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ARTICLES

Activated Mutant of $G\alpha_{12}$ Enhances the Hyperosmotic Stress Response of NIH3T3 Cells

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Abstract Heterotrimeric G protein G12 stimulates diverse physiological responses including the activities of Na^+/H^+ exchangers and Jun kinases. We have observed that the expression of the constitutively activated, GTPase-deficient mutant of $G\alpha_{12}$ ($G\alpha_{12}QL$) accelerates the hyperosmotic response of NIH3T3 cells as monitored by the hyperosmotic stress-stimulated activity of JNK1. The accelerated response appears to be partly due to the increased basal activity of JNK since cell lines—such as NIH3T3 cells expressing JNK1—in which JNK activity is elevated, show a similar response. NIH3T3 cells expressing $G\alpha_{12}QL$ also display heightened sensitivity to hyperosmotic stress. This is in contrast to JNK1–NIH3T3 cells that failed to enhance sensitivity although they do exhibit an accelerated hyperosmotic response. Reasoning that the increased sensitivity seen in $G\alpha_{12}QL$ cells is due to a signaling component other than JNK, the effect of dimethyamiloride, an inhibitor of Na^+/H^+ exchanger in this response, was assessed. Treatment of vector control NIH3T3 cells with 50 μM dimethylamiloride potently inhibited their hyperosmotic response whereas the response was only partially inhibited in $G\alpha_{12}QL$-NIH3T3 cells. These results, for the first time, identify that NHEs are upstream of the JNK module in the hyperosmotic stress-signaling pathway and that $G\alpha_{12}$ can enhance this response by modulating either or both of these components namely, JNKs and NHEs in NIH3T3 cells. J. Cell. Biochem. 81:1–8, 2001.

Key words: G proteins; $G\alpha_{12}$; JNK; p38MAPK; ERK; hyperosmotic stress; cell volume regulation

Maintenance of cellular volume, largely represented by cell water content, is fundamental to all aspects of regulated metabolic and cell-physiological functions including cell division, growth, and apoptosis [Bortner and Cidlowski, 1998; Lang et al., 1998]. Although the regulatory mechanism(s) involved in cell volume regulation is not fully understood, it appears to include heterotrimeric G proteins, small GTPases, and protein kinases. The best-studied mechanisms are the regulatory volume decrease, or RVD, which follows hypotonic medium-induced cell swelling and the regulatory volume increase (RVI) that follows hypertonic medium induced cell-shrinking [Hoffmann and Simonsen, 1989; Grinstein and Foskett, 1990; Bussolati et al., 1996]. In mammalian cells, activation of Na^+/H^+ exchangers (NHEs) has been observed during RVI as well as RVD [Grinstein et al., 1993; MacLeod and Hamilton, 1996]. Furthermore, recent studies have indicated that the activation of extracellular signal-regulated kinases (ERKs), Jun kinases (JNKs) and/or p38MAP kinases (p38MAPKs) are closely associated with the signaling pathways governing RVI [Terada et al., 1994; Matsuda et al., 1995; Schliess et al., 1995; Shrode et al., 1997]. It has also been observed that the expression of JNK or p38MAPK can

Abbreviations used: G protein, heterotrimeric guanine nucleotide binding protein; JNK, Jun kinase; ERK, extracellular-signal regulated kinase; p38MAPK, p38-mitogen activated protein kinase; MEKK, MAPK/ERK kinase kinase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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readily rescue yeast cells deficient in HOG-1 kinase, which is involved in the osmotic response pathway [Galcheva-Gargova et al., 1994; Han et al., 1994] suggesting a critical role for these kinases in the regulation of cell volume. In this context, it is interesting to note that $G\alpha_{12}$ can stimulate NHEs as well as JNKs in many diverse cell-types [Vara Prasad et al., 1995; Wadsworth et al., 1997]. However, the role of $G\alpha_{12}$ during hyperosmotic stress and volume control has not been investigated. In this report, we present our results showing that JNKs are more potently activated in response to hyperosmotic stress in NIH3T3 cells and that the expression of $G\alpha_{12}$ dramatically accelerates the kinetics of the hyperosmotic response through its activation of JNKs. We also describe our finding that the activation of JNK in response to hyperosmotic stress is partially dependent on the activity of dimethylamiloride-sensitive NHEs. These results, for the first time, identify that NHEs are upstream of the JNK module in the hyperosmotic stress-signaling pathway and that $G\alpha_{12}$ can enhance this response by modulating either or both of these components namely, JNKs and NHEs.

