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Mihail S. Iordanov

*Oregon Health Sciences University*, [miordanov@cu-portland.edu](mailto:miordanov@cu-portland.edu)

Bruce E. Magun

*Oregon Health Sciences University*

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## Loss of Cellular K<sup>+</sup> Mimics Ribotoxic Stress

INHIBITION OF PROTEIN SYNTHESIS AND ACTIVATION OF THE STRESS KINASES SEK1/MKK4, STRESS-ACTIVATED PROTEIN KINASE/c-Jun NH<sub>2</sub>-TERMINAL KINASE 1, AND p38/HOG1 BY PALYTOXIN\*

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Mihail S. Iordanov and Bruce E. Magun‡

From the Department of Cell and Developmental Biology, Oregon Health Sciences University, Portland, Oregon 97201

The tumor promoter palytoxin has been found to activate the stress-activated protein kinase/c-Jun NH<sub>2</sub>-terminal kinase 1 (SAPK/JNK1), and it also potentiates, as demonstrated here, the p38/HOG1 mitogen-activated protein kinase and the upstream activator of SAPK/JNK1, SEK1/MKK4. In search of possible mechanisms for both the cytotoxicity and the activation of stress kinases by palytoxin, we found that palytoxin is a potent inhibitor of cellular protein synthesis. The inhibition of translation by palytoxin does not result from its direct binding to the translational apparatus. We have previously demonstrated that ribotoxic stressors (Iordanov, M. S., Pribnow, D., Magun, J. L., Dinh, T.-H., Pearson, J. A., Chen, S. L.-Y., and Magun, B. E. (1997) *Mol. Cell Biol.* 17, 3373–3381) signal the activation of SAPK/JNK1 by binding to or covalently modifying 28 S rRNA in ribosomes that are active at the time of exposure to the stressor. Palytoxin acted as a ribotoxic stressor, inasmuch as it required actively translating ribosomes at the time of exposure to activate SAPK/JNK1. Palytoxin has been shown to augment ion fluxes by binding to the Na<sup>+</sup>/K<sup>+</sup>-ATPase in the plasma membrane of cells. To determine whether altered fluxes of either Na<sup>+</sup> or K<sup>+</sup> could be responsible for the effects of palytoxin on translation and on activation of SAPK/JNK1, cells were exposed to palytoxin in modified culture medium in which a major portion of the Na<sup>+</sup> was replaced by either K<sup>+</sup> or by choline<sup>+</sup>. The substitution of Na<sup>+</sup> by K<sup>+</sup> strongly inhibited the ability of palytoxin both to inhibit protein translation and to activate SAPK/JNK1, whereas the substitution of Na<sup>+</sup> by choline<sup>+</sup> did not. These results suggest that palytoxin-induced efflux of cellular K<sup>+</sup> mimics ribotoxic stress by provoking both translational inhibition and activation of protein kinases associated with cellular defense against stress.

Palytoxin is a non-peptide water-soluble marine toxin that is a potent tumor promoter in the mouse skin carcinogenesis model (2–4). Many tumor-promoting compounds that are effective in the skin of carcinogen-initiated mice also produce an inflammatory reaction (5–15). Inflammatory mediators such as interleukin-1 (IL-1)<sup>1</sup> and tumor necrosis factor- $\alpha$  are potent

activators of stress kinases such as SAPK/JNK1 and p38/HOG1 (for a review, see Ref. 16). Kuroki *et al.* (17) have shown that Swiss 3T3 cells exposed to concentrations of palytoxin as low as 0.1 nM display an abundant activation of the stress-activated kinase SAPK/JNK1. Stimulation of SAPK/JNK1 has been shown to lead to activation of AP-1, a dimeric transcription factor composed of Jun (c-Jun, JunB, JunD) and Fos (c-Fos, FosB, Fra-1, Fra-2) family members (for a review, see Ref. 18). SAPK/JNK1 phosphorylates c-Jun at serines 63 and 73 in its NH<sub>2</sub> terminus, thereby increasing the transcription activating potential of AP-1 (19, 20). Stimulation of SAPK/JNK1 also leads to the phosphorylation and activation of the transcription factor Elk-1 (21, 22), which operates on the regulatory region of *c-fos* (for reviews, see Refs. 23 and 24), and to the phosphorylation of activating transcription factor-2 (25, 26), which, as an activating transcription factor-2/c-Jun heterodimer, operates on the promoter of *c-jun* (27). Thus, SAPK/JNK1 increases AP-1 activity in the nucleus by activating pre-existing AP-1 complexes and by transcriptionally inducing the expression of the components of AP-1, c-Jun, and c-Fos.

The cellular effects of palytoxin, which include ionic disequilibria (28–30), increased production of prostaglandins from arachidonic acid (31–36), and alterations in the affinity of the EGF receptor (37–39), have been attributed to the ability of palytoxin to bind to the Na<sup>+</sup>/K<sup>+</sup>-ATPase situated in the plasma membrane. Direct binding of palytoxin to the Na<sup>+</sup>/K<sup>+</sup>-ATPase, also known as the sodium pump, transforms the pump into a permanently open ion channel that permits the outward flux of K<sup>+</sup> and the inward flux of Na<sup>+</sup> and that is independent of ATP hydrolysis (28–30). Conclusive demonstration that palytoxin acts through the sodium pump has come from heterologous expression of the sodium pump in *Saccharomyces cerevisiae*, which lose intracellular K<sup>+</sup> following exposure to palytoxin (29, 30).

