Rapid activation of sodium–proton exchange and extracellular signal-regulated protein kinase in fibroblasts by G protein-coupled 5-HT_{1A} receptor involves distinct signalling cascades

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These experiments tested the hypothesis that signalling elements involved in the activation of the extracellular signal-regulated protein kinase (ERK) mediate rapid activation of sodium–proton exchange (NHE) in fibroblasts when both signals are initiated by a single G protein-coupled receptor, the 5-HT_{1A} receptor. Similarities between the two processes were comparable concentration-response curves and time-courses, and overlapping sensitivity to some pharmacological inhibitors of tyrosine kinases (staurosporine and genistein), and phosphoinositide 3′-kinase (wortmannin and LY204002). Activation of NHE was much more sensitive to the phosphatidylcholine-specific phospholipase inhibitor (D609) than was ERK. Neither pathway was sensitive to manoeuvres designed to block PKC. In contrast, Src or related kinases appear to be required to activate ERK, but not NHE. Transfection of cDNA constructs encoding inactive mutant phosphoinositide 3′-kinase, Grb2, Sos, Ras, and Raf molecules were successful in attenuating ERK, but had essentially no effect upon NHE activation. Finally, PD98059, an inhibitor of mitogen activated/extracellular signal regulated kinase kinase, blocked ERK but not NHE activation. Thus, in CHO fibroblast cells, activation by the 5-HT_{1A} receptor of ERK and NHE share a number of overlapping features. However, our studies do not support a major role for ERK, when activated by the 5-HT_{1A} receptor, as a short-term upstream regulator of NHE activity.

INTRODUCTION

The purpose of this study was to test the hypothesis that the extracellular signal-regulated protein kinase type (ERK) of mitogen-activated protein kinase (MAPK) is an immediate upstream regulator of fibroblast Na^+/H^+ exchanger (NHE) activity. The rationale for this hypothesis is based upon several known similarities between the regulation of ERK signalling pathways and the regulation of electroneutral Na^+/H^+ exchangers. 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 [1–4]. Those similarities provide evidence to suggest that ERK may be a direct proximal component of an NHE regulatory pathway [5,6]. There is a growing awareness that tyrosine phosphorylation cycles are critical in regulating NHE activities in a number of cell types [6–10], as has also been shown for ERK [11]. Other studies have demonstrated that NHE and ERK activities are modulated by overlapping upstream enzymes, including phosphoinositide 3′-kinase (PI-3K), phospholipase Cγ, and PKC [12–19]. In aggregate, those studies implicate G proteins, lipid-recognizing enzymes, tyrosine kinases, and NHEs as playing interrelated roles along with ERK in cell growth [2,5,6,11,20].

NHEs are expressed at the surface of all mammalian cells, serving to regulate cell volume, intracellular pH, and transepithelial transport of Na^+ and acid-base equivalents [1,2]. Salient to the current hypothesis are studies showing that microinjection of activated Ras [21] or transfection of the Ha-Ras oncogene [22–24] stimulates NHE activity in fibroblasts. The classical effect of GTP-bound Ras is the activation of the ERK1 and ERK2 [11]. 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. However, it was not clear from the previous studies whether the regulation of NHE activity occurred in the short term, or was mediated mainly by an upregulation of NHE message and protein.

The possibility that ERK rapidly regulates NHE activity was recently tested in platelets by Aharonovitz and Granot [6], 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 [6]. The study of Aharonovitz and Granot [6]

Abbreviations used: AVP, arginine vasopressin; Csk, C terminal Src family kinase; D609, tricyclodecan-9-yl xanthogenate (D609), a putatively specific inhibitor of PC-PLC; ECAR, extracellular acidification rate which in this study is synonymous with NHE activity; ERK, extracellular signal-regulated protein kinase; G protein, guanine nucleotide-binding regulatory protein; genistein, 5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one; H-7, (±)-1-(5-isoquinolinesulphonyl)-2-methylpiperazine; 5-HT, 5-hydroxytryptamine, serotonin; MEK, MAPK/ERK kinase; PDGF, platelet-derived growth factor; PTP1D, cytosolic phosphotyrosine phosphatase; PI-3K, phosphoinositide 3′-kinase; PKC, protein kinase C; PLC, phospholipase C; PC-PLC, phosphatidylcholine-specific phospholipase C.

