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MAPK signaling pathways are needed for survival of H9c2 cardiac myoblasts under extracellular alkalosis

Konstantina Stathopoulou, Isidoros Beis, and Catherine Gaitanaki

Department of Animal and Human Physiology, School of Biology, Faculty of Sciences, University of Athens, Panepistimioupolis, Athens, Greece

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Stathopoulou K, Beis I, Gaitanaki C. MAPK signaling pathways are needed for survival of H9c2 cardiac myoblasts under extracellular alkalosis. *Am J Physiol Heart Circ Physiol* 295: H1319–H1329, 2008. First published July 25, 2008; doi:10.1152/ajpheart.01362.2007.—pH is one of the most important physiological parameters, with its changes affecting the function of vital organs like the heart. However, the effects of alkalosis on the regulation of cardiac myocyte function have not been extensively investigated. Therefore, we decided to study whether the mitogen-activated protein kinase (MAPK) signaling pathways [c-Jun NH_2-terminal kinases (JNKs), extracellular signal-regulated kinases (ERKs), and p38 MAPK] are activated by alkalosis induced with Tris-Tyrode buffer at two pH values, 8.5 and 9.5, in H9c2 rat cardiac myoblasts. These buffers also induced intracellular alkalinization comparable to that induced by 1 mM NH_4Cl. The three MAPKs examined presented differential phosphorylation patterns that depended on the severity and the duration of the stimulus. Inhibition of Na^+/H^+ exchanger (NHE)1 by its inhibitor HOE-642 prevented alkalinization and partially attenuated the alkalosis (pH 8.5)-induced activation of these kinases. The same stimulus also promoted c-Jun phosphorylation and enhanced the binding at oligonucleotides bearing the activator protein-1 (AP-1) consensus sequence, all in a JNK-dependent manner. Additionally, mitogen- and stress-activated kinase 1 (MSK1) was transiently phosphorylated by alkalosis (pH 8.5), and this was abolished by the selective inhibitors of either p38 MAPK or ERK pathways. JNKs also mediated Bcl-2 phosphorylation in response to incubation with the alkaline medium (pH 8.5), while selective inhibitors of the three MAPKs diminished cell viability under these conditions. All these data suggest that alkalosis activates MAPKs in H9c2 cells and these kinases, in turn, modify proteins that regulate gene transcription and cell survival.

signal transduction; cardiomyoblast; cell death

PROPER CELL FUNCTION DEPENDS on the maintenance of intracellular and extracellular physicochemical parameters within certain physiological limits. Such an important parameter is pH, which affects the conformation of proteins and hence their activity (35). Changes of pH toward more alkaline values can be induced systemically by a decline in arterial P_{CO_2} (respiratory alkalosis) (35) or during the action of growth factors and hormones (11, 49), and it has been found to affect cell decisions between survival and death (26).

In cardiac cells, increases in intracellular pH (pH_i) are observed after activation of Na^+/H^+ exchanger (NHE)1 by factors such as endothelin-1 (24) and α_1-adrenergic agonists (20). In addition, cardiomyocyte alkalinization affects cell metabolism by enhancing protein synthesis rate (10) and has a potent positive inotropic action, since it has been associated with increases in cardiac cell contractility (31). Nevertheless, little is known about the intracellular molecular events that alkalosis induces in this type of cell.

Cells respond to alterations in their inner or outer environment by triggering a series of signal transduction pathways, which regulate cellular physiology through gene transcription and posttranslational modifications of proteins important for metabolism. Among the most important signaling pathways are those involving the mitogen-activated protein kinases (MAPKs). MAPKs comprise a superfamily of Ser/Thr kinases and are divided into four families: extracellular signal-regulated kinases (ERKs), c-Jun NH_2-terminal kinases (JNKs), p38 MAPK, and big MAP kinase 1 (BMK1/ERK5) (for review, see Ref. 25). MAPKs are activated by a variety of stimuli and stressful conditions and are involved in the regulation of cell growth, differentiation, survival, and death, but the outcome of their activation is highly cell type- and stimulus specific (25).

The effects of MAPKs are mediated through their substrates, among which other kinases and transcription factors are included. A kinase known to be a downstream target for both ERKs and p38 MAPK is mitogen- and stress-activated kinase 1 (MSK1) (35), which has been implicated in the regulation of gene transcription by phosphorylating histone H3 (39). In addition, MAPKs can directly phosphorylate transcription factors, whose activation has been related to either the inhibition or the induction of cell death; an example is c-Jun, a component of the transcription factor activator protein-1 (AP-1) (6). c-Jun is phosphorylated by JNKs at its NH_2 terminal (residues Ser63 and Ser73), where its transactivation domain resides, and these modifications induce its transcriptional activity (6).

In addition, members of the Bcl-2 family, which consists of proteins with either pro- or antiapoptotic function, are regulated by MAPKs. The well-established antiapoptotic member of this family, Bcl-2, can be phosphorylated at multiple sites, and it has been reported that JNKs mediate its phosphorylation at Ser70 (36). However, the importance of this specific phosphorylation for Bcl-2 function has not been clarified yet, since there are conflicting data on whether it promotes or diminishes its protective activity (19, 36, 47).

In the present study, we investigated the effect of alkalosis on the activation of JNKs, ERKs, and p38 MAPK signaling pathways in H9c2 rat cardiomyoblasts, which are morphologically similar to embryonic cardiomyocytes and are recognized as a model well suited for the study of cardiomyocyte biology (17). Alkalosis was induced by using Tris-Tyrode buffer at two pH values, 8.5 and 9.5. Previous studies in adult rat cardiac

Address for reprint requests and other correspondence: C. Gaitanaki, Dept. of Animal and Human Physiology, School of Biology, Faculty of Sciences, Univ. of Athens, Panepistimioupolis, Athens 157 84 Greece (e-mail: cgaitan@biol.uoa.gr).

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myocytes and the isolated, perfused rat heart have shown that the specific experimental approach induces a $\Delta pH_i/\Delta$extracellular pH (pH_o) of ~0.25 units (10). Our results provide for the first time evidence that in H9c2 cells alkalosis differentially activates all three MAPK pathways examined and that this activation is associated with the regulation of important cell functions, such as cell survival.

MATERIALS AND METHODS

Materials. Most biochemicals used were from Applichem (Darmstadt, Germany). The enhanced chemiluminescence (ECL) kit was from Amersham International (Uppsala, Sweden). Bradford protein assay reagent was from Bio-Rad (Hercules, CA). Nitrocellulose (0.45 μm) was purchased from Schleicher & Schuell (Keene, NH). Propidium iodide (PI; stock solution) and poly(dI-dC) were purchased from Sigma (St Louis, MO). Hoechst 33258, nigericin, and 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) were from Molecular Probes (Carlsbad, CA). HOE-642 was kindly provided by Sanofi-Aventis Deutschland (Frankfurt am Main, Germany). SP-600125 and PD-98059 were purchased from Calbiochem-Novabiochem (La Jolla, CA). SB-203580 was obtained from Alexis Biochemicals (Lausen, Switzerland). Stock solutions of SB-203580, SP-600125, PD-98059, and HOE-642 were prepared in dimethyl sulfoxide (DMSO). [γ-^{32}P]ATP was from Hartmann Analytic (Braunschweig, Germany). AP-1 consensus oligonucleotide and T4 polynucleotide kinase were obtained from Promega (Madison, WI).

Rabbit polyclonal antibodies specific for the total or dually phosphorylated forms of p38 MAPK (nos. 9212 and 9211, respectively), JNKs (nos. 9252 and 9251, respectively) or ERKs (nos. 9102 and 9101, respectively), phosphorylated Bcl-2 (no. 2871), phosphorylated MSK1 (no. 9565), and total or phosphorylated c-Jun (nos. 9162 and 9261, respectively) were purchased from Cell Signaling Technology (Beverly, MA). Anti-actin antibody (A2103) was from Sigma. Prestained molecular mass markers were from New England Biolabs (P7708S). Biotinylated anti-rabbit antibody was from DAKO (Glostrup, Denmark). Super RX film was purchased from Fuji Photo Film (Dusseldorf, Germany).

Cell culture supplies were from PAA Laboratories (Pasching, Austria). Dulbecco's modified Eagle's medium (DMEM) was from Sigma.

Tyrode buffers. In this study HEPES- and Tris-Tyrode buffers were used. The HEPES-Tyrode buffer (pH 7.5) consisted of (in mM) 10 HEPES, 140 NaCl, 6 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, and 25 glucose. For the preparation of this buffer, NaCl was added at an initial concentration of 120 mM, and after pH adjustment to 7.5 with NaOH the appropriate quantity of NaCl was added so as its final concentration would be 140 mM. The Tris-Tyrode buffer consisted of (in mM) 10 Tris, 140 NaCl, 6 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, and 25 glucose, and pH was adjusted to 8.5 or 9.5 with HCl. Both HEPES- and Tris-Tyrode buffers were gassed with 100% O_2 for 5 min and warmed to 37°C before their use. pH of these buffers remained constant throughout experiments.

Cell culture and treatments. H9c2 rat cardiac myoblasts (purchased from the American Type Culture Collection, Rockville, MD) were grown in DMEM supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum and antibiotics under a humidified atmosphere of 95% air-5% CO_2 at 37°C. Experiments were carried out with mononucleated myoblasts after serum had been withdrawn for at least 16 h. In all experiments, DMEM was discarded and cells were equilibrated for 3 h, at 37°C and under an atmosphere of 100% air, with a HEPES-Tyrode buffer of normal pH value (7.5). After the 3-h equilibration period, HEPES-Tyrode buffer was discarded and H9c2 cells were incubated for increasing time periods either with fresh HEPES-Tyrode buffer or with an alkaline (pH 8.5 or 9.5) Tris-Tyrode buffer. As controls, we used cells that were only equilibrated for 3 h with the HEPES-Tyrode buffer. Equilibration of cells with a normal Tris-Tyrode buffer (pH 7.5) instead of the HEPES-Tyrode buffer (pH 7.5) did not affect the results described in this study (data not shown).

When pharmacological inhibitors were used, they were added 30 min before treatment and were present throughout the experiment. Control experiments of the same duration were also performed by incubating cells with HEPES-Tyrode buffer containing the respective inhibitors. In some experiments, after the 3-h equilibration period, cells were incubated for 30 min with 0.5 M sorbitol diluted in HEPES-Tyrode buffer and used as positive controls. For the experiments with NH_4Cl, a stock 41 mM solution of this substance was prepared in HEPES-Tyrode buffer pH 7.5, and after the 3-h equilibration period the appropriate quantity was added in the incubation medium so as to obtain a final concentration of 1 mM NH_4Cl.

Protein extraction. Cells were extracted in *buffer G* [in mM: 50 HEPES pH 7.5, 20 β-glycerophosphate, 2 EDTA, 10 benzamidine, 20 NaF, 0.2 Na_3VO_4, and 5 DTT, with 200 μM leupeptin, 10 μM E-64, 300 μM PMSF, and 0.5% (vol/vol) Triton X-100] on ice for 20 min. Samples were centrifuged (10,000 *g*, 4°C, 10 min), and the supernatants were boiled with 0.33 volumes of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer [0.33 M Tris·HCl pH 6.8, 10% (wt/vol) SDS, 13% (vol/vol) glycerol, 20% (vol/vol) 2-mercaptoethanol, 0.2% (wt/vol) bromophenol blue]. Protein concentrations were determined with the Bio-Rad Bradford assay.

Subcellular fractionation. Cytosolic and nuclear extracts were prepared as previously described (22) with minor modifications. Briefly, cells were harvested in *buffer A* (in mM: 10 HEPES pH 7.9, 10 KCl, 0.1 EDTA, 0.1 EGTA, 1.5 $MgCl_2$, 10 NaF, 1 Na_3VO_4, 20 β-glycerophosphate, 1 DTT, and 0.5 PMSF, with 4.2 μM leupeptin and 4 μg/ml aprotinin) and incubated on ice for 15 min. Samples were centrifuged (1,400 *g*, 4°C, 10 min), and the supernatants, containing the cytosolic fraction, were boiled with 0.33 vols of SDS-PAGE sample buffer. Pellets were incubated on ice with *buffer A* containing 0.6% (vol/vol) Nonidet P-40 (10 min) and centrifuged (1,400 *g*, 4°C, 10 min). Supernatants were discarded, and pellets were resuspended in *buffer B* (in mM: 20 HEPES pH 7.9, 400 NaCl, 1 EGTA, 0.1 EDTA, 1.5 $MgCl_2$, 10 NaF, 1 Na_3VO_4, 20 β-glycerophosphate, 0.2 DTT, and 0.5 PMSF, with 4.2 μM leupeptin and 4 μg/ml aprotinin) and incubated under rotation for 1 h at 4°C. After centrifugation (11,000 *g*, 4°C, 10 min) the supernatants, consisting of the nuclear proteins, were either stored at −80°C for use in the electrophoretic mobility shift assay (EMSA) or boiled with 0.33 vols of SDS-PAGE sample buffer for use in Western blotting assays. Protein concentrations were determined with the Bio-Rad Bradford assay.

Immunoblotting. Proteins were separated by SDS-PAGE on 10% (wt/vol) or 15% (wt/vol) polyacrylamide gels and transferred electrophoretically onto nitrocellulose membranes (0.45 μm). Nonspecific binding sites were blocked by incubating membranes with Tris-buffered saline-Tween [TBS-T; 20 mM Tris·HCl pH 7.6, 137 mM NaCl, 0.05% (vol/vol) Tween 20] containing 1% (wt/vol) bovine serum albumin (BSA) at room temperature. Subsequently, membranes were incubated with the appropriate primary antibody according to the manufacturer's instructions. After being washed in TBS-T (4 × 5 min), membranes were incubated with horseradish peroxidase-conjugated secondary antibody [1:5,000 dilution in TBS-T containing 1% (wt/vol) BSA, 1 h at room temperature]. Bands were detected with the ECL reaction and exposure to Super RX films. Blots were quantified by laser scanning densitometry (Gel Analyser v. 1.0).

Electrophoretic mobility shift assay. The assay was performed as previously described (22). Briefly, oligonucleotides (3.5 pmol) corresponding to the binding consensus sequence of AP-1 were 5′ end-labeled with [γ-^{32}P]ATP by incubation for 30 min at 37°C with 10 U of T4 polynucleotide kinase and 15 μCi of [γ-^{32}P]ATP. Subsequently, nuclear extracts (10 μg) were incubated (15 min, 4°C) in binding buffer [5 mM $MgCl_2$, 34 mM KCl, and 0.15 μg/μl poly(dI-dC)], and then the samples were incubated for 30 min at 4°C with 100,000 cpm of labeled oligonucleotides. DNA-protein complexes were resolved in

4% (wt/vol) polyacrylamide (29:1 acrylamide-bis-acrylamide) gels in 0.5× TBE buffer (in mM: 890 Tris·HCl pH 8.0, 890 boric acid, 20 EDTA). Gels were dried and exposed to Super RX photo film at −80°C for 24 h with an intensifying screen.

Intracellular pH measurement. For measuring pH_i, cells were grown in appropriate 96-well plates (10,000 cells/well), equilibrated for 3 h with HEPES-Tyrode buffer (pH 7.5), and then loaded with the fluorescent pH-sensitive dye BCECF-AM (5 μM) for 30 min in the dark at room temperature. After two washes with HEPES-Tyrode buffer (pH 7.5), cells were incubated for 30 min with the same buffer and then subjected to treatments. When treatments included either HOE-642 or DMSO alone, cells were preincubated for 30 min with these compounds. Immediately after treatments, loaded cells were excited at 490 and 440 nm, and emitted fluorescence was detected at 535 nm with the TECAN Safire^2 plate reader (Tecan Trading). pH_i was calculated from the fluorescence intensity ratio (490/440), and a pH calibration curve built with the nigericin technique (42), using at least three calibration solutions in the pH range of 6.8–7.8. The calibration solutions contained (in mM) 146 KCl, 10 HEPES, 1 $MgCl_2$, and 1.8 $CaCl_2$, with 10 μg/ml nigericin.

Cell viability assay. Viability was assessed by staining cells with PI, which penetrates damaged membranes and intercalates to nuclear DNA. H9c2 cells were grown on cell culture coverslips, equilibrated for 3 h with HEPES-Tyrode buffer (pH 7.5), and treated with the alkaline medium (pH 8.5) for 4 h, in the absence or presence of selective MAPK inhibitors. Control cells were incubated for 4 h, after the equilibration period, with HEPES-Tyrode buffer, in the presence or absence of these inhibitors. Thirty minutes before the end of treatments, PI was added at a dilution of 1:2,000. Cell nuclei were counterstained for 10 min with 10 μg/ml Hoechst 33258. Coverslips were then washed three times in phosphate-buffered saline (PBS), mounted, and observed under a Zeiss Axioplan fluorescence microscope equipped with an AxioCam MRc5.

Statistical evaluation. All data are presented as means ± SE from at least three independent experiments. Statistical evaluation was performed with one-way analysis of variance followed by Dunnett's multiple comparison test. A value of $P < 0.05$ was considered to be statistically significant. In all proteins examined, phosphorylation levels were normalized against total protein levels and in "control" cells were set at 1, whereas the stimulated kinase and protein phosphorylation in treated cells was expressed as "fold" activation or increase over control cells.

RESULTS

Extracellular alkalosis induces intracellular alkalinization in H9c2 cells. The steady-state pH_i of cardiac myocytes can be conventionally manipulated by cell exposure to buffers of varying pH (12). Therefore, we incubated H9c2 cells with buffers of increasing pH values (8.5 and 9.5) and measured the respective pH_i values, using BCECF-AM. The results obtained revealed that extracellular alkalosis induces significant intracellular alkalinization with intensity depending on the pH_o value [from 7.10 ± 0.01 to 7.46 ± 0.07 ($P < 0.001$) or 7.68 ± 0.06 ($P < 0.001$) for pH_o 8.5 or pH_o 9.5, respectively] (Table 1). The increase in pH_i value by the mild alkaline (pH 8.5) Tris-Tyrode buffer was prevented by 5 μM HOE-642, a specific NHE1 inhibitor (21), while DMSO alone did not affect the cytosolic alkalinization of H9c2 cells induced by this buffer (Table 1). Furthermore, NH_4Cl, which was used as a positive control for intracellular alkalinization (38), increased pH_i (7.40 ± 0.06, $P < 0.01$) to values comparable to those induced by the alkaline Tris-Tyrode buffers (Table 1).

Table 1. *Measurement of intracellular pH values under different conditions*

Condition	pH_i
pH_o 7.5 (control)	7.10±0.01
pH_o 8.5	7.46±0.07†
pH_o 9.5	7.68±0.06†‡
pH_o 8.5 + 5 μM HOE-642	7.06±0.03§
pH_o 8.5 + 0.1% (vol/vol) DMSO	7.55±0.06†
pH_o 7.5 + 1 mM NH_4Cl	7.40±0.06*

Intracellular pH (pH_i) values are presented as means ± SE for $n = 8$ experiments. pH_o, extracellular pH. *$P < 0.01$ vs. control; †$P < 0.001$ vs. control; ‡$P < 0.05$ vs. pH_o 8.5; §$P < 0.001$ vs. pH_o 8.5.

Extracellular alkalosis induces phosphorylation of MAPKs in H9c2 cells. The MAPK families of JNKs, ERKs, and p38 MAPK are activated by concomitant Thr and Tyr phosphorylation within a conserved Thr-X-Tyr motif that exists in the activation loop of the kinases (25). For this reason, we performed immunoblot analysis using antibodies that specifically recognize these dually phosphorylated forms of the kinases in order to determine their activation. It is of note that the three JNK genes (in cardiac cells *JNK1* and *JNK2* genes are mainly expressed) produce alternatively spliced transcripts that encode multiple protein isoforms with apparent molecular masses of 54 and 46 kDa. These isoforms cannot be discriminated by the immunoblotting method used in this study, and we will refer to them as the p54 JNK and p46 JNK isoforms, respectively.

Incubation of H9c2 cells with the mild alkaline Tris-Tyrode buffer (pH 8.5) differentially activated all three MAPK families examined; the phosphorylation of JNKs and ERKs was rapid, intense, and prolonged, whereas p38 MAPK activation was also immediate but transient and moderate. In particular, alkalosis at pH 8.5 induced a strong phosphorylation of all JNK isoforms (p54 JNK and p46 JNK) that was evident from the second minute of treatment, maximized within 15 min, and was persistent for at least 60 min. Of these JNK isoforms, p46 JNK presented the greatest activation [at 15 min: p46 JNK 5.52 ± 0.47-fold relative to control values ($P < 0.01$), p54 JNK 3.17 ± 0.31-fold ($P < 0.01$)] (Fig. 1, *A*, *top*, and *B*).

ERK phosphorylation followed a similar pattern: maximal activation was also observed at the 15-min time point, with p44 ERK exhibiting more intense phosphorylation (3.45 ± 0.38-fold relative to control values, $P < 0.01$) than p42 ERK (2.50 ± 0.39-fold, $P < 0.01$) (Fig. 1, *C*, *top*, and *D*). As a positive control, a sample from cells treated with 0.5 M sorbitol for 30 min was used.

By contrast, alkalosis at pH 8.5 induced a rapid but transient p38 MAPK phosphorylation. Maximal activation was observed at 5 min of incubation with the alkaline medium (2.04 ± 0.38-fold relative to control values, $P < 0.05$), reaching control values after 15 min of treatment (Fig. 1, *E*, *top*, and *F*).

Equal protein loading was verified by reprobing the membranes with antibodies against phosphorylation state-independent levels of the respective protein kinases (Fig. 1, *A*, *C*, and *E*, *bottom*).

At more severe alkaline conditions (pH 9.5), JNKs were also significantly activated. Maximal phosphorylation was attained at 15 min of incubation for p46 JNK (3.03 ± 0.70-fold, $P < 0.05$) and at 30 min for p54 JNK (3.16 ± 0.50-fold, $P < 0.05$) and declined thereafter (Fig. 2, *A*, *top*, and *B*). On the contrary,

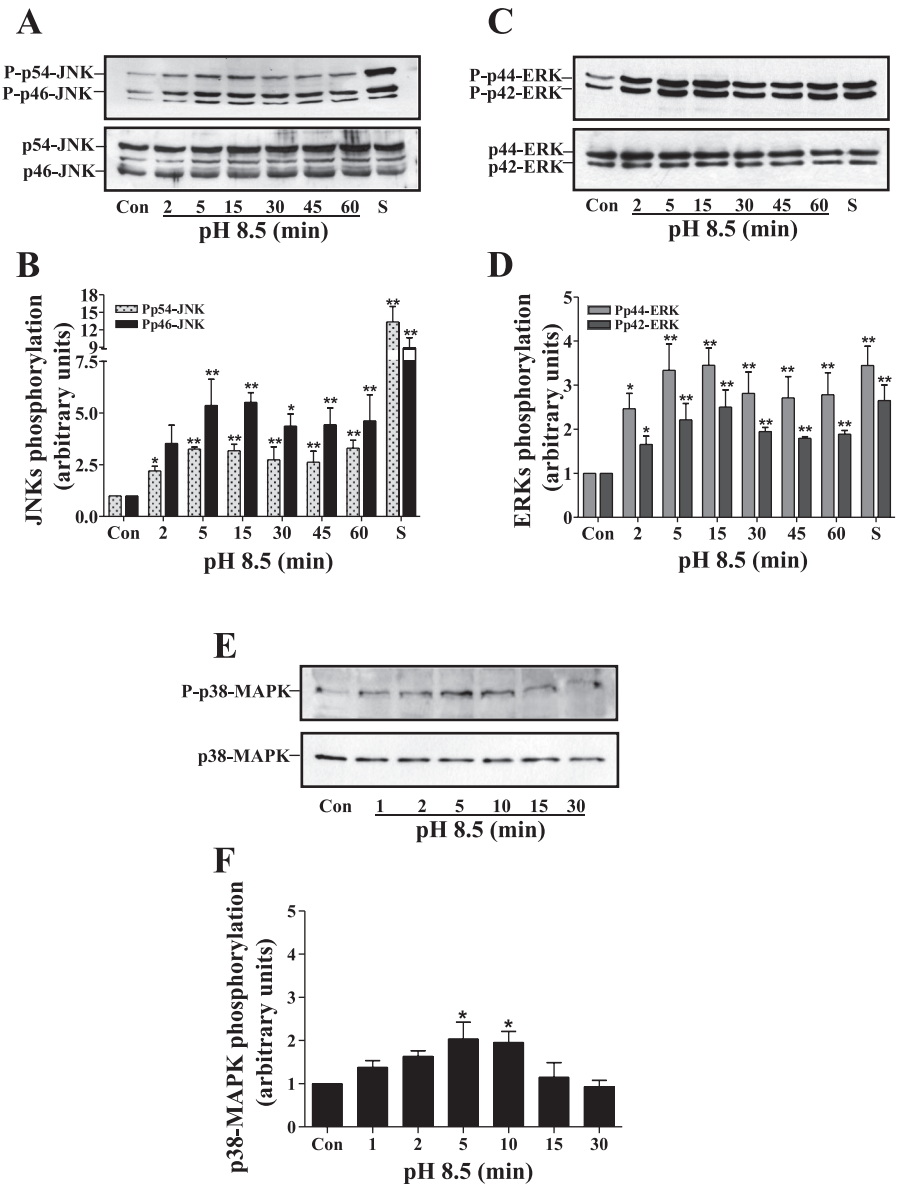

Fig. 1. Effect of alkalosis (pH 8.5) on c-Jun NH_2-terminal kinases (JNK), extracellular signal-regulated kinases (ERK), and p38 mitogen-activated protein kinase (MAPK) phosphorylation. *A*, *C*, and *E*: protein (20 μg) from H9c2 cells incubated without (Con) or with Tris-Tyrode buffer pH 8.5 for the times indicated was assessed by immunoblot analysis with antibodies specific for the phosphorylated (P; *top*) or phosphorylation state-independent (*bottom*) forms of JNKs (*A*), ERKs (*C*), and p38 MAPK (*E*). Extracts from cells incubated with 0.5 M sorbitol for 30 min (S) were used as a positive control (*A* and *C*, *top*). *B*, *D*, and *F*: densitometric analysis of phospho-JNK (*B*), phospho-ERK (*D*), and phospho-p38 MAPK (*F*) bands by laser scanning. Values are means ± SE of at least 3 independent experiments. $*P < 0.05$, $**P < 0.01$ vs. control values.

even though p44 ERK was significantly activated after 5 min of incubation with the alkaline medium (pH 9.5) (2.19 ± 0.24-fold, $P < 0.05$), ERK phosphorylation levels fell significantly below basal after 45 min of treatment (Fig. 2, *C*, *top*, and *D*). p38 MAPK was not considerably activated under these extreme alkaline conditions (data not shown). Phosphorylation state-independent levels of each MAPK were detected in order to confirm equal protein loading (Fig. 2, *A* and *C*, *bottom*).

Incubation of H9c2 cells with the control buffer (HEPES-Tyrode, pH 7.5) for increasing time periods varying between 5 and 60 min showed no significant MAPK phosphorylation (data not shown).

Alkalosis-induced MAPK activation was confirmed by incubating cells with NH_4Cl. In particular, 1 mM NH_4Cl induced a strong phosphorylation of p54 JNK and p46 JNK within 15 min [3.97 ± 0.54-fold ($P < 0.05$) for p54 JNK and 2.77 ± 0.52-fold ($P < 0.05$) for p46 JNK] (Fig. 3, *A*, *top*, and *B*) and a moderate phosphorylation of ERKs [1.82 ± 0.31-fold ($P < 0.05$) for p44 ERK at 5 min and 1.71 ± 0.06-fold ($P < 0.01$) for p42 ERK at 15 min] (Fig. 3, *C*, *top*, and *D*) and p38 MAPK (2.32 ± 0.23-fold, $P < 0.05$ at 5 min) (Fig. 3, *E*, *top*, and *F*).

Alkalosis-induced phosphorylation of MAPKs needs an active NHE1. NHE1 is one of the most important pH regulators in cardiac cells. Addition of HOE-642 (5 μM) into the alkaline (pH 8.5) Tris-Tyrode buffer prevented intracellular alkalinization of H9c2 cells (Table 1). Therefore, we examined whether NHE1 is involved in JNK, ERK, and p38 MAPK activation by alkalosis. For this purpose, H9c2 cells were incubated with the alkaline (pH 8.5) medium for 15 min (time point for JNK and ERK maximum phosphorylations) or 5 min (time point for p38 MAPK maximum phosphorylation) in the absence or presence of HOE-642 (5 μM). As shown in Fig. 4, HOE-642 differentially attenuated the alkalosis-induced phosphorylation of all three MAPKs examined. Taking into consideration the activation of MAPKs induced by the inhibitor alone, HOE-642 partially inhibited JNK activation (by ~56% for p54 JNK and ~34% for p46 JNK) (Fig. 4*A*, *top*, and *B*), strongly attenuated ERK phosphorylation (by ~63% for p44 ERK and ~84% for

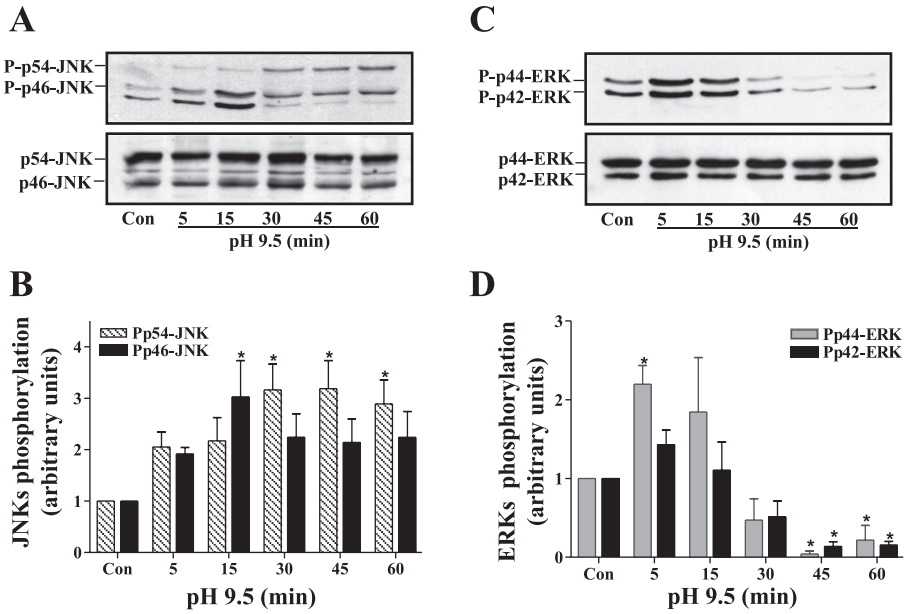

Fig. 2. Phosphorylation of JNKs and ERKs under extreme alkaline conditions (pH 9.5). *A* and *C*: phosphorylation of JNKs (*A*, *top*) and ERKs (*C*, *top*) was detected in extracts (20 μg of protein) from control H9c2 cells (Con) or cells incubated with Tris-Tyrode buffer pH 9.5 for the times indicated. Total JNKs (*A*, *bottom*) and ERKs (*C*, *bottom*) were detected in identical samples as a control for equal loading. *B* and *D*: densitometric analysis of phospho-JNK (*B*) and phospho-ERK (*D*) bands by laser scanning. Values are means ± SE of at least 3 independent experiments. $*P < 0.05$ vs. control values.

p42 ERK) (Fig. 4*C*, *top*, and *D*), and abolished the alkalosis-induced p38 MAPK phosphorylation (Fig. 4*E*, *top*, and *F*). The above results suggest that activation of MAPKs by alkalosis is at least in part mediated by NHE1. Equal protein loading was verified by probing identical samples with antibodies against the respective total MAPK protein levels (Fig. 4, *A*, *C*, and *E*, *bottom*).

Alkalosis increases c-Jun phosphorylation and AP-1 DNA binding activity. Alkalosis at pH 8.5 also induced the Ser63 phosphorylation of c-Jun (at 15 min: 2.44 ± 0.13-fold relative to control values, $P < 0.05$) (Fig. 5, *A*, *top*, and *B*), a component of the AP-1 transcription factor and one well-established substrate of JNKs (6, 25). This phosphorylation was abolished by the JNK inhibitor SP-600125 (10 μM) (Fig. 5, *A*, *top*, and *B*). This result was corroborated by EMSA experiments in which we observed that alkalosis increased the binding activity at oligonucleotides containing the AP-1 consensus sequence. In agreement with the immunoblot analysis, the JNK inhibitor SP-600125 (10 μM) abolished this alkalosis-induced increase in the DNA binding activity of AP-1 (Fig. 5*C*). Total c-Jun as well as actin protein levels remained constant under these experimental conditions (Fig. 5*A*, *middle* and *bottom*, respectively).

MSK1 phosphorylation is enhanced under alkaline conditions in a p38 MAPK- and ERK-dependent manner. MSK1 is a downstream member of both the p38 MAPK and ERK pathways and is also implicated in the regulation of gene transcription (15, 22). Therefore, MSK1 phosphorylation was also investigated under alkaline conditions (pH 8.5) in H9c2 cells. Immunoblot analysis was performed with an antibody that recognizes MSK1 when phosphorylated at Thr581. Alkalosis induced this MSK1 phosphorylation within 2 min of

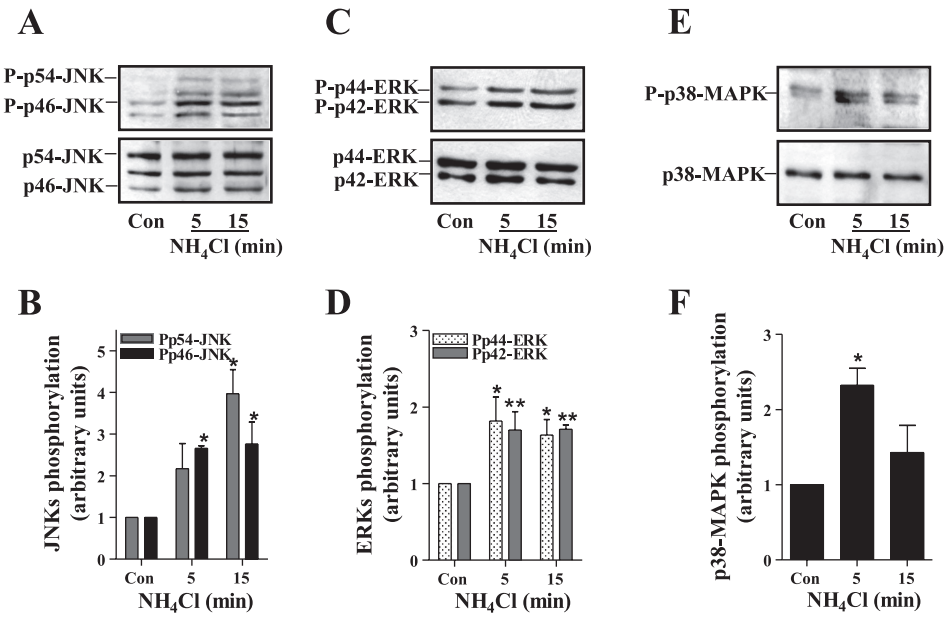

Fig. 3. Effect of NH_4Cl on JNK, ERK, and p38 MAPK phosphorylation. *A*, *C*, and *E*: protein (20 μg) from H9c2 cells incubated without (Con) or with 1 mM NH_4Cl for the times indicated was assessed by immunoblot analysis using antibodies specific for the phosphorylated (*top*) or total (*bottom*) forms of JNKs (*A*), ERKs (*C*), and p38 MAPK (*E*). *B*, *D*, and *F*: densitometric analysis of phospho-JNK (*B*), phospho-ERK (*D*), and phospho-p38 MAPK (*F*) bands by laser scanning. Values are means ± SE of 3 independent experiments. $*P < 0.05$, $**P < 0.01$ vs. control values.

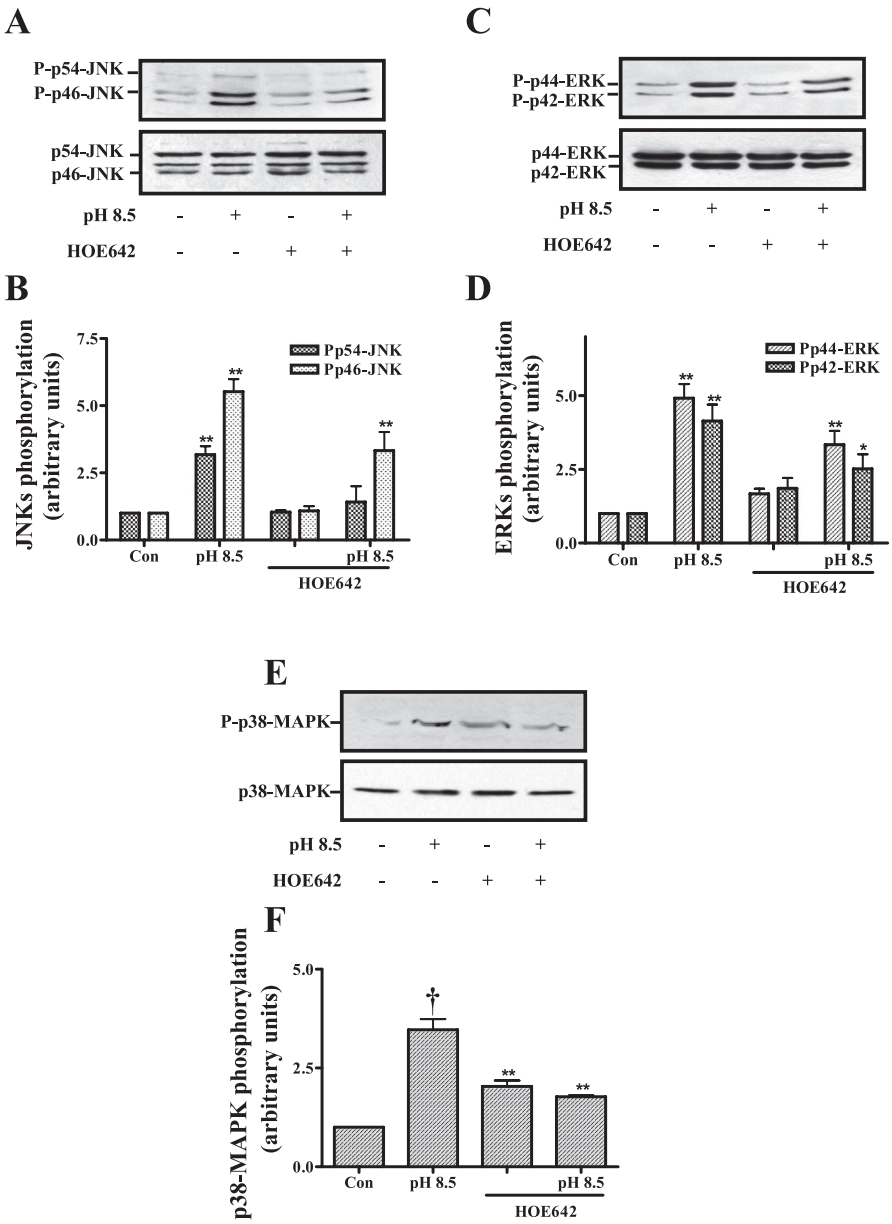

Fig. 4. Effect of inhibition of Na^+/H^+ exchanger (NHE)1 on the alkalosis-induced phosphorylation of JNKs, ERKs, and p38 MAPK. *A*, *C*, and *E*: protein (20 μg) from cells incubated under alkaline conditions (pH 8.5) for 15 min (*A*, *C*) or 5 min (*E*), in the presence or absence of 5 μM HOE-642, was immunoblotted against phospho-JNKs (*A*, *top*), phospho-ERKs (*C*, *top*), and phospho-p38 MAPK (*E*, *top*). Phosphorylation state-independent MAPK protein levels (*A*, *C*, and *E*, *bottom*) were detected as a control for equal loading with the corresponding antibodies. *B*, *D*, and *F*: densitometric analysis of phospho-JNK (*B*), phospho-ERK (*D*), and phospho-p38 MAPK (*F*) bands by laser scanning. Values are means ± SE of 3 independent experiments. $*P < 0.05$, $**P < 0.01$, $\dagger P < 0.001$ vs. control values.

treatment, but this response was transient since it was diminished to control levels after 45 min. Maximum phosphorylation was detected after 5 min of incubation with the alkaline medium (5.71 ± 0.93-fold relative to control values, $P < 0.01$) (Fig. 6, *A*, *top*, and *B*). This phosphorylation pattern resembles the activation pattern of p38 MAPK, and for this reason we performed experiments using the selective p38 MAPK inhibitor SB-203580 (10 μM), so as to delineate whether this kinase is implicated in MSK1 phosphorylation in our experimental model. Our results showed that inhibition of p38 MAPK activity abolished the alkalosis-induced phosphorylation of MSK1 at Thr581 (Fig. 6*C*, *top*, *D*). In addition, similar treatment with 25 μM PD-98059, which restrains ERK activation, also attenuated MSK1 phosphorylation (Fig. 6*C*, *top*, *D*), indicating that in H9c2 cells and under alkaline conditions these two MAPK pathways converge at the level of MSK1.

Equal protein loading was assessed by probing identical samples with an anti-actin antibody (Fig. 6, *A*, *bottom*, and *C*, *bottom*).

JNKs mediate alkalosis-induced Bcl-2 phosphorylation at Ser70. We also investigated whether alkalosis (pH 8.5) alters the phosphorylation status of the anti-apoptotic protein Bcl-2. In particular, Bcl-2 phosphorylation at Ser70 was examined by immunoblot analysis using an antibody specific for this phosphorylated form of Bcl-2. Our results showed that alkalosis induced a rapid and prolonged increase in Ser70 phosphorylation. This posttranslational modification was evident from the fifth minute after the onset of the experiment (2.71 ± 0.66-fold relative to control values, $P < 0.05$), remained at the same levels for at least 45 min (2.60 ± 0.50-fold, $P < 0.01$), and declined thereafter, reaching control levels after 6 h of treatment (Fig. 7, *A*, *top*, and *B*).

Previous studies suggest that Ser70 Bcl-2 phosphorylation is mediated by JNKs (29, 47), and this was also tested in our experimental model. Therefore, H9c2 cells were incubated with the alkaline medium (pH 8.5) for 45 min in the absence or presence of the JNK inhibitor SP-600125 (10 μM). Ser70

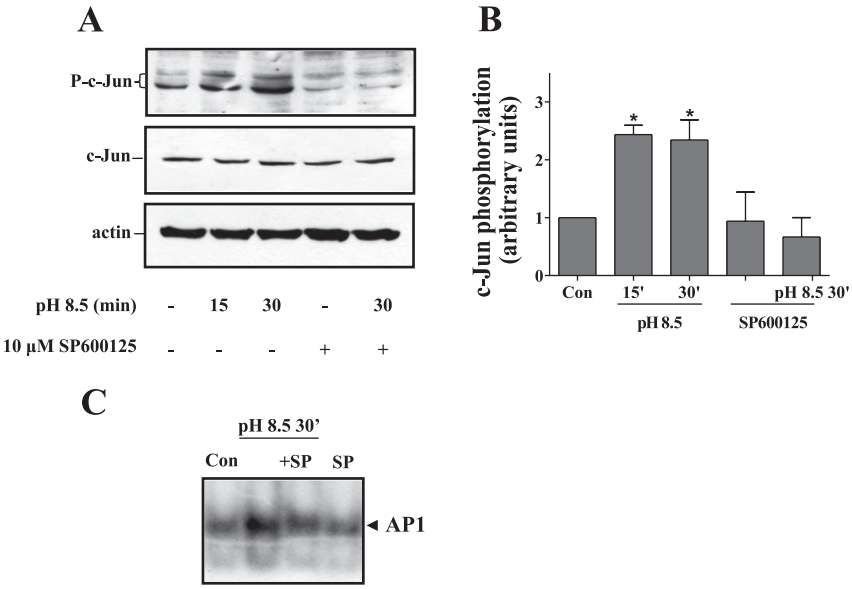

Fig. 5. Effect of alkalosis on c-Jun phosphorylation and activator protein-1 (AP-1) DNA binding. *A*: nuclear extracts (25 μg of protein for phospho-c-Jun and actin or 15 μg for total c-Jun) from control cells or cells subjected to alkalosis (pH 8.5), in the absence or presence of the JNK inhibitor SP-600125 (10 μM), were assayed with antibodies specific for the phosphorylated form of c-Jun at Ser63 (*top*), the total levels of c-Jun (*middle*), and actin protein levels (*bottom*). *B*: densitometric analysis of phospho-c-Jun bands by laser scanning. Values are means ± SE of at least 3 independent experiments. $*P < 0.05$ vs. control values. *C*: nuclear extracts prepared from control cells (Con) and cells treated under alkaline conditions (pH 8.5, 30 min), in the absence or presence of 10 μM SP-600125 (SP), were assayed for their ability to bind to oligonucleotides bearing the AP-1 consensus sequence. Image is representative of 3 independent experiments.

phosphorylation of Bcl-2 was abolished by JNK inhibition (Fig. 7, *C*, *top*, and *D*), indicating that these kinases mediate this modification in H9c2 cells. Equivalent protein loading was verified by probing identical samples with an antibody recognizing actin protein levels (Fig. 7, *A*, *bottom*, and *C*, *bottom*).

MAPKs are involved in cell survival under alkaline conditions. To examine whether alkalosis affects cell survival as well as the possible involvement of the three MAPK families in the latter, we assessed cell viability under alkaline conditions (pH 8.5, 4 h) in the absence or presence of MAPK inhibitors. Using the PI staining method, we observed that, compared with control cells, cells subjected to alkalosis did not show any decrease in viability (Fig. 8). When the selective ERK inhibitor PD-98059 (25 μM) and the p38 MAPK inhibitor SB-203580 (10 μM) were used along with the alkaline stimulus, a significant increase of cell death was observed (Fig. 8). Cells were also incubated with the respective inhibitors at normal pH (7.5; Fig. 8) or with the respective amount of DMSO alone (data not shown), with no apparent change in their viability. On the other hand, 10 μM SP-600125, a selective JNK inhibitor, induced cell death to some extent under control conditions but massive cell death under alkaline conditions (Fig. 8). When this inhibitor was used at an even lower concentration (5 μM), no cell death was observed under normal conditions, whereas its effect under alkaline conditions was quite similar to that induced by 10 μM (Fig. 8). The above results indicate that all three MAPK signaling pathways examined have a beneficial role, promoting cell survival under the alkaline conditions tested. To verify the presence of cells in the respective fields, nuclei were simultaneously stained with Hoechst 33258 (Fig. 8).

DISCUSSION

In the mammalian heart, the MAPK signaling pathways are activated by a variety of stimuli including different kinds of stress, growth factors, cytokines, and hormones (1, 7, 13, 25) and are related to physiological responses like cardiac cell hypertrophy, survival, or death (5). Some of the factors that activate these signal transduction pathways in cardiomyocytes and other cell types can also induce a rise in pH_i. Changes in pH_i toward alkaline values have been observed in response to osmotic shock (3), hormones like epinephrine and insulin (11, 49), and growth factors such as the epidermal growth factor (27). In rat heart, acute α_1-adrenergic stimulation has been demonstrated to increase pH_i, and this correlated with an enhancement of cardiac protein synthesis rate (11), whereas insulin stimulated cytosolic alkalinization, which activated glucose transport in rat cardiomyocytes (49).

In the present study, we investigated the effect of alkalosis on the induction of the three well-established MAPK families (JNKs, ERKs, and p38 MAPK) in H9c2 rat cardiac myoblasts. Alkalosis was exerted by using Tris-Tyrode buffer at two pH values (8.5 and 9.5). Even though pH_o changes of such magnitude are difficult to produce in vivo, with the exception of some pathological states, these buffers were used in order to alkalinize H9c2 cells (Table 1). All three MAPKs examined were differentially activated by alkalosis, and their phosphorylation patterns largely depended on the intensity of the stimulus (Figs. 1 and 2), pointing out that changes in cellular H^+ concentration ($[H^+]$) can activate the mechanisms of signal transduction. The results obtained from these experiments could not be attributed to the effect of Tris-base on cells, as Motz et al. suggested in a recent paper (30), since even though both alkaline (8.5 and 9.5) Tris-Tyrode buffers have the same concentration of Tris-base (10 mM) they induce different phosphorylation patterns of all three MAPKs examined in our experimental model (Figs. 1 and 2). Nevertheless, we confirmed that alkalosis activates MAPKs by using another alkalinizing factor, NH_4Cl (Fig. 3), indicating that the activation of the MAPK signal transduction pathways is a specific response of H9c2 cells to pH_i increases.

Our results are in agreement with previous studies reporting activation of MAPKs by alkalosis. In particular, JNKs and p38 MAPK were activated by intracellular alkalinization induced by the weak bases NH_4Cl, trimethylamine, and triethylamine in U937 cells (38). p38 MAPK was also activated by extracellular alkalosis in the isolated, perfused amphibian heart (40), and a similar response of ERKs was observed in vascular smooth

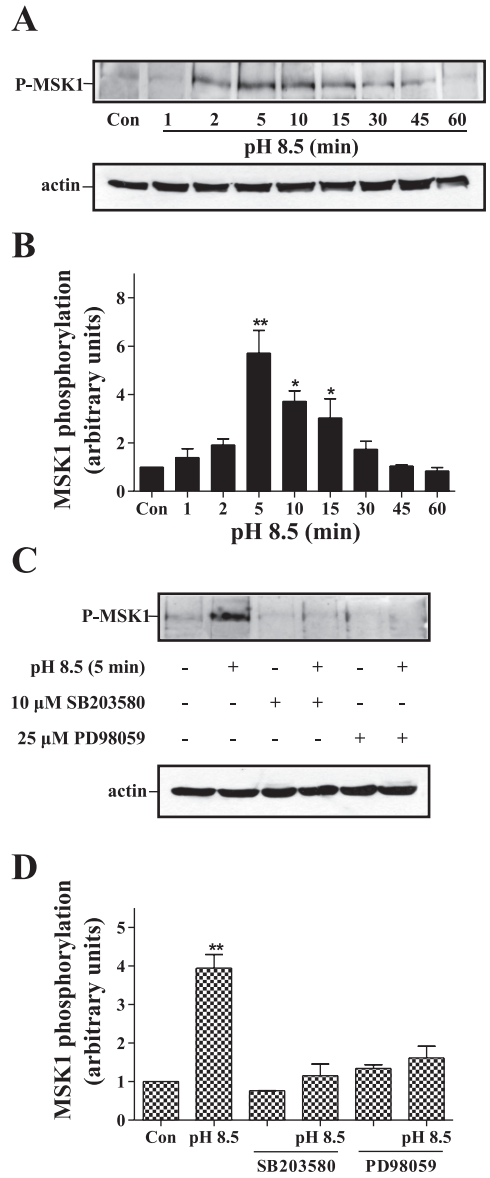

Fig. 6. Mitogen- and stress-activated kinase 1(MSK1) phosphorylation in cells exposed to alkaline conditