogenous intrarenal kinin hormone bradykinin (BK) that exerts its multiple pathophysiological functions via two known receptors, the bradykinin B_1 (BK B_1) and bradykinin B_2 (BK B_2) which belong to the superfamily of G protein-coupled receptors (GPCR) (Hess et al., 1992; Menke et al., 1994; Bagate et al., 2001). BK plays a significant role as a modulator of renal function such as electrolyte and water excretion (Mukai et al., 1996) as well as in renal cell growth and proliferation (El-Dahr et al., 1998; Jaffa et al., 1997). We have previously reported that BK activates NHE1 in a kidney cell line derived from the inner medullary collecting duct of mice (mIMCD-3 cells) (Mukhin et. al., 2001) via the similar pathway that Ang II and serotonin employ to activate NHE1 activity in VSMC, which involves the activation of Jak2, tyrosine phosphorylation of Ca^{2+}/calmodulin, and binding of calmodulin (CaM) to NHE-1 (Garnovskaya et al., 2003). We have also described that BK is a potent mitogenic factor for mIMCD-3 cells, and demonstrated that BK-induced cell proliferation was dependent on activation of epidermal growth factor receptor (EGFR) tyrosine kinase and subsequent activation of mitogen- and extracellular signal-regulated kinase kinase (MEK) and (Mukhin et al., 2003; Mukhin et al., 2006; Kramarenko et al., 2010). However, we were not able to establish the relationship between NHE1 and ERK in mIMCD-3 cells. Our data did not support either the hypothesis that ERK is a proximal short-term regulator of NHE or the hypothesis that NHE1 is necessary for the BK-induced ERK activation in normal kidney mIMCD-3 cells (Garnovskaya, unpublished data). Because there is evidence linking BK to the cancerogenic process (Bhoola et al., 2001; Chan et al., 2002), and because NHE1 has been proposed to play role in cancer cells growth (Cardone et al., 2005), we wanted to explore the possibility that BK exerts its mitogenic effects via activation of NHE in cancer cell lines. The expression of BK receptors has been demonstrated in clinical speciments of adenocarcinoma, squamous carcinoma, lymphoma, hepatoma and carcinoid, and in experimental mouse sarcoma 180 and colon adenocarcinoma 38 (Wu et al., 2002), in small cell and non-small cell carcinomas of the lung (Chee et al., 2008), and in oesophageal squamous cell carcinoma (Dlamini et al., 2005). The mitogenic effects of BK have been reported in primary cultured epithelial breast cancer cells and in MCF-7 breast cancer cell line, where BK stimulated cell proliferation through ERK activation (Greco et al., 2005; Greco et al., 2006). Because there were limited studies on the role of BK in renal cell carcinomas, we have chosen to use A498 cells, a transformed cell line derived from primary undifferentiated kidney carcinoma (Giard et al., 1973), which represents a widely used model for studying of renal carcinomas. Our results demonstrated that NHE1 is involved in BK-induced ERK activation and proliferation of A498 cells, and that BK B_2 receptor-induced ERK activation in A498 cells depends on NHE activity (Garnovskaya et

al., 2008), suggesting that the critical role of NHE1 in GPCR-induced ERK activation is not restricted to one specific cell type and receptor. Previously, NHE1-mediated intracellular alkalinization has been proposed to play role in cancer cells growth because it has been shown that increased pH_i of tumor cells is associated with increased *in vivo* tumor growth, DNA synthesis, and cell-cycle progression, suggesting that over-expression of NHE1 contributes to the transformed phenotype of multiple cancer cells (reviewed by Cardone et al., 2005). The cellular alkalinization of tumor cells induced by hyperactivation of NHE1 has been shown to be directly related to increased protein synthesis and tumor cell growth (Cardone et al., 2005; Harguindey et al., 2005). It has been suggested that the mechanism of NHE1-mediated tumor cell growth and metastasis does not depend of its ion-transporting activities but rather employs NHE1 as a scaffolding protein to directly regulate cytoskeletal dynamics (Cardone et al., 2005). Further it has been shown that NHE1 antisense gene suppresses cell growth, induces cell apoptosis, and partially reverses the malignant phenotypes of human gastric carcinoma cells (Liu et al., 2008). Similarly, silencing of NHE1 gene by siRNA interference and /or inhibition of NHE1 activity by amiloride analogs effectively blocked the invasiveness of human hepatocellular carcinoma cells (Yang et al., 2011). Thus, inhibition of NHE1 might result in an antiproliferative effect, and NHE1 may be a potential target for chemotherapeutics to treatment of renal carcinoma.

3. Conclusion

The elucidation and understanding of the relationship between NHE1 and ERK cascade has been one of the most active areas in biological research over the past few years. As discussed above, experimental studies have strongly implicated a role for NHE1 in the regulation of ERK activity, although the precise pathway, which leads from the activation of NHE1 to ERK regulation still has to be defined. One possibility is that GPCR-induced NHE1-dependent ERK activation depends on NHE1-mediated Na^+/H^+ exchange. In that sense, Grinstein et al have demonstrated uneven distribution of NHE1 molecules throughout the cell surface with the focal accumulation at or near terminal edges of fibroblasts and CHO cells, and the areas of increased NHE1 density closely corresponded to sites of accumulation of cytoskeletal proteins (Grinshtein et al., 1993). It is possible that NHE1 regulates ERK by altering Na^+ or H^+ concentrations in cellular microdomains, thereby affecting enzyme activity or protein-protein interactions. Another possibility is that NHE1 acts as a plasma membrane scaffold (Baumgartner et al., 2004) in the assembly of signaling complexes independent of its ion exchange activity bringing together GPCRs, and the members of ERK-activation cascade. Regardless of the mechanisms, the critical role of NHE1 as an upstream molecule in GPCR-induced ERK activation could have significant physiological and pathophysiological relevance.

Because ERK-dependent cell proliferation is thought to be a critical component in many pathologic conditions, and NHE is involved in a variety of complex physiological and pathological events that include regulation of intracellular pH, cell movement, heart disease, and cancer, improved understanding of the molecular mechanisms that regulate NHE and ERK may allow alternative approaches to the therapeutic manipulation of ERK and NHE activity to be developed.

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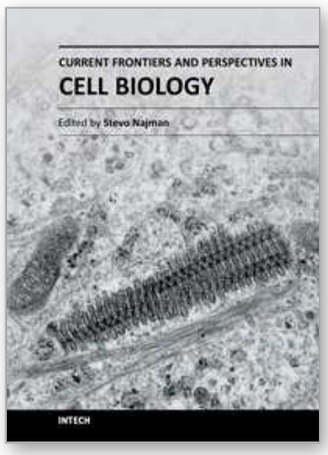

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