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specifically supported a role for MEK and a tyrosine kinase in the activation of platelet NHE by AVP, but did not address the role of other molecules in the ERK cascade in this process. More recently, Bianchini et al. [25] presented data to support a role for ERK in activating NHE type 1 in fibroblast cells. 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 half. Because the study of Bianchini et al. [25] used mixtures of growth factors, the generality of the relationship between ERK and short-term NHE activation has yet to be determined, particularly for specific types of G protein-coupled receptors.

We chose to investigate the relationship between NHE and ERK in CHO-K1 fibroblasts which were transfected with the human 5-HT_{1A} receptor, a prototypical $G_{i\alpha}$-linked receptor [26,27]. The relatively selective nature of the coupling of this receptor to G proteins would reduce potential ambiguities that might result from a more complex pattern of G protein coupling. This receptor has also been shown to stimulate both ERK [28,29] and NHE activities [30], and in the main, those data are consistent with a mutual regulatory pathway for NHE and ERK. However, it has been noted that rapid activation of ERK, but not of NHE, is sensitive to sequestration of G protein $\beta\gamma$-subunits, suggesting that ERK might not always be a proximal regulator of NHE in CHO cells over a short-time frame [29,30]. Thus, the current studies were undertaken to establish whether ERK, when activated by the 5-HT_{1A} receptor, is a rapid upstream regulator of NHE in CHO fibroblast cells.

EXPERIMENTAL

Materials

CHO-K1 cells expressing 5-HT_{1A} receptors ($\approx$ 1 pmol of receptor/mg of protein) were obtained as previously described [29]. Cell culture supplies were obtained from Gibco–BRL (Grand Island, NY), the Comprehensive Cancer Center at Duke University, or Corning Costar (Cambridge, MA). EIPA, MIA, and 5-HT were from Research Biochemicals International (Natick, MA). Genistein, daidzein, staurosporine, D609, and H-7 were from LC Laboratories (Woburn, MA). Wortmannin, pertussis toxin and myelin basic protein were from Sigma (St. Louis, MO), and LY294002 was from Biomol (Plymouth Meeting, PA). GF 109 203X (Bis-indolylmaleimide I) was from Sigma (St. Louis). Protein A-agarose and rabbit polyclonal anti-rat MAPK (ERK-1 carboxyl terminus) were from Upstate Biotechnology Inc.

DNA constructs

DNA constructs were obtained from the following sources: dominant negative $p21^{Ras}$ which incorporates an S $\rightarrow$ N mutation at residue 17 that reduces the affinity for GTP [Ras^{N17} [31] was from Drs. D. Aultschuler and M. Ostrowski (Columbus)]. ΔmSos-1, from which the guanine nucleotide exchange domain is deleted, was obtained from Dr. M. Sakaue (Kobe, Japan) [32]. Sos-Pro [33], which contains only the proline-rich carboxy-terminal fragment of mSos1, and Csk [34] were from T. van Biesen and R. J. Lefkowitz (Duke University). Dominant negative Grb2 (ΔNGrb2), in which the N-terminal SH3 domain is deleted, was from A. M. Pendergast (Duke University) [35]. Dominant negative $p74^{Raf\text{-}1}$, (NΔRaf) which only contains the N-terminal regulatory domain of Raf-1 (residues 1–258) [36], inactive p110 (p110Δkin) with a mutated inactive ATP site [37], and constitutively activated p110 (p110*) in which the p85 inter-SH2 domain was ligated to the amino terminus of p110, were from Dr. L. T. Williams (San Francisco) [38]. A dominant negative mutant of p85 (Δp85) which lacks a p110 binding site (residues 479–513) was from Dr. M. Sakaue [39]. DN-SHPT1D, a mutant phosphotyrosine phosphatase type 1D which incorporates a carboxy terminal deletion of 60 amino acids required for its tyrosine phosphorylation, was from Dr. Ben Neel (Beth Israel Hospital) [40]. Wild-type Src, dominant negative Src (K298MSrc) [41] and constitutively activated Src (Src Y527F) which lacks a key inhibitory phosphorylation site [42] were from Dr. L. Luttrell (Duke University).