Recently, we reported on the identification of the 28 S ribosomal RNA as a specific sensor for stress induced by a subset of agents that inhibit protein synthesis (1). Some inhibitors of translation are strong activators of SAPK/JNK1, whereas other equally effective inhibitors of translation are unable to activate SAPK/JNK1. The translational inhibitors that activated SAPK/JNK1, termed ribotoxic stressors (1), either bind to the 28 S rRNA in the peptidyl transferase center (40) (*e.g.* anisomycin and blasticidin S) or cause specific damage to 28 S rRNA (*e.g.* ricin A chain and  $\alpha$ -sarcin) within a conserved loop involved in binding of the elongation factors EF-1 and EF-2 (for a review, see Ref. 41). Activation of SAPK/JNK1 and of its upstream activator SEK1/MKK4 by this group of ribotoxins can occur only when ribosomes are actively translating at the time of exposure to the ribotoxic stressor. Prior inhibition of trans-

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‡ To whom correspondence should be addressed: Tel.: 503-494-7811; Fax: 503-494-4253; E-mail: magunb@OHSU.edu.

<sup>1</sup> The abbreviations used are: IL, interleukin; SAPK/JNK, stress-activated protein kinase/c-Jun NH<sub>2</sub>-terminal kinase; p38/HOG1, mammalian homolog of the *S. cerevisiae* high osmolarity glycerol response kinase-1; SEK1/MKK4, SAPK/extracellular signal-regulated kinase-1/mitogen-activated protein kinase kinase-4; AP-1, activator protein-1;

GST-Elk-1, glutathione S-transferase/Elk-1 fusion protein; MEM, modified Eagle's medium; EGF, epidermal growth factor.

lation by nonactivating agents such as diphtheria toxin, T-2 toxin, pactamycin, or emetine for as little time as 2 min abrogate the ability of the ribotoxic stressors, but not of IL-1 $\alpha$  or osmotic stress, to stimulate SAPK/JNK1 activity. We concluded that the sensors for ribotoxic damage are ribosomes, which can transduce signals that activate SEK1/MKK4 and SAPK/JNK1 only when they are translationally active at the time of induced ribotoxicity. Although many stress signals ultimately converge to activate the stress kinases, signals arising during ribotoxicity are initially conveyed through a pathway distinct from those used by IL-1 $\alpha$  and osmotic stress (1). The transduction of stress signals through ribosomes is a feature that eukaryotes share with prokaryotes, whose ribosomes respond to some translational inhibitors by recapitulating cellular responses characteristic of either heat shock or cold shock (42).

In the experiments described here, we found that, like ribotoxic stressors, palytoxin potently inhibited protein synthesis in the concentration range that leads to activation of the stress kinases SEK1/MKK4, SAPK/JNK1, and p38/HOG1. Similar to ribotoxic stressors, the ability of palytoxin to activate SAPK/JNK1 depended on the presence of actively translating ribosomes at the time of exposure to palytoxin. However, unlike ribotoxic stressors, palytoxin did not inhibit protein synthesis as a consequence of direct binding to the translational apparatus. To determine whether the effects of palytoxin on translation and SAPK/JNK1 activation resulted from altered ionic fluxes, a major portion of the Na<sup>+</sup> in the culture medium was replaced by either K<sup>+</sup> or choline<sup>+</sup>. The substitution of Na<sup>+</sup> by K<sup>+</sup>, but not by choline<sup>+</sup>, inhibited the ability of palytoxin both to inhibit protein translation and to activate SAPK/JNK1, suggesting that the efflux of cellular K<sup>+</sup> may be responsible for both translational inhibition and activation of SAPK/JNK1. Thus, loss of K<sup>+</sup> from stressed cells may lead to the activation of stress kinases through ribosome-mediated signaling.

#### EXPERIMENTAL PROCEDURES

**Chemicals, Cytokines, and Ribotoxins**—Anisomycin, emetine, D-sorbitol, and choline were from Sigma. IL-1 $\alpha$  (recombinant mouse) was from Genzyme (Cambridge, MA). Palytoxin was from Calbiochem. Anisomycin, emetine, and palytoxin were dissolved in (H<sub>3</sub>C)<sub>2</sub>SO. In all the cases when (H<sub>3</sub>C)<sub>2</sub>SO was used as a vehicle, corresponding control cells received the same amount of the vehicle alone (typically not more than 0.2% (v/v)). D-Sorbitol was dissolved in Dulbecco's modified Eagle's medium (DMEM) as a 3 M stock solution. All radiochemicals were from NEN Life Science Products.

**Cell Culture**—Rat-1 cells were maintained as described previously (43). The derivative cell line FC<sub>2</sub>-Rat1 has been described by Rodland *et al.* (44). All experiments presented here were performed using confluent, quiescent cultures obtained through serum deprivation for typically 24 h. DMEM and MEM were from Life Technologies, Inc.

**Immunoprecipitation of SAPK/JNK1 and Extracellular Signal-regulated Kinase and Immunocomplex Kinase Assays**—All immunoprecipitations and immunocomplex kinase reactions were performed as described for SAPK/JNK1 in Ref. 1. For immunoprecipitation of SAPK/JNK1, the antibody sc-474 was used, and for immunoprecipitation of extracellular signal-regulated kinase 1, the antibody sc-93-G was used (both from Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

**Western Blot Analysis of SEK1/MKK4 Phosphorylation**—The analysis of threonine 223 phosphorylation of SEK1/MKK4 was performed with the antibody 9151S, and the analysis of tyrosine 182 phosphorylation of p38/HOG1 was performed with the antibody 9211S (both from New England Biolabs Inc., Beverly, MA) as described in Ref. 1.

**Measurement of Protein Synthesis**—Incorporation of [<sup>3</sup>H]leucine was performed as described in Ref. 1. Determination of protein synthesis using FC<sub>2</sub>-Rat1 cells and a chloramphenicol acetyltransferase (CAT) assay has been described by Rodland *et al.* (44). The measurement of luciferase mRNA translation was performed with the Rabbit Reticulocyte Lysate System (catalog number L4960) as described by the manufacturer (Promega, Madison, WI).

**Measurement of Na<sup>+</sup>/K<sup>+</sup>-ATPase Activity**—The activity of the Na<sup>+</sup>/K<sup>+</sup>-ATPase in membranal preparations from Rat-1 cells was determined as described by Brotherus *et al.* (51) with modifications. Briefly,

S100 microsomal pellets (20  $\mu$ g of total protein/experimental point) were resuspended in 0.6 ml of assay solution (pH 7.2; 0.6 mM EGTA, 156 mM NaCl, 24 mM KCl, 3.6 mM MgCl<sub>2</sub>, 3.6 mM ATP, 60 mM imidazole, 10 mM Na<sub>3</sub>N; with or without 0.5 mM ouabain) in the presence of varying concentrations of palytoxin. After 60 min of incubation at 37 °C, the reactions were stopped by the addition of 1.5 ml of ice-cold stopping solution (made by the sequential addition on ice of 21.3 ml of 1 M HCl, 18.3 ml of H<sub>2</sub>O, 1.29 g of L-ascorbic acid, 2.13 ml of 10% ammonium molybdate, and 3.3 ml of 20% SDS). After 10 min of incubation on ice, the color development was achieved by the addition of 1.5 ml of a solution containing 2% (w/v) sodium meta-arsenite, 2% (w/v) sodium citrate, and 2% (v/v) glacial acetic acid. After incubation for 10 min at room temperature, the absorption of the samples was measured at 850 nm, and the amount of P<sub>i</sub> in each sample was determined using a standard containing 50  $\mu$ M Na<sub>3</sub>PO<sub>4</sub>.

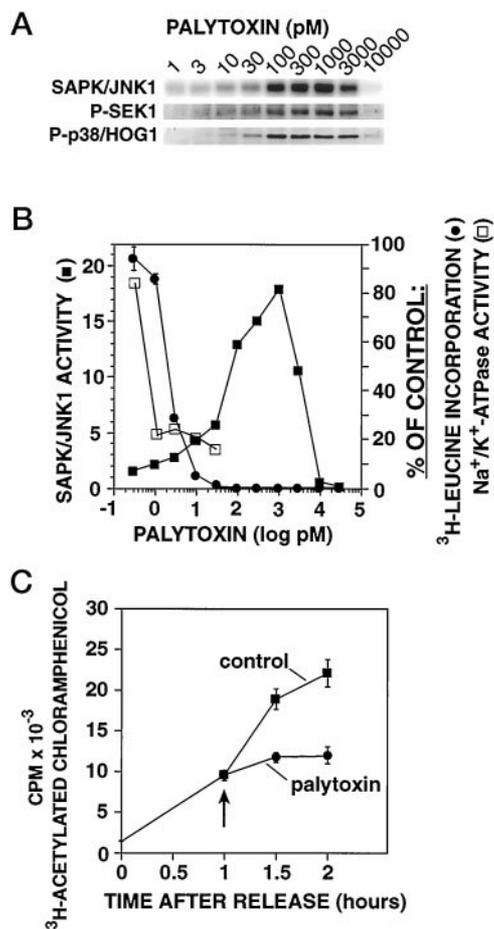
**PhosphorImager and Statistical Analyses**—The quantification of <sup>32</sup>P<sub>4</sub><sup>3-</sup> transferred from ATP onto GST-Elk-1 in experiments measuring SAPK/JNK1 kinase activity was performed using the PhosphorImager apparatus and the IPLab Gel<sup>®</sup> software from Molecular Dynamics (Sunnyvale, CA). Statistical analyses were performed using the StatView<sup>®</sup> software from Abacus Concepts, Inc. (Berkeley, CA).

#### RESULTS

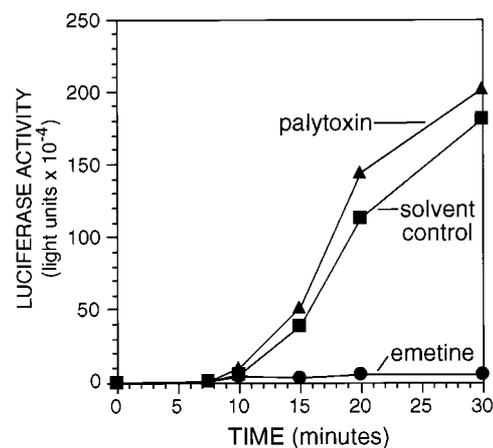
**Activation of SAPK/JNK1, SEK1/MKK4, and p38/HOG1 by Palytoxin**—The addition of palytoxin to serum-deprived Rat-1 cells potently induced the activation of SAPK/JNK1 when examined 30 min later (Fig. 1, A and B). As reported previously by Kuroki *et al.* (17), the ability of palytoxin to activate SAPK/JNK1 was biphasic. At concentrations greater than 1 nM, cells displayed a decreased responsiveness to palytoxin; concentrations of palytoxin greater than 10 nM were ineffective in activating SAPK/JNK1.

SEK1/MKK4 is a kinase directly responsible for activation of SAPK/JNK1 (45–47). Palytoxin showed the same dose dependence in causing the phosphorylation of SEK1/MKK4 at threonine 223, an event indicative of the activation of this kinase (48) (Fig. 1A). These data suggest that activation of SAPK/JNK1 is mediated by kinase-directed signals generated upstream of SEK1/MKK4. Palytoxin showed a similar dose dependence in the activation of p38/HOG1, a member of the proline-directed stress kinases that bears functional and structural homology to SAPK/JNK1 (Fig. 1A). As previously reported, palytoxin did not induce the activation of the MAP kinase extracellular signal-regulated kinase 1 (data not shown).

**Translational Inhibition by Palytoxin**—Previously we reported that some adverse agents that activate SAPK/JNK1 require ribosomes actively engaged in translation at the the moment of exposure to do so, while simultaneously inhibiting protein synthesis. These agents were termed ribotoxic stressors (1). To determine if it could be acting as a ribotoxic stressor, palytoxin was tested for its ability to inhibit the incorporation of [<sup>3</sup>H]leucine into proteins in Rat-1 cells. Indeed, palytoxin inhibited the incorporation of [<sup>3</sup>H]leucine in a dose-dependent manner (Fig. 1B). The ability of palytoxin to both activate stress kinases (Fig. 1, A and B) and repress [<sup>3</sup>H]leucine incorporation (Fig. 1B) became apparent at concentrations of the toxin that were inhibitory for the Na<sup>+</sup>/K<sup>+</sup>-ATPase in membranal preparations isolated from Rat-1 cells (Fig. 1B; see "Experimental Procedures," and see below). Because palytoxin is known to act on membrane-associated functions, we considered the possibility that transport of [<sup>3</sup>H]leucine into cells is impaired following exposure to palytoxin. To avoid this potential complication, we applied a method for measuring cellular protein synthesis that is independent of transport and incorporation of amino acids. A derivative of the parental Rat-1 cells, the cell clone FC<sub>2</sub>-Rat1 bears a stably integrated CAT reporter gene under the control of a 1-kilobase promoter sequence of the human *c-fos* gene (44). The expression of CAT mRNA is kept at very low levels in quiescent cells, but it can be



**FIG. 1. Palytoxin-induced inhibition of protein synthesis and activation of the stress kinases SEK1/MKK4, SAPK/JNK1, and p38/HOG1.** *A*, activation of SAPK/JNK1 and phosphorylation of SEK1/MKK4 and p38/HOG1 following treatment of Rat-1 cells with palytoxin. Rat-1 cells ( $3 \times 10^6$ ) were serum-deprived in DMEM for 24 h prior to stimulation with palytoxin concentrations as shown. Cells were harvested in lysis buffer 30 min after palytoxin addition, and the activity of SAPK/JNK1 was determined in immunocomplex assays using an anti-SAPK/JNK1 antibody to precipitate SAPK/JNK1 and using GST-Elk-1 recombinant protein to serve as a substrate for phosphorylation (see "Experimental Procedures"). The phosphorylation states of SEK1/MKK4 and p38/HOG1 were assessed in Western blots using antibodies that recognize the respective phosphorylation sites associated with activation (see "Experimental Procedures"). *B*, effect of varying concentrations of palytoxin on  $\text{Na}^+/\text{K}^+$ -ATPase activity, on  $[^3\text{H}]$ leucine incorporation and on SAPK/JNK1 activity. For the  $\text{Na}^+/\text{K}^+$ -ATPase activity, membranes isolated from Rat-1 cells (20  $\mu\text{g}$  of total protein) were incubated in an assay solution (see "Experimental Procedures") with the indicated concentrations of palytoxin, in the presence or in the absence of 0.5 M ouabain at 37 °C. Sixty min later, the reactions were stopped (see "Experimental Procedures"), and the degree of ATP hydrolysis was determined as described under "Experimental Procedures." The activity of the  $\text{Na}^+/\text{K}^+$ -ATPase (defined as the difference between the total ATPase activities without and with ouabain) in the absence of palytoxin (indicated as 100% in the graph) was 17  $\mu\text{mol}$  of hydrolyzed ATP/1  $\mu\text{g}$  of protein/1 h. The values for SAPK/JNK1 activity were determined following quantitative PhosphorImager analysis of the data in *A* (above). The effect of palytoxin on  $[^3\text{H}]$ leucine incorporation into acid-insoluble material was determined in serum-deprived Rat-1 cells that were pulse-labeled with  $[^3\text{H}]$ leucine (1  $\mu\text{Ci}/\text{ml}$ ) in DMEM from 15 to 30 min following the addition of palytoxin. *C*, palytoxin-induced inhibition of CAT mRNA translation in FC<sub>2</sub>-Rat1 cells. Serum-deprived cells were stimulated for 2 h with EGF (40 ng/ml) in the presence of cycloheximide (25  $\mu\text{g}/\text{ml}$ ). Thereafter, both EGF and cycloheximide were removed from the medium by extensive washout (indicated as *release*,  $t = 0$  min). The accumulation of CAT protein was monitored by measuring CAT activity 0, 60, 90, and 120 min after the release. Palytoxin (1 nM) was applied 60 min after the release (arrow). Squares represent CAT activity in cells treated at 60 min with solvent alone; circles represent CAT activity in the palytoxin-treated cells. S.D. was obtained from experimental points in triplicate.



**FIG. 2. Effect of palytoxin on the translation of luciferase mRNA in a rabbit reticulocyte lysate assay.** The mRNA for luciferase (0.5  $\mu\text{g}$ ), palytoxin (30 nM), or solvent ( $\text{H}_3\text{C}_2\text{SO}$ ) was added at  $t = 0$  min. Emetine (10  $\mu\text{g}/\text{ml}$ ) was added at  $t = 8$  min. At designated times, aliquots of the lysate were withdrawn and analyzed for luciferase activity. The assay was performed with a reticulocyte lysate kit, supplemented with amino acids, as described by the manufacturer (Promega).

induced up to 100-fold upon stimulation with EGF in the presence of cycloheximide (not shown). The presence of cycloheximide prevents the translation of the CAT mRNA into CAT protein. Release from the cycloheximide-induced translational arrest (Fig. 1C,  $t = 0$  min) allows efficient translation of the accumulated CAT mRNA. The accumulation of CAT protein increased linearly within 2 h following the washout of cycloheximide as measured by a CAT activity assay (Fig. 1C). Since EGF was also removed from the medium together with cycloheximide, the increase of CAT activity resulted solely from translation of the CAT mRNA that had accumulated before the washout. Treatment of the cells with palytoxin 60 min after the release from translational arrest resulted in a substantial decrease in CAT activity detectable both 30 and 60 min after the addition of palytoxin (Fig. 1C). We therefore conclude that exposure of cells to palytoxin indeed interferes with the process of translation.

Many inhibitors of protein synthesis alter ribosomal activity by binding directly to ribosomes (49, 50). The actions of these inhibitors are readily detected following their addition to reticulocyte lysates that contain ribosomes and all of the necessary ingredients to allow the initiation and elongation of translation *in vitro*. To test whether palytoxin could inhibit protein synthesis by directly modifying the functionality of ribosomes *in vitro*, we added palytoxin to a reticulocyte lysate preparation engaged in protein synthesis (Fig. 2). The addition to the reticulocyte lysate of mRNA for luciferase reporter protein (Fig. 2,  $t = 0$  min) and subsequent monitoring of luciferase activity demonstrated the effectiveness of the lysate in promoting both initiation and elongation. The addition of the elongation inhibitor emetine at 8 min completely blocked the further accumulation of luciferase activity. Palytoxin (30 nM) was completely ineffective in inhibiting translation of luciferase when added prior to the addition of luciferase mRNA. These data demonstrate that neither translational initiation nor translational elongation were affected by the addition of palytoxin to a cell-free translational system. We therefore conclude that the inhibition of translation induced by palytoxin *in vivo* is unlikely to be mediated by direct interaction between palytoxin and the translational apparatus.

**Translational Inhibitors Interfere with Palytoxin-induced Activation of SAPK/JNK1**—The demonstration that palytoxin is a potent *in vivo* inhibitor of protein synthesis prompted us to test whether palytoxin shares some of the properties of ribo-

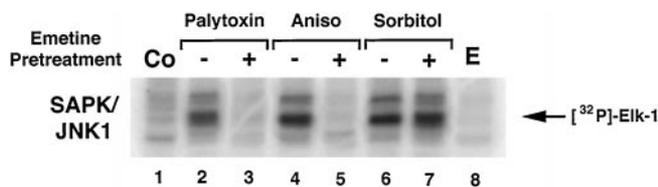


FIG. 3. Inhibition of the palytoxin-induced activation of SAPK/JNK1 by pretreatment of Rat-1 cells with emetine. Serum-deprived Rat-1 cells were left untreated (lanes 1, 2, 4, and 6) or were exposed to emetine (lanes 3, 5, 7, and 8) for 15 min prior to the addition of palytoxin (1 nM; lanes 2 and 3), anisomycin (10  $\mu$ g/ml; lanes 4 and 5), or sorbitol (600 mM; lanes 6 and 7). Cells were harvested in lysis buffer 30 min later, and SAPK/JNK1 activity was determined by immunocomplex assay using GST-Elk-1 as substrate. Co, control cells. E, emetine-treated cells.

toxic stressors, which both activate SAPK/JNK1 and inhibit protein translation (1). We previously showed that the activation of SAPK/JNK1 by ribotoxic stressors is rapidly suppressed in cells whose protein synthesis had been previously blocked by translational inhibitors that are incapable of activating SAPK/JNK1 (1). Mediation of SAPK/JNK1-activating signals could only occur in ribosomes that are active at the time of exposure of ribotoxic stressors. The following experiments test whether activation of SAPK/JNK1 by palytoxin similarly depends on the presence of active ribosomes.

Pretreatment of cells for 15 min with emetine, an inhibitor of translational elongation, suppressed the palytoxin-induced activation of SAPK/JNK1 measured 30 min after the addition of palytoxin (Fig. 3, lanes 2 and 3). A similar suppression of SAPK/JNK1 activation was observed when cells were preincubated with pactamycin, a specific inhibitor of translational initiation (data not shown). Emetine also suppressed the activation of SAPK/JNK1 by the ribotoxic stressor anisomycin (1) (Fig. 3, lanes 4 and 5), but not by sorbitol, an osmotic stressor that does not act through ribosomal toxicity (1) (Fig. 3, lanes 6 and 7).

In studying the effects of ribotoxic stressors, we reported that inhibiting protein translation by emetine as soon as 1 min prior to the addition of anisomycin was sufficient to block transduction of the signals that lead ultimately to activation of SAPK/JNK1 (1). This rapid action of emetine was taken as evidence that prior inhibition of translation by emetine was unlikely to exert its blockade of SAPK/JNK1 activation by blocking the synthesis of a protein with rapid decay kinetics (1). The experiment presented in Fig. 4 similarly demonstrates that the addition of emetine 1 min prior to the addition of palytoxin substantially suppressed the activation of SAPK/JNK1. Emetine failed to suppress palytoxin-induced activation of SAPK/JNK1 when emetine was added 3 min after the addition of palytoxin. The ability of a translational inhibitor to block palytoxin-induced signaling within such a short time suggests that active ribosomes are necessary for signal transduction and furthermore argues against the likelihood that a protein with kinetics of rapid decay could explain the emetine-induced block to SAPK/JNK1 activation. The inability of emetine to inhibit SAPK/JNK1 signaling when added 3 min after the palytoxin addition suggests that the critical ribosome-mediated events that are required to transduce the signal have already occurred by 3 min. These data demonstrate that, similar to ribotoxic stressors, palytoxin requires the presence of active ribosomes at the time of addition to activate SAPK/JNK1.

**Effects of Palytoxin on Translation and Signaling in Media Containing Modified  $[Na^+]$ ,  $[K^+]$ , or  $[Choline^+]$** —Several lines of evidence indicate that the biological effects of palytoxin are mediated through its interaction with the  $Na^+/K^+$ -ATPase (28–30). The most convincing evidence derives from the intro-

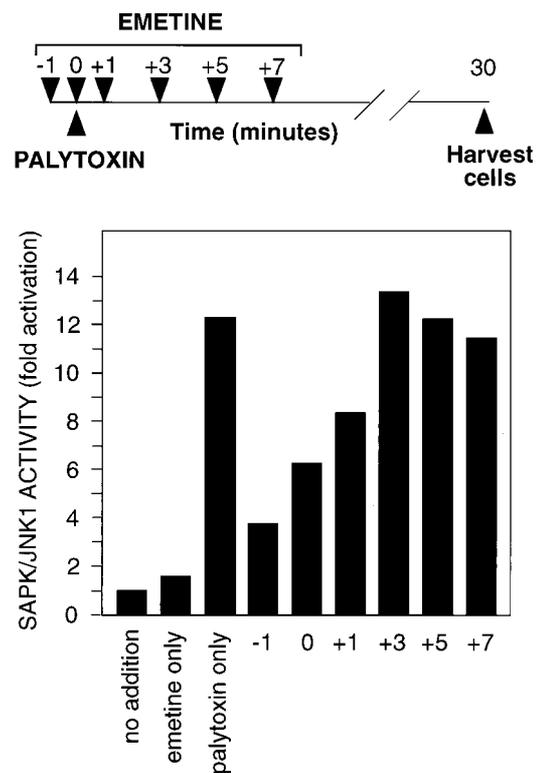


FIG. 4. Effect of emetine on the activation of SAPK/JNK1 by palytoxin in Rat-1 cells. Graphic representation of the data from immunocomplex kinase assays. Serum-deprived Rat-1 cells were treated as follows (from left to right) with the following: solvent alone; emetine (100  $\mu$ g/ml) alone; palytoxin (1 nM) alone; or palytoxin under conditions of prior, contemporaneous, or subsequent treatment with emetine as denoted in the scheme (top). SAPK/JNK1 activity was determined 30 min after the addition of palytoxin.

duction of a mammalian  $Na^+/K^+$ -ATPase into yeast, which demonstrated that palytoxin interacts directly with this molecule to facilitate the simultaneous entry of  $Na^+$  and exit of  $K^+$  from cells (29, 30). Since it appeared that the ribotoxic activity of palytoxin could not be related to direct interaction with the translational machinery, we tested whether the ribotoxic actions of palytoxin could have resulted from ionic disequilibria produced in cells as a consequence of poisoning of the  $Na^+/K^+$ -ATPase. To test this possibility, we conducted experiments in which 75% of the 154 mM NaCl present in MEM culture medium was replaced by either KCl (116 mM KCl; K-MEM) or choline chloride (116 mM; Cho-MEM). Experiments were conducted to determine whether the modified isotonic MEM containing either  $K^+$  or choline $^+$  would reduce the ability of palytoxin to inhibit protein synthesis or to activate SAPK/JNK1. Cells were far more sensitive to inhibition of protein synthesis by palytoxin in MEM, compared with K-MEM, which shifted the dose-response curve 2 decades to the right (Fig. 5). By contrast, cells were 3-fold more sensitive in Cho-MEM than in MEM. The ability of increased extracellular  $K^+$ , but not choline $^+$ , to diminish substantially the ability of palytoxin to inhibit translation is consistent with the conclusion that efflux of  $K^+$  may be responsible for the effects of palytoxin. The inability of extracellular choline $^+$  to reduce the translational inhibition by palytoxin is consistent with the notion that influx of  $Na^+$  is unlikely to be the cause of the effects of palytoxin.

To determine whether the potential of palytoxin to inhibit protein translation in MEM, K-MEM, or Cho-MEM correlates with the potential to activate SAPK/JNK1, we exposed cells to standard or modified MEM for 30 min prior to the addition of palytoxin. The results demonstrate that, compared with MEM,

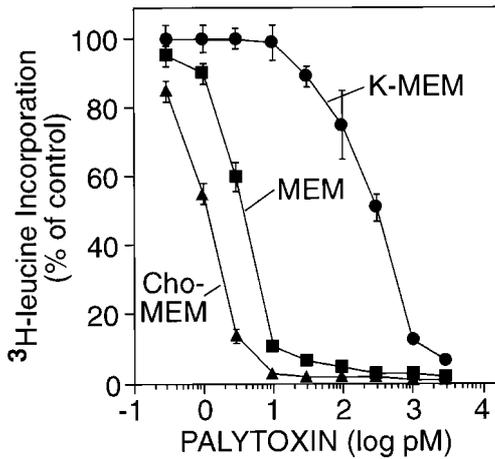


FIG. 5. Effect of palytoxin on the incorporation of [ $^3\text{H}$ ]leucine following replacement of  $\text{Na}^+$  in MEM by  $\text{K}^+$  or choline $^+$ . Serum-deprived Rat-1 cells were incubated in standard MEM (containing 154 mM  $\text{Na}^+$ ) or in MEM containing 38 mM  $\text{Na}^+$  and 116 mM of either  $\text{K}^+$  (K-MEM) or choline $^+$  (Cho-MEM). After the addition of varying concentrations of palytoxin, cells were pulse-labeled with 5  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]leucine from 10 to 20 min. S.D. was obtained from experimental points in triplicate.

K-MEM suppressed the ability of palytoxin to activate SAPK/JNK1, and that cells incubated in Cho-MEM showed a more pronounced activation of SAPK/JNK1 at equivalent palytoxin concentrations (Fig. 6A). The cells in K-MEM were still capable of responding to other ribotoxic and nonribotoxic stressors, since the activity of SAPK/JNK1 was strongly elevated in cells exposed to K-MEM containing anisomycin (a ribotoxic stressor), sorbitol, or IL-1 $\alpha$  (Fig. 6B). These data demonstrate that, in addition to its diminished ability to inhibit protein translation in K-MEM, palytoxin was also unable to activate SAPK/JNK1. These data also demonstrated that the inability of palytoxin to activate SAPK/JNK1 in K-MEM does not result from the general inhibition by K-MEM of pathways leading to SAPK/JNK1 activation.

We next tested whether cells exposed to palytoxin in K-MEM were capable of responding to the toxin when transferred from K-MEM to standard MEM (Fig. 7). The increase in SAPK/JNK1 activity in cells exposed for 30 min to 0.1 nM palytoxin in standard MEM was inhibited in K-MEM (Fig. 7, lanes B and D). When cells incubated for 30 min in K-MEM containing palytoxin were rinsed with palytoxin-free K-MEM and transferred to standard MEM without palytoxin (Fig. 7, lane F), SAPK/JNK1 activity was increased to at least the same levels achieved by palytoxin in standard MEM. These data suggest that K-MEM impeded the transduction of a signal generated by palytoxin; shifting cells to standard MEM supplied the conditions necessary to complete transduction of the signal that leads to the activation of SAPK/JNK1. The increase in SAPK/JNK1 activity following the exchange of K-MEM for standard MEM again suggests that  $\text{K}^+$  efflux may be responsible for initiating the activation of SAPK/JNK1 in palytoxin-treated cells.

#### DISCUSSION

The ability of palytoxin to both inhibit protein synthesis and stimulate SAPK/JNK1 activity (Fig. 1) led us to investigate whether palytoxin acts as a ribotoxic stressor. Previously identified ribotoxic stressors share the following characteristics: (i) they inhibit translational elongation by interacting directly with 28 S rRNA in the region of the peptidyl transferase center or the S/R loop; (ii) they rapidly induce the activation of the stress kinases SEK1/MKK4, SAPK/JNK1, and p38/HOG1; and (iii) they induce the activation of stress kinases only in cells that contain actively translating ribosomes (1). Other nonribo-

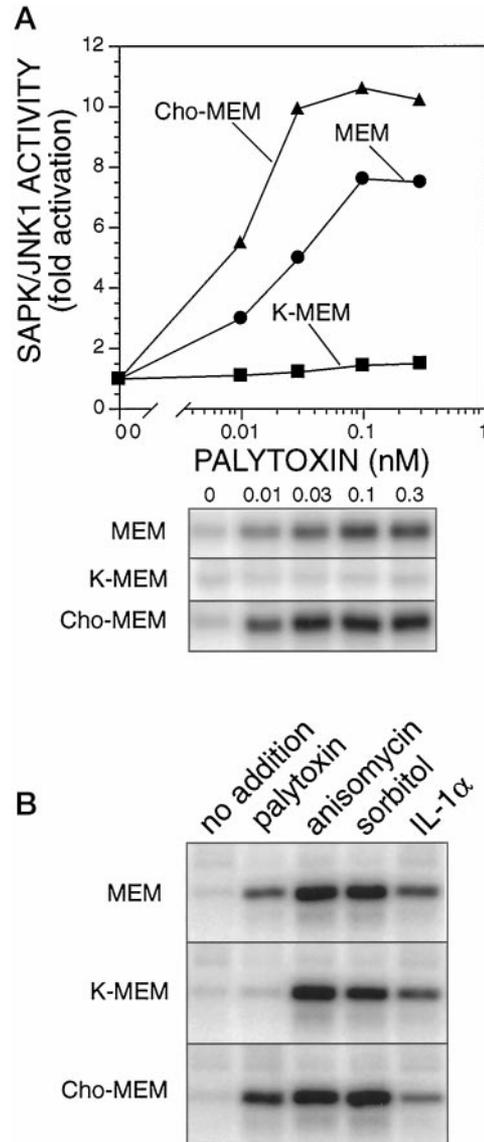
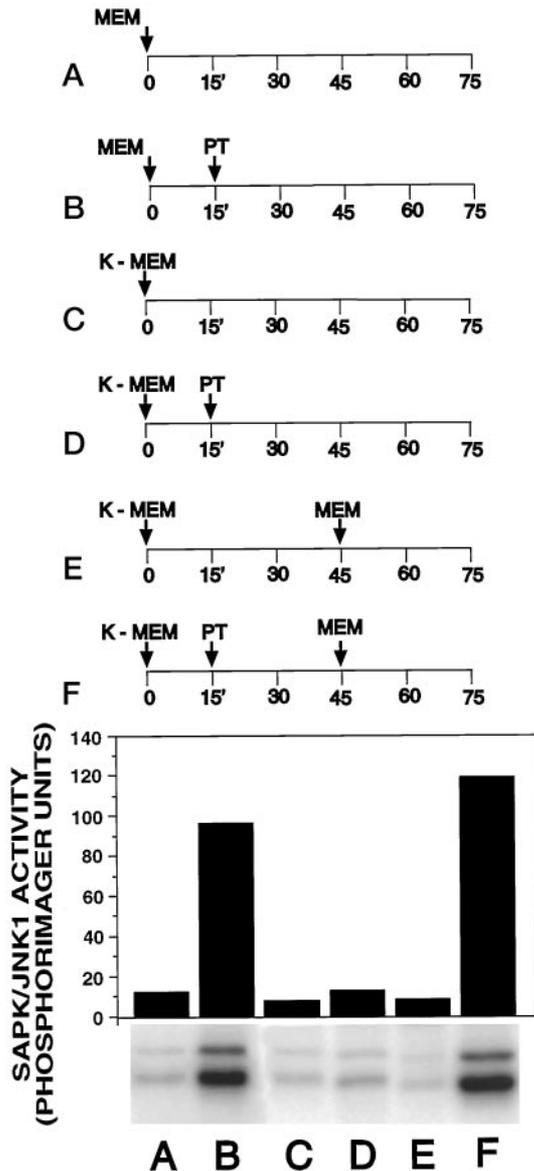


FIG. 6. Effect of palytoxin and other agents on SAPK/JNK1 activation following the replacement of  $\text{Na}^+$  in MEM by  $\text{K}^+$  or choline. A, effect of palytoxin on SAPK/JNK1 activation. Serum-deprived Rat-1 cells were incubated in MEM, K-MEM, or Cho-MEM (as in Fig. 5) for 10 min prior to the addition of varying doses of palytoxin. Thirty min later, cells were harvested, and the lysate was analyzed for activated SAPK/JNK1 by an immunocomplex assay. The PhosphorImager data is displayed in the lower part; the graphic representation of this data is displayed in the upper part. B, effectiveness of several agents to activate SAPK/JNK1 following replacement of  $\text{Na}^+$  in MEM by  $\text{K}^+$  or choline $^+$ . The experiment is similar to the one presented in A. Cells were incubated in MEM, K-MEM, or Cho-MEM and exposed to palytoxin (0.1 nM), anisomycin (10  $\mu\text{g/ml}$ ), sorbitol (600 mM) for 30 min or to IL-1 $\alpha$  (25 ng/ml) for 10 min prior to harvesting and analysis of SAPK/JNK1 activation by the immunocomplex assay.

toxic stressors that activate stress kinases, such as proinflammatory cytokines and osmotic stress, are distinguished from ribotoxic stressors by their ability to induce signaling to the stress kinases even in the absence of translationally active ribosomes. Although experiments demonstrated that palytoxin was ineffective as a translational inhibitor when added directly to actively translating ribosomes *in vitro* (Fig. 2), palytoxin nevertheless required translating ribosomes to transduce signals that activate SAPK/JNK1 *in vivo*. The rapid inhibition of SAPK/JNK1 activation by emetine, which substantially blocked the activation when added just 1 min prior to palytoxin (Fig. 3), was also observed when emetine was added just prior



**FIG. 7. Activation of SAPK/JNK1 when Rat-1 cells incubated in K-MEM plus palytoxin are postincubated in MEM without palytoxin.** A, cells were incubated in MEM for 75 min. B, cells were incubated in MEM for 15 min prior to the addition of palytoxin for an additional 60 min. C, cells were incubated in K-MEM for 75 min. D, cells were incubated in K-MEM for 15 min prior to the addition of palytoxin. E, cells were incubated in K-MEM for 45 min; cells were then rinsed several times in K-MEM and placed into fresh MEM for an additional 30 min. F, cells were incubated in K-MEM for 15 min prior to the addition of palytoxin. At 45 min, the medium was removed, cells were rinsed several times in palytoxin-free K-MEM, and fresh MEM was added for an additional 30 min. All cells were harvested at 75 min, and cell lysates were analyzed for activation of SAPK/JNK1 by an immunocomplex assay. Palytoxin (PT) was added at 0.1 nM.

to ribotoxic stressors such as anisomycin and ricin (1). The rapid action of emetine in this context suggests that actively translating ribosomes are required for palytoxin-initiated signals to be transduced to the kinase(s) upstream of SEK1/MKK4 and SAPK/JNK1. That cells exposed to palytoxin became refractory to emetine when emetine was added 3 min after palytoxin suggests that the critical events of ribosome-mediated transduction are completed by 3 min. A similar time course was observed for anisomycin (1).<sup>2</sup> Since the effects of palytoxin

include translational inhibition, it appears plausible that the signaling is self-terminating and that the involvement of ribosomes in this process is completed within this short period of time.

The binding of palytoxin to Na<sup>+</sup>/K<sup>+</sup>-ATPase results in an "open" ion channel that permits free passage of Na<sup>+</sup> and K<sup>+</sup> into or out of cells, depending on the ion gradient on both sides of the cell membrane (28–30). Kuroki *et al.* (17) reported that in Swiss 3T3 fibroblasts DMEM in which Na<sup>+</sup> was replaced by K<sup>+</sup> suppressed the ability of palytoxin to activate SAPK/JNK1; from these data they concluded that palytoxin-activated Na<sup>+</sup> influx was responsible for the activation of SAPK/JNK1. Our data in Rat-1 fibroblasts are in agreement with those of Kuroki *et al.* (17) and furthermore demonstrated that replacement of Na<sup>+</sup> by K<sup>+</sup> strongly suppressed the translational inhibition by palytoxin (Figs. 5 and 6). However, neither SAPK/JNK1 activation nor translational inhibition was diminished when Na<sup>+</sup> was replaced by choline<sup>+</sup>, a nonpenetrating cation used to maintain osmotic balance, and in fact both SAPK/JNK1 activation and translational inhibition were increased in the choline<sup>+</sup>-containing medium. These data suggest that palytoxin-induced efflux of K<sup>+</sup>, rather than influx of Na<sup>+</sup>, was responsible for both SAPK/JNK1 activation and translational inhibition. The inability of K-MEM to suppress the activation of SAPK/JNK1 mediated by other agents such as anisomycin, sorbitol, or IL-1 $\alpha$  suggests that replacement of Na<sup>+</sup> by K<sup>+</sup> did not generally inhibit the transduction of stress-generated signals from other ribotoxic or nonribotoxic stressors. Additionally, the activation of SAPK/JNK1 and suppression of translation that occurred in palytoxin-treated cells following the removal of K-MEM and replacement by palytoxin-free MEM demonstrates that the exposure of cells to K-MEM did not impair the ability of the cells to respond when placed in an appropriate ionic environment.

Palytoxin is the most potent nonproteinaceous toxin that has been identified. Although the ability of palytoxin to alter cation fluxes has been well documented, to date there has been no explanation for its severe toxicity. The demonstration in this paper that palytoxin can inhibit protein synthesis at picomolar concentrations (IC<sub>50</sub> = 1 pM, Fig. 1B) places palytoxin among the most potent translational inhibitors, and this may account for its potent toxicity.

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