MATERIALS AND METHODS

Plasmids, Cells, and Culture Conditions

NIH3T3 cells (ATCC) were maintained as previously described [Dhanasekaran et al., 1994; Vara Prasad et al., 1995]. The expression vectors pcDNA3-α_{12}Q229L ($G\alpha_{12}$QL) have been described before [Vara Prasad et al., 1995; Wadsworth et al., 1997]. The establishment of $G\alpha_{12}$QL-NIH3T3 cells has been previously described [Vara Prasad et al., 1995]. Stable expression of HA-JNK1 in NIH3T3 cells involved the shuttling of HA-JNK1 cDNA insert from pSRα vector [Vara Prasad et al., 1995] into pcDNA3 vector using the 5′ *Hin*dIII and 3′ *Bam*HI cloning sites. HA-JNK1-pcDNA3 was electroporated into NIH3T3 cells following the previously described methods. Functional expression of HA-JNK1 in NIH3T3 cells was established by immunoblot and activity analyses (Fig. 4A). RasQL-NIH3T3 and vSrc-NIH3T3 cells were kindly provided by Dr. Scott K. Shore, Fels Institute, Philadelphia, PA. Actively growing cells were seeded in 100-mm dishes containing DMEM with 10% calf serum. After 24 h, the cells were washed with PBS (pH 7. 2) and hyperosmotic stress was given by exposing the cells to varying concentrations of NaCl in phosphate buffer (pH 7.2). The cells were harvested at different time points and the kinase assays were carried out following previously published procedures [Vara Prasad et al., 1995; Wadsworth, et al., 1997].

Immunocomplex/Solid-Phase Kinase Assays

Cell extracts were prepared as described previously [Vara Prasad et al., 1995]. ERK2 and p38MAPK were immunoprecipitated using ERK2 (sc-154) or p38MAPK antibodies from 100 μg of cell lysate for 1 h followed by an additional incubation with 20 μl of protein-A Sepharose (Pharmacia) for 1 h. Similarly, solid-phase JNK assays were performed by incubation of 100 μg of protein lysate with glutathione sepharose 4B-bound GST-cJun(1–79) for 2 h as described previously [Vara Prasad et al., 1995]. The kinase assays were carried out with the protein- or immuno-complex bound to glutathione sepharose 4B or protein-A Sepharose beads respectively, using the appropriate substrates [Vara Prasad et al., 1995; Wadsworth, et al., 1997]. The phosphrylated GST-cJun(1–79), GST-ATF2(2-105), or MBP were separated by SDS-PAGE and the radioactive bands were excised and quantified in a scintillation counter. pGEX-5X-3 vector expressing GST-ATF2(2–105) was a kind gift from Dr. Lynn Heasley, University of Colorado Health Sciences Center, Denver, CO. While ERK2 antibodies were from a commercial vendor (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), p38MAPK antibodies raised against the C-terminal peptide were kindly provided by Gary L. Johnson, University of Colorado Health Sciences Center, Denver, CO.

RESULTS

Hyperosmotic Stress Differentially Regulates MAP Kinases in NIH3T3 Cells

Osmotic stress results in the activation of JNKs, p38MAPKs, or ERKs in a cell-type-specific manner [Terada et al., 1994; Matsuda et al., 1995; Schliess et al., 1995; Krump et al., 1997; Shrode et al., 1997; van der Wijk et al., 1998; Parrott and Templeton, 1999]. Hyperosmotic stress stimulates both JNKs and p38MAPKs in many different cells whereas only ERK is activated in some cell-types [van der Wijk et al., 1998]. Therefore, we sought to define the kinase module that is stimulated

upon hyperosmotic stress in NIH3T3 cells. NIH3T3 cells were subjected to hyperosmotic stress by treating them with 600 mOsm NaCl and the activities of JNK, p38MAPK, and ERK were monitored in response to such hyperosmotic stress. In response to the hyperosmotic stress, JNKs were potently activated whereas p38MAPKs were weakly activated (Fig. 1). By contrast, ERK activity was drastically attenuated by the hyperosmotic stress in these cells (Fig. 1).

Expression of the Activated Mutant $G\alpha_{12}$ Accelerates the Hyperosmotic Response of NIH3T3 Cells

Expression of the constitutively activated mutant of $G\alpha_{12}$ ($G\alpha_{12}QL$) greatly enhanced the activity of JNKs in response to hyperosmotic stress compared to the control NIH3T3 cells. Kinetic analysis of the activation of JNKs in response to hyperosmotic stress indicated that the cells expressing $G\alpha_{12}QL$ showed a persistent, stronger response to hyperosmotic stress (Fig. 2).

Since the expression of $G\alpha_{12}$ leads to the potent activation of JNK in NIH3T3 cells, the basal activity of JNKs in cells expressing $G\alpha_{12}QL$ is considerably higher than that of the control cells. Hence, it is possible that the rapid response seen in $G\alpha_{12}QL$-NIH3T3 cells is due to the increased activity of JNK. Alternatively, the increased response could be a function of other JNK-independent signaling pathway(s) regulated by $G\alpha_{12}QL$. To investigate whether

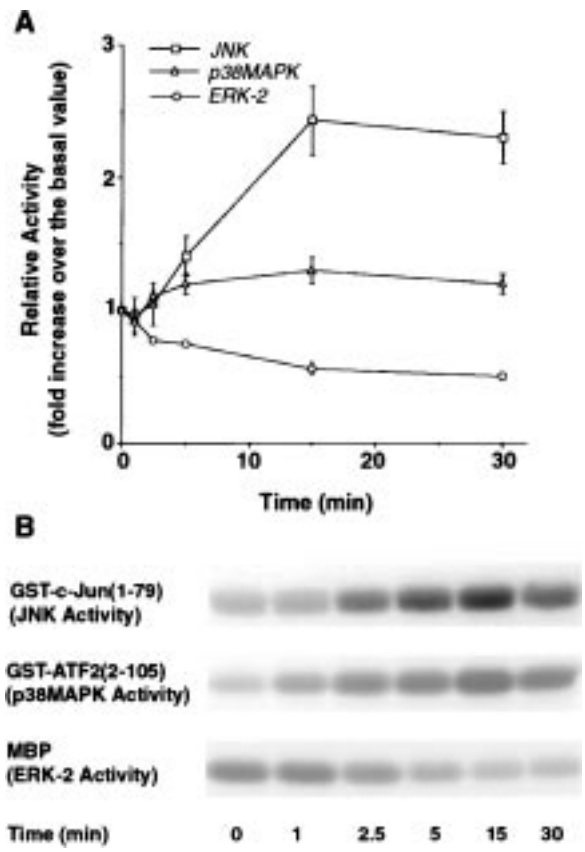

Fig. 1. Effect of 600 mOsm hyperosmotic stress on the activities of JNKs, p38MAPKs, and ERKs in NIH3T3 cells. NIH3T3 cells were treated with 300 mM NaCl and the activities of JNK, p38MAPK, and ERK2 were assayed at different time points according to the methods described under Experimental Procedures. The values are expressed as the fold-increase over basal (time 0) JNK activities. Mean ± SE values determined from three independent experiments are shown (**A**). A representative autoradiogram shows the phosphorylation profiles of the respective substrates at the specific time points (**B**).

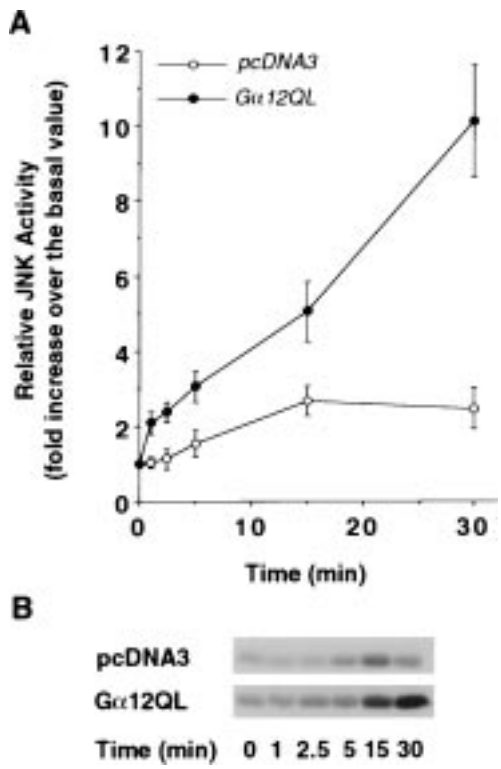

Fig. 2. Effect of $G\alpha_{12}QL$ expression on the hyperosmotic stress response of NIH3T3 cells. NIH3T3 cells expressing $G\alpha_{12}QL$ along with vector-control group were treated with 300 mM NaCl and JNK activity was monitored at different time points following the hyperosmotic stress as described under Experimental Procedures. The values are expressed as the fold increase over basal (time 0) JNK activities in each cell line (**A**). The values represent the mean ±SE of three different experiments. An autoradiogram from a typical experiment is also shown (**B**).

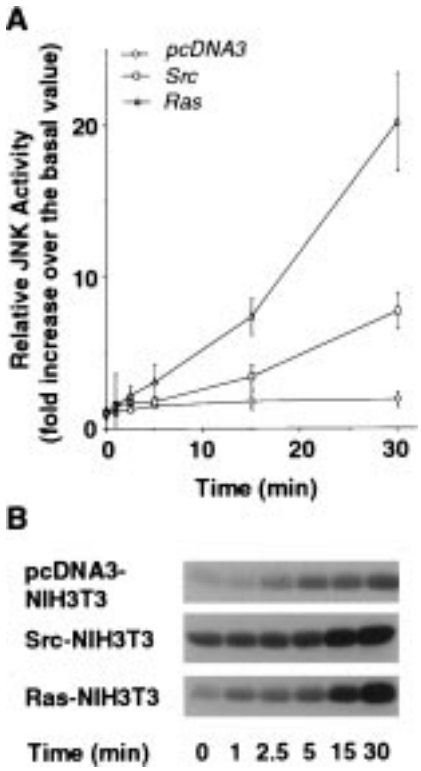

Fig. 3. Effect of RasQL and vSrc expression on the hyperosmotic stress response of NIH3T3 cells. JNK activities in NIH3T3 cells expressing RasQ61L or v-Src along with vector control cells were monitored at different time points following treatment with 300 mM NaCl. The values are expressed as the fold increase over basal (time 0) JNK activities of each cell line (**A**). The values represent the mean ±SE of three independent experiments and the autoradiographic profile of a representative experiment is presented in Panel **B**.

the increased response to hyperosmotic stress is a function of elevated basal JNK activity, we examined the hyperosmotic response of JNK in other cell lines in which the basal levels of JNK activity have been elevated by signaling pathways other than that of $G\alpha_{12}$. It has been observed that JNKs are constitutively activated in NIH3T3 cells transformed by Ras or vSrc (Fig. 3B). When these cells were subjected to hyperosmotic stress and the activation profiles of JNKs were monitored, the results indicated that both Ras- and vSrc-transformed cells showed a hyperosmotic response similar to that of $G\alpha_{12}$ (Fig. 3A). Since the basal activity of JNK is elevated in all of these cell lines, a plausible hypothesis is that the accelerated response to hyperosmotic stress may be due to the elevated basal activity of JNK. However, these NIH3T3 cell lines along with $G\alpha_{12}$QL transfectants display a transformed phenotype. Therefore, it can be argued that the accelerated response is a function of the transformed phenotype. To delineate further that the accelerated response is not a function of the transformed phenotype, we examined the hyperosmotic response of NIH3T3 cells in which JNK1 was over-expressed. While the NIH3T3 cells over-expressing epitope-tagged JNK1 (HA-JNK1-NIH3T3) show an elevated level of JNK activity (Fig. 4A), they are not transformed and show a normal phenotype. When

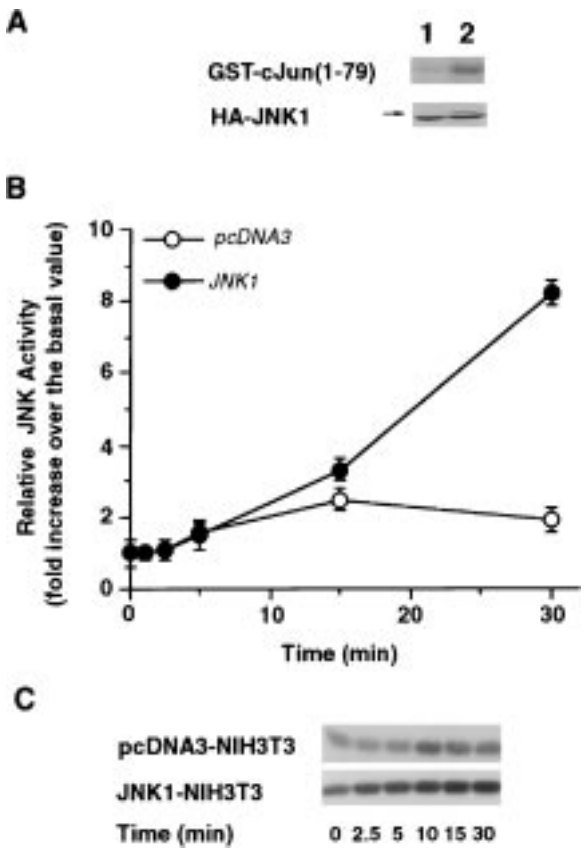

Fig. 4. Effect of JNK1 expression on the hyperosmotic stress response of NIH3T3 cells. Lysates from pcDNA3-NIH3T3 (**Lane 1**) and HA-JNK1-NIH3T3 cells (**Lane 2**) were utilized for immunoblotting and solid-phase JNK assays. Western blotting using anti-HA antibodies demonstrates that NIH3T3 cells stably expressing hemagglutinine epitope-tagged JNK1 were established. The resultant increased JNK activity in these cells is shown by increased phosphorylation of GST-cJun(1–79) using a solid-phase kinase assay as described under Experimental Procedures (**A**). These cells along with vector control group were treated with 300 mM NaCl for the indicated times and solid-phase JNK assays were performed. The JNK activity was expressed as fold increase over the basal (time 0) levels of each cell line. Mean ± SE values from three independent experiments are shown (**B**). An autoradiographic profile from a typical experiment is presented in Panel **C.**

these HA-JNK1-NIH3T3 cells were subjected to hyperosmotic shock, they showed a similar increased response to hyperosmotic stress (Fig. 4B,C). Together, these results suggest that the increased JNK activity sensitizes NIH3T3 cells to respond more avidly to the hyperosmotic stress stimuli.

Expression of the $G\alpha_{12}$QL Enhances the Sensitivity of NIH3T3 Cells to Hyperosmotic Stress

In *S. cerevisiae*, the response to hyperosmotic stress has been shown to involve two signaling components, namely a sensing component and a regulatory component [Davenport et al., 1995; Wurgler-Murphy and Saito, 1997; Posas et al., 1998]. While PBS2 and HOG1 kinases form part of the regulatory component, distinct osmosensors such as SLN1 and SHO1 define the independent sensing components. An analogous paradigm can be envisioned in mammalian cells in which the JNK- or p38MAPK-signaling module is involved in the regulation of adaptive responses that include the expression of genes involved in osmoregulation and volume changes. Although the osmosensory components involved in these pathways remain to be identified, it is possible that $G\alpha_{12}$ enhances the hyperosmotic responses of NIH3T3 cells by stimulating both the osmosensory and osmoregulatory components of this pathway. Therefore, we sought to examine whether $G\alpha_{12}$ enhances the hyperosmotic response by regulating either or both the components of this signaling pathway.

To define whether the enhanced responsiveness to hyperosmotic stress also involves the stimulation of the putative "osmosensor," we investigated whether these cells showed an increased sensitivity to hyperosmotic stress. The cells expressing $G\alpha_{12}$QL were exposed to increasing strengths of hyperosmotic solutions ranging from 300 to 1200 mOsm/kg H_2O. The control cells were responsive only at 600–900 mOsm, whereas the cells expressing $G\alpha_{12}$QL showed increased sensitivity by exhibiting a potent JNK response from 400 mOsm onward (Fig. 5). In contrast to $G\alpha_{12}$QL-NIH3T3 cells, the cells expressing JNK1 failed to display such an increased sensitivity to hyperosmotic stress. Although the magnitude of hyperosmotic response was higher, they were similar to the control cells in terms of sensitivity (Fig. 5). Thus, the increased sensitivity to hyperosmotic stress

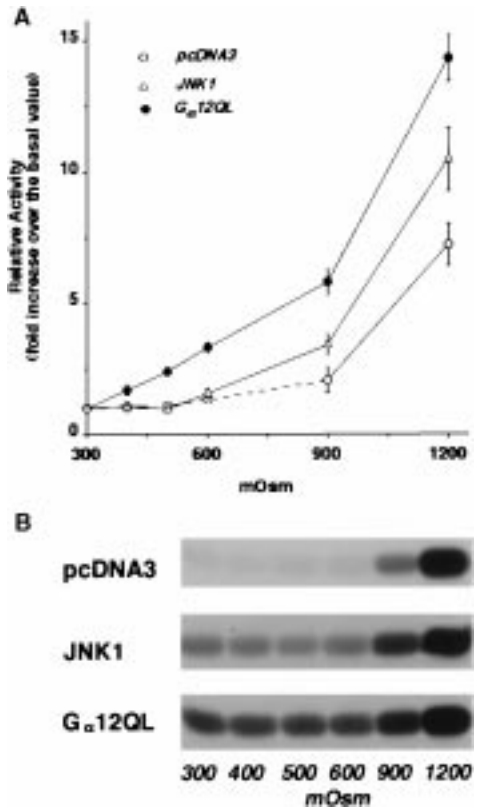

Fig. 5. Effect of $G\alpha_{12}$QL expression on the hyperosmotic stress sensitivity of NIH3T3 cells. NIH3T3 cells expressing $G\alpha_{12}$QL, HA-JNK1, or empty vector (pcDNA3) were treated with varying hyperosmotic concentrations of NaCl. At 10 min following hyperosmotic stress, JNK activity was monitored and quantified according to the methods described before. The JNK activities expressed as fold increase over the basal (time 0) values of each cell line represent the mean ±SE of three independent experiments (**A**). An autoradiographic profile from a typical experiment is shown (**B**).

observed in $G\alpha_{12}$QL-NIH3T3 cells must be due a pathway distinctly different from that of JNK1. These results suggest the possibility that $G\alpha_{12}$ stimulates the regulatory as well as the sensory components of hyperosmotic response.

Inhibition of NHEs Blunts the Hyperosmotic Response of NIH3T3 Cells

Our previous studies have shown that $G\alpha_{12}$ potently activates NHEs in diverse cell types including NIH3T3 cells and that 50 μM dimethylamiloride (DMA) can effectively inhibit their activities [Dhanasekaran et al., 1994; Wadsworth et al., 1997]. The expression of constitutively activated $G\alpha_{12}$ in NIH3T3 cells results in an increased intracellular pH compared to the vector-control cells (7.49 ± 0.03 vs.

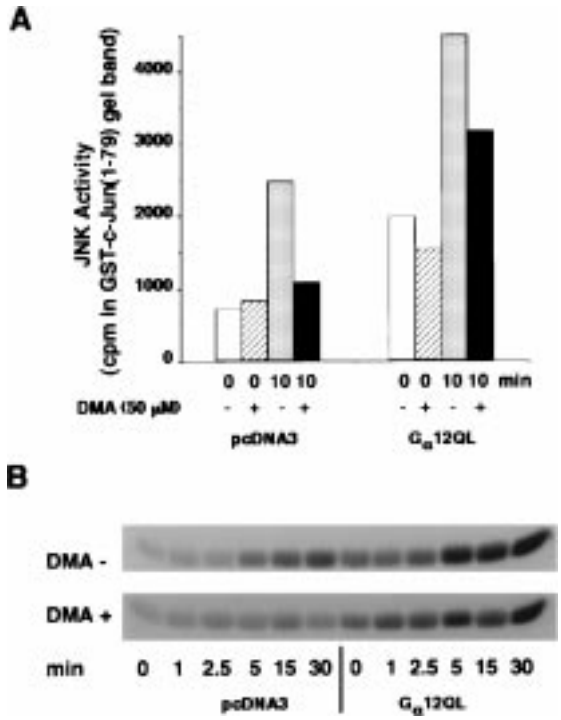

Fig. 6. Effect of DMA on the hyperosmotic response of NIH3T3 cells. NIH3T3 cells expressing $G\alpha_{12}QL$ or control vector (pcDNA3) were treated with 50 μM dimethylamiloride for 5 min prior to treatment with phosphate-buffered 300 mM NaCl (pH 7.2). Cells not treated with DMA were included as controls. At the indicated times following hyperosmotic stress the cells were lysed and JNK activities were monitored by solid-phase kinase assay using c-*Jun* (1–79) as substrate. (**A**) shows the actual cpm values from a typical experiment where the cells were treated for 0 or 10 min with 300 mM NaCl. A representative autoradiogram of a longer time course is presented in (**B**). The experiment was repeated three times with similar activity profiles.

6.51 ± 0.05) presumably through the persistent activation of NHEs. In this context, it should be noted that NHEs are stimulated upon hyperosmotic stress and involved in the control of RVI and RVD [Grinstein et al., 1993]. Based on these observations, we reasoned that NHEs may form a component of the "osmosensor" and that the upregulation of NHEs by $G\alpha_{12}$ therefore enhances the sensitivity of the osmosensor. If indeed NHEs play a role in osmosensing, inhibition of NHEs should in turn inhibit the hyperosmotic response of the cells. To test this, the cells were subjected to hyperosmotic stress following pre-treatment with 50 μM DMA (along with the non-treated control groups) and JNK activity was monitored. The results indicated that DMA potently inhibited the hyperosmotic stress response of JNK in control cells (56%) but had little or no effect on basal activity. By contrast, the inhibition was only to a limited extent (30%) in $G\alpha_{12}QL$-NIH3T3 cells (Fig. 6). Since $G\alpha_{12}QL$-NIH3T3 cells have a higher basal level of JNK activity, these results appear to suggest that DMA was able to inhibit only the hyperosmotic stress-induced JNK activity in $G\alpha_{12}QL$–NIH3T3 cells.

DISCUSSION

Previous studies have demonstrated that hyperosmotic stress activates MAP kinases and NHEs [Terada et al., 1994; Matsuda et al., 1995; Schliess et al., 1995; Krump et al., 1997; Shrode et al., 1997; van der Wijk et al., 1998; Parrott et al., 1999]. It has also been shown that NHEs can be activated by both phosphorylation-dependent and -independent mechanisms [Grinstein et al., 1992]. Although it has been shown that p42/44 ERKs play a predominant role in the activation of NHE1 by growth factors, none of these kinases including JNK appear to have a critical role in the activation of NHEs by hyperosmotic stress [Grinstein et al., 1992; Bianchini et al., 1997]. In this context, our results for the first time reveal an upstream role for NHE in hyperosmotic stress-activated JNK.

In addition, several novel inferences can be derived from the results presented here. In *S. cerevisiae*, where the osmo-signaling pathway has been relatively well characterized, the activation of HOG1 is required for the osmoadaptive responses including the expression of osmoregulatory genes. Likewise, the activation of JNK in NIH3T3 cells can prime these cells by increasing the expression of osmoadaptive, volume-regulatory gene products and due to the prior accumulation of these factors via the activation of JNK, $G\alpha_{12}QL$ transfectants respond more acutely to hyperosmotic shock. However, the increased JNK activity does not contribute to an increase in the levels or the activities of the putative "osmosensor" since the expression of JNK1 alone does not enhance the sensitivity of the cells to osmo-signals. In contrast, $G\alpha_{12}$ enhances the hyperosmotic response by stimulating the activities of both the sensing and the regulatory components of the osmo-signaling pathway.

While the identity of the osmosensor is not known, our observation that the inhibition of NHEs results in the inhibition of hyperosmotic stress-induced JNK activity suggests that a

DMA-sensitive isoform of NHE may form at least a part of the osmosensing component in NIH3T3 cells. The findings that DMA potently inhibits hyperosmotic stress-induced JNK activation in controls cells and only partially in the $G\alpha_{12}$QL–NIH3T3 cells also suggest that the activation of JNK by NHE is through a mechanism distinctly different from that of $G\alpha_{12}$. Presently, the mechanism by which NHEs activate JNK in this cell line is not known. However, a striking similarity can be seen between yeast and mammalian signaling. In *S. cerevisiae*, SLN1 transmits osmotic as well as oxidative stress signals to PBS2-Hog2 through the MAPKKK SSK22, whereas SHO1 activates PBS2-Hog-1 via an alternate MAPKKK, STE11 [Davenport et al., 1995; Wurgler-Murphy and Saito, 1997; Posas et al., 1998]. Likewise, the activation of JNK during hyperosmotic stress via NHE may involve an alternate pathway distinctly different from the one activated by $G\alpha_{12}$.

In analyzing the interrelationship between the two responses activated by hyperosmotic stress, namely NHEs and JNKs, it was observed that the intracellular alkalinization, a function that can be regulated by NHEs, could stimulate the activity of JNK in U937 cells [Shrode et al., 1997]. However, the inhibition of NHEs by DMA had little or no effect on the osmotic activation of JNK in these cells. This is different from our results in which the inhibition of NHEs by DMA decreases the hyperosmotic activation of JNK in NIH3T3 cells. The simplest explanation for this discrepancy is possibly due to the different types of cells used in the two studies. While there is ample evidence to support such cell type-specific differences in hyperosmolar responses [Terada et al., 1994; Krump et al., 1997; van der Wijk et al., 1998; Bode et al., 1999; Parrott et al., 1999], the physiological significance of such differences remains to be identified. Similarly, the physiological role of NHEs in the hyperosmotic activation of JNKs needs to be further clarified.

It has been speculated that the parallel activation of NHEs and JNK/p38MAPKs is likely to be coordinated by a "common early event" such as the G12-family of G proteins [Shrode et al., 1997]. Our results presented here provide the first evidence for such a paradigm in which $G\alpha_{12}$ coordinately regulates the activities of both NHEs and JNKs. Thus, $G\alpha_{12}$ enhances the hyperosmotic response of NIH3T3 cells by regulating two distinct loci. The enhancement of hyperosmotic response by $G\alpha_{12}$ may involve the NHE-mediated intracellular alkalinization as well as the upregulation of the expression of osmoadptive genes via JNKs. By modulating NHE and JNK—two distinct but interrelated components of the hyperosmotic stress-signaling pathway—$G\alpha_{12}$ can greatly enhance the sensitivity as well as the rapidity of hyperosmotic response. Such a control mechanism will have great physiological significance. Since NHEs have been shown to be involved in cell volume regulation, by modulating NHEs and JNKs, $G\alpha_{12}$ may initiate cell volume changes—independently of hyperosmotic stress—in other physiological contexts, such as cell shape changes, cell motility, cell division, differentiation, and apoptosis. Considering the variety of signaling pathways stimulated by $G\alpha_{12}$, it is likely that the integrated response regulated by NHE and JNK may play a predominant role in $G\alpha_{12}$ signaling. The specific role of the individual components of $G\alpha_{12}$ signaling pathway in this complex pathway leading to cell volume and shape changes is presently being investigated in the laboratory.

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