Microphysiometry

NHE activity was measured in real time as the rate of decrease in extracellular pH in intact cells placed in an eight chamber Cytosensor™ microphysiometer (Molecular Devices Corporation, Sunnyvale, CA). We have previously extensively validated that the 5-HT_{1A} receptor changes in extracellular acidification rate (ECAR) in CHO cells are due almost completely to activation of NHE by the following criteria: (i) Sodium-dependence of the proton efflux; (ii) ability of amiloride analogues to block the increases in ECAR; (iii) lack of dependence upon glycolytic substrate, and (iv) ability of 5-HT to increase ECAR over a range of intracellular pH ($\approx$ 6.6, 7.0, and 7.2) [30].

Expression of signalling constructs

Transient expression of the cDNA constructs was achieved by transfection in the presence of 10–15 μl of lipofectAMINE™ (2 h in serum- and antibiotic-free medium). Two days prior to assays, cells were transfected with 1 μg of the various plasmids. Transfection efficiency ($> 90\,\%$) was evaluated by co-transfecting with 0.2 μg of a humanized version of green fluorescent protein from the jellyfish *Aequorea victoria* (Clontech) as a marker.

ERK assays

ERK activity was measured in immune complexes using myelin basic protein as the substrate [29].

RESULTS

There is ample published justification for positing overlap between the signalling pathways involved in activating NHE and ERK, and in CHO cells both processes are completely sensitive to pertussis toxin when initiated by the 5-HT_{1A} receptor. Moreover, the concentration–response curves and time courses for the two processes are virtually superimposable (Figure 1). The EC_{50} values for 5-HT-activated ERK and NHE were 36 ± 5 and 44 ± 10 nM, respectively. Those data support the hypothesis that the same signalling pathway regulates NHE and ERK.

Lack of role for PKC

We recently showed that ERK activation in CHO cells by the 5-HT_{1A} receptor does not depend upon phorbol ester-sensitive PKC types [29]. Because PKC types are frequently implicated in NHE regulation [1,2], we tested their involvement in our model. Figure 2A shows that 1 μM 5-HT further stimulates NHE in the presence of 1 μM PMA. If 5-HT stimulates NHE primarily through a PKC-dependent-pathway, no response would be expected when PKC is maximally activated. Further support for the non-essential role of PKC in the 5-HT response is shown in Figure 2B. Two PKC inhibitors, 50 μM H-7 [43] and 1 μM GF 109 203X [44], did not alter the stimulatory effect of 1 μM 5-HT

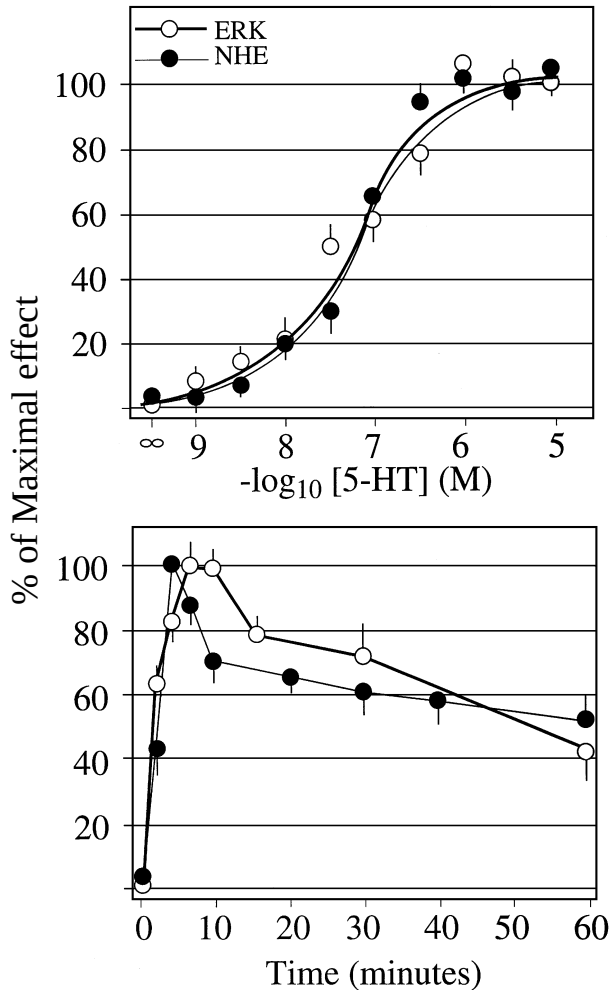

Figure 1 Concentration–response curves and time-courses for 5-HT$_{1A}$ receptor-activated ERK and NHE

Experiments were performed at least three times in duplicate for ERK (○) and NHE (●) as described under Experimental. Data are presented as the mean % of the maximal effect seen in each assay. The bars represent the standard errors from the means of the individual experiments. EC_{50} values were calculated using a non-weighted least-squares fit (Inplot, San Diego).

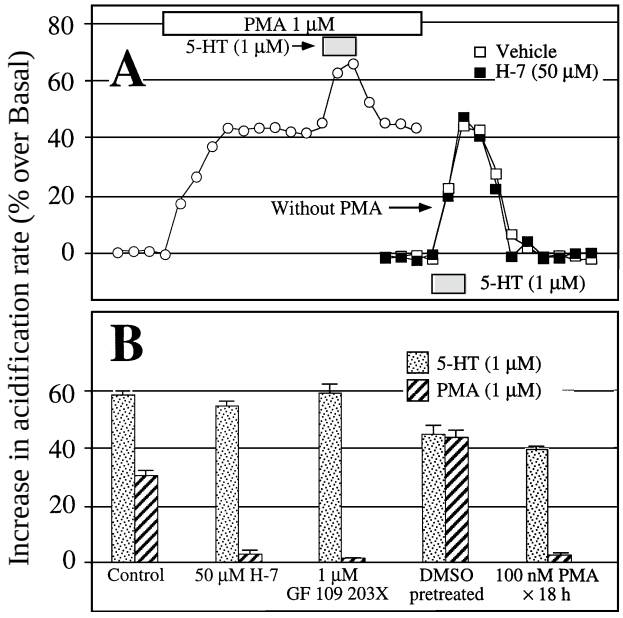

Figure 2 Lack of involvement of PKC in 5-HT-mediated increases in NHE

(**A**) 5-HT further increases extracellular acidification rate in the presence of 1 μM PMA. The righthand panel depicts a representative plot showing that the cyclic nucleotide-dependent kinase inhibitor, H-7, does not inhibit the 5-HT increase in NHE activity. (**B**) This bar graph shows that two inhibitors of PKC (H-7 and GF 109 203X) do not block NHE activation by 5-HT. Overnight pre-treatment with PMA to downregulate PKC inhibits subsequent activation of NHE by PMA, but has no effect upon the increase in NHE induced by 5-HT. Overnight incubation with the vehicle (DMSO) had no effect on the ability of 5-HT and PMA to activate NHE. Experiments were performed in Ham's F-12 medium (bicarbonate- and serum-free) at least three different times in at least two chambers for each condition. Bars depict standard errors.

($n = 8$ and 3, respectively). Neither compound altered basal acidification rates, but both markedly attenuated the stimulation of NHE induced by 1 μM PMA. Finally, prolonged PMA treatment (100 nM overnight) did not alter the ability of 5-HT to increase ECAR while completely eliminating the ability of PMA (1 μM) to increase ECAR (Figure 2B). Thus, like ERK, NHE activation by the 5-HT$_{1A}$ receptor does not involve a phorbol ester-sensitive PKC.

Role of tyrosine kinases

Because activation of NHE and ERKs typically requires activation of phosphorylation cascades, we examined the roles of tyrosine kinases. We previously showed that 5-HT$_{1A}$ receptor activation of ERK could be inhibited by staurosporine and genistein, two broad spectrum protein kinase inhibitors [29]. Staurosporine (100 nM), a broad spectrum kinase inhibitor with effects on both serine/threonine and tyrosine kinases, was able to effectively suppress the effect of 5-HT upon both ERK and NHE, suggesting that a kinase is involved in both pathways. Moreover, because H-7 and GF 109 203X treatment and PKC depletion have no effect on the signals, the effects of staurosporine are most likely secondary to inhibition of tyrosine kinase(s). Genistein, a broad-spectrum tyrosine kinase inhibitor, markedly attenuated the ability of 5-HT to activate ERK and NHE, whereas the structurally similar but inactive compound, daidzein, had no effect, supporting the involvement of tyrosine kinase(s) in both pathways (Table 1). PTP1D is a cytosolic phosphotyrosine phosphatase which has been shown to be a critical positive regulator of tyrosine kinase signals (DNA synthesis and ERK activation) initiated by both growth factor and G protein-coupled receptors [38–49]. Transfection of a cDNA construct encoding a mutant inactive PTP1D (ΔPTP1D) molecule effectively blocked 5-HT-stimulated ERK, but had no effect on NHE activity. Transfection of empty vector, a control used for all of the cDNA experiments, had no effect on the activity of 5-HT in either the ERK or NHE assays. This set of studies strongly supports the involvement of tyrosine phosphorylation cycles in the regulation of NHE and ERK by the 5-HT$_{1A}$ receptor, but shows that there may be differential involvement in the activation of the two effectors. Nevertheless, those studies do not identify the specific tyrosine kinase pathways involved in ERK and NHE activation.

In that regard, recent work has focused upon the roles of Src family nonreceptor tyrosine kinases in mediating $G_{i/o}$ protein $\beta\gamma$-subunit activation of ERK [50], and in modulating NHE activity in renal cells [52]. We used several cDNA constructs to examine the potential role of Src family kinases in the 5-HT-induced

Table 1 Comparative effects of inhibitors of candidate molecules upon activation of NHE and ERK by 5-HT

All data are expressed as the mean % inhibition of the effects on 5-HT activated NHE and ERK Higher percentage indicates more effective blockade. Experiments were performed at least three times in duplicate for each assay condition; the numbers of duplicate repetitions for each experiment are indicated in parentheses. N.E. means no effect ($\leqslant 5\%$). ECAR means extracellular acidification rate, a measure of NHE activity.

Inhibitor or cDNA construct	% Inhibition of 5-HT-stimulated ERK phosphorylation	% Inhibition of 5-HT-stimulated ECAR
Chemical inhibitors		
Staurosporine (100 nM)	67 ± 9 (5)	84 ± 7 (8)
Genistein (100 μM)	82 ± 3 (7)	90 ± 3 (7)
Daidzein (100 μM)	N.E. (5)	N.E. (8)
Wortmannin (100 nM)	71 ± 8 (7)	65 ± 4 (6)
LY294002 (100 μM)	81 ± 2 (3)	77 ± 2 (3)
D609 (50 μM)	N.E. (3)	73 ± 2 (3)
D609 (100 μM)	13 ± 1 (3)	76 ± 1 (3)
PD98059 (20 μM)	64 ± 4 (5)	N.E. (4)
PD98059 (100 μM)	81 ± 5 (5)	8 ± 1 (4)
cDNA constructs		
Empty vector	N.E. (6)	N.E. (6)
Csk	81 ± 4 (3)	N.E. (3)
Wild-type Src	N.E. (3)	N.E. (3)
K298M-Src	68 ± 2 (3)	N.E. (3)
Src-Y527F	N.E. (3)	10 ± 1 (3)
ΔPTP-1D	70 ± 2 (3)	N.E. (4)
Δp85	84 ± 5 (5)	N.E. (3)
p110ΔKin	83 ± 5 (4)	N.E. (3)
p110*	N.E. (3)	N.E. (3)
ΔNGrb2	60 ± 7 (4)	N.E. (3)
Sos-Pro	66 ± 6 (4)	N.E. (3)
ΔmSos	71 ± 5 (3)	N.E. (3)
Ras^{N17}	90 ± 1 (7)	N.E. (3)
NAF	85 ± 4 (5)	N.E. (4)

activation of NHE and ERK. First, transfection with Src slightly augmented the 5-HT-induced activation of ERK, while having no effect on NHE. Second, we transfected cells with an inactive Src mutant (K298M-Src) and a constitutively activated Src mutant (Src-Y527F). K298M-Src greatly attenuated the activation of ERK by 5-HT while having no effect on NHE activation by 5-HT. Src-Y527F neither attenuated nor augmented the effect of 5-HT on ERK and NHE, but did significantly increase the basal ERK ($252 \pm 34\%$) and NHE ($72 \pm 10\%$) activities. Third, we transfected cells with Csk, a protein kinase that inactivates Src family tyrosine kinases by phosphorylating a tyrosine residue in the carboxyl terminus of the Src molecule [51]. Transfection of Csk had no effect on NHE while it nearly completely attenuated the ability of 5-HT to activate ERK (Table 1). Those studies support a role for Src family kinase(s) in the activation by the 5-HT_{1A} receptor of ERK, but not of NHE activity in CHO cells.

Role of phospholipids

Phospholipid products have been implicated as important regulators of NHE and ERK activity. In particular, much work has focused upon the products of phospholipase C (PLC) and phosphoinositide-3′-kinase (PI-3K). Specifically for the 5-HT_{1A} receptor expressed in CHO cells, evidence for the involvement of a phosphatidylcholine specific PLC (PC-PLC) [28] and a PI-3K [28,29] in the activation of ERK has been presented. Therefore, we studied the involvement of those enzymes in the activation of NHE activity. Two structurally distinct PI-3K inhibitors, wortmannin and LY294002 each inhibited 5-HT activation of ERK and NHE in concentrations at which each is reported to be highly specific for PI-3Ks [52–54] (Table 1). To further confirm the involvement of PI-3K in the regulation of NHE and ERK, we transfected cDNAs encoding a mutated p85 subunit of PI-3K (Δp85), a kinase-deficient mutant of the catalytic p110 subunit of PI-3K (p110Δkin). Transfection of both plasmids (but not the empty plasmid) greatly attenuated the response of ERK to 5-HT, but did not affect NHE activity. In contrast, transfection of a constitutively activated form of p110 (p110*) had no effect on either activity. Therefore, this set of experiments supports an important role for PI-3K(s) as critical participants in the ability of the 5-HT_{1A} receptor to activate ERK and NHE, but also provides evidence for differential involvement in each pathway.

In the next set of experiments, tricyclodecan-9-yl xanthogenate (D609), a putatively specific inhibitor of PC-PLC [55] was used. Incubation with D609 effectively inhibited activation of NHE, and had little effect upon the activation of ERK by the 5-HT_{1A} receptor (Table 1), further distinguishing between activation of NHE and ERK.

Role of known elements that convey the $\beta\gamma$-mediated signal to stimulate ERK

Other experiments were designed to examine the role of the known elements that convey the $\beta\gamma$-mediated signal to stimulate ERK, those elements being the adaptor molecule Grb2, the Ras-GTPase-activating factor Sos, and the kinases Ras, Raf and MEK [12,29,37,50]. For those studies we relied primarily upon dominant negative cDNA constructs for Grb2 (ΔNGrb2), for Sos (ΔmSos and Sos-Pro), Ras (Ras^{N17}), and Raf (NΔRaf). In order to test the involvement of MEK, we used a specific MEK inhibitor, PD98059 [56]. The results of those experiments show that all of the manoeuvres blocked ERK, whereas they had no effect on NHE (Table 1) Those studies effectively rule out a major role for the proximal components of the $\beta\gamma \rightarrow$ ERK pathway in the short-term regulation of NHE in the CHO cell, at least when ERK and NHE are activated by the 5-HT_{1A} receptor.

DISCUSSION

The major finding of this study is that the signalling pathways that increase ERK and NHE activity in CHO cells diverge when both are stimulated by the 5-HT_{1A} receptor. This finding was unexpected based on work with other growth-modulating receptors, and thus provides evidence that ERK does not always serve as a short-term regulator of NHE activity, even in settings in which both are activated. Thus, this work clarifies and refines an evolving paradigm.

NHE and ERK effector pathways are associated with growth and differentiation of many cell types, although each effector may serve other purposes. Many similarities have been noted between the regulation of NHE and ERK activities, leading to the generation of the hypothesis that ERK is a proximal regulator of NHE [5,6]. There is little doubt that sustained activation of ERK can increase NHE activity. Transfection of the Ha-Ras oncogene [22–24] stimulate sodium-dependent NHE activity in fibroblasts. This effect is most likely mediated through activation of transcription cascades that upregulate the NHE message/protein or modulate expression of key regulators of NHE activity. Moreover, recent work suggests that ERK might regulate NHE activity in the short term, as well.

The recent study of Aharonovitz and Granot [6] showed that AVP and PMA rapidly activated NHE by a pathway which was sensitive to PD98059, a specific inhibitor of MEK1. The in-

volvement of MEK-ERK was interpreted to occur through a pathway that does not require transcription or new protein synthesis, because platelets do not have nuclei or machinery for protein synthesis, and the effects of AVP were studied over a very short time frame (minutes). Their study [6] specifically supported a role for MEK and a tyrosine kinase in the activation of platelet NHE by AVP. Bianchini et al. [25] went further to characterize the role of ERK when activated by combinations of growth factors or serum in regulating NHE. They used a dominant negative construct of p44 ERK and one encoding a wile-type MAPK phosphatase (MKP-1), to show an $\approx 50\,\%$ reduction in ERK activity by growth factors. They obtained similar results with the MEK inhibitor PD98059. It was not certain that their results could be generalized to other receptors or other cell types.

The current studies were designed to test the role of a number of signalling molecules as proximal mediators of NHE and ERK activities initiated by a single receptor subtype, the 5-HT_{1A} receptor, expressed in CHO fibroblast cells. The CHO cell is particularly convenient in that extremely high transfection efficiencies ($> 90\,\%$) are possible with a simple protocol, allowing the use of transiently expressed inhibitory cDNA constructs as well as cell-permeable pharmacological inhibitors. Although we were able to demonstrate that there is some regulatory overlap between the ERK and NHE pathways, the majority of our data do not support the hypothesis that ERK is a proximal short-term regulator of NHE in CHO cells, at least when the signal is initiated by the $G_{i/o/z}$ protein-coupled 5-HT_{1A} receptor.

Although it appears that tyrosine kinases may be involved in the activation of both NHE and ERK by the 5-HT_{1A} receptor, studies with overexpression of the Src inhibitory kinase, Csk, and various Src constructs support a role for Src family kinases in ERK, but not NHE, activation.

PI-3K activities have been implicated in the activation of NHE-1 fibroblasts [60], as well as in ERK activation [29,50]. In the current study, two structurally distinct PI-3K inhibitors blocked both pathways, whereas dominant negative cDNAs encoding mutant p85 and p110 subunits of PI-3K blocked only the ERK signal. Does this mean that PI-3K is not involved in the activation of NHE initiated by the 5-HT_{1A} receptor? Not necessarily. One possibility is that distinct PI-3Ks mediate the two responses. In that regard, PI-3K species vary in their regulation by G protein subunits. A number of cells have recently been shown to have cytosolic PI-3K activities that can be activated by G protein $\beta\gamma$-subunits, but $G_{i\alpha}$-subunits can also stimulate certain PI-3K activities [57–59]. It is also conceivable that two distinct PI-3Ks are involved in activation of ERK and NHE, one being both stimulated by $\beta\gamma$ and sensitive to expression of Δp85 and p110Δkin, and the other being activated by α-subunits of the G_i proteins and insensitive to expression of Δp85 and p110Δkin. A second possibility is that the same PI-3Ks are involved, but that their roles in each process are distinct. For example, perhaps PI-3K serves as a signalling platform or adaptor in one pathway which does not require its kinase activity *per se*, whereas the other pathway requires PI-3K activity to yield an important biological intermediate product like phosphatidylinositol 3,4,5-trisphosphate. In such a scenario, kinase defective p110 could block ERK, but not NHE activation. A third possibility is that NHE is more efficiently coupled to PI-3K than is ERK, and that neutralization of more PI-3K molecules is required to attenuate NHE than ERK activity. This possibility is unlikely in that both processes are inhibited by similar half maximal concentrations of wortmannin ($\approx$ 20–50 nM). A fourth possibility is that the PI-3K activities are compartmentalized such that the mutated signalling molecules do not have access to a specific pool of PI-3K that mediates the NHE activation. Such a compartmentalization of PI-3K effects has been shown in 3T3-L1 adipocytes when stimulated with insulin and platelet-derived growth factor [61]. A fifth possibility is that the chemical inhibitors are blocking another enzyme which is distinct from PI-3K, such as the target of rapamycin [62] or PI-4 kinase [63]. Although our results do not clarify which of those mechanisms is operant, they do provide further evidence for differential regulation of ERK and NHE by the 5-HT_{1A} receptor.

The current studies extend a previous study [28] showing that the 5-HT_{1A} receptor activates ERK through a pathway that is partially sensitive to $\approx 200\ \mu$M D609, a putative specific inhibitor of PC-PLC [55], by showing that D609 also effectively blocks activation of NHE by the 5-HT_{1A} receptor. Although the specificity of D609 is far from established, the remarkably different concentration-dependency and efficacy of D609 to inhibit both processes suggests that the pathways are not identical.

A final set of experiments examined the role of the known elements that convey the $\beta\gamma$-mediated signal to stimulate ERK in the activation of NHE. Dominant negative cDNA constructs for Grb2 (ΔNGrb2), Sos (ΔmSos and Sos-Pro), Ras (Ras^{N17}), and Raf (NΔRaf), as well as a specific MEK inhibitor, PD98059 [56] blocked ERK, whereas they had no effect on NHE. Moreover, PD98059 was not effective in the current studies even when used at a concentration (100 μM) that was 10-fold higher than in the study by Bianchini et al. [25]. The current studies effectively ruled out a major role for the proximal components of the $\beta\gamma \rightarrow$ ERK pathway in the short-term regulation of NHE in the CHO cell when stimulated through the transfected human 5-HT_{1A} receptor.

The differences in our results and those of Aharonovitz and Granot [6] can probably be explained either by cell- or receptor-specific differences, or possibly by different methods of NHE measurement. Although they relied mainly on the efficacy of PD98059 to support a role for ERK as a rapid upstream regulator of NHE stimulation, their platelet model allowed them to rule out the requirement for transcription or new protein synthesis because platelets do not have nuclei or machinery for protein synthesis. It is possible that there are fundamental differences in the ways that adherent cells such as CHO cells, and circulating cells such as platelets might activate NHE. Our measurements of NHE activity were done with a microphysiometer, which allowed the measurement of NHE activation in relatively unperturbed (non-acid-loaded) cells in monolayer. This protocol allowed for normal cell–cell contact, which through integrin-mediated signals might exert important modulatory signals on both NHE and ERK.

It is somewhat more difficult to explain the discrepancies between the current report and the study of Bianchini et al. Both studies used multiple methods including cDNA constructs and pharmacological tools to investigate the involvement of ERK as a rapid upstream regulator of NHE [25]. Like the current report, the protocol of Bianchini et al. [25] used adherent cells, but they measured NHE activity using a [^{14}C]benzoic acid redistribution technique that required a prolonged preincubation with a buffer at pH 7.0. One possible explanation for the discrepancy between their results and ours it that their protocol may have primed cells to respond to the ERK pathway. However, this explanation seems unlikely in that the host cell used for their transfection studies (PS120) has been shown to have a resting cell pH_i of about 7.2 when incubated in bicarbonate-free extracellular medium with pH_o of 7.0 [64], whereas our cells have a resting pH_i of 7.23 under conditions used for the microphysiometry studies [30].

In summary, this group of experiments tested the hypothesis that ERK, or signalling elements involved in its activation, could

mediate rapid, short-term activation of NHE activity in fibroblasts when both signals were initiated by a single G protein-coupled receptor. 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). Neither pathway was sensitive to manoeuvres designed to block PKC. However, major differences in the regulation of the two pathways were noted. Src or related kinases appear to be required for conveyance of the ERK stimulation, but not for NHE activation. Definitive studies designed to block signalling molecules possessing well-defined roles in activating ERK through the 5-HT_1 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. Finally, PD98059, a MEK inhibitor, blocked ERK, but not NHE activation mediated by the 5-HT_{1A} receptor. Thus, in CHO fibroblast cells, activations by the 5-HT_{1A} receptor of ERK and NHE share a number of overlapping features. However, these studies do not support a major role for ERK, when activated by the 5-HT_{1A} receptor, as a short-term upstream regulator of NHE activity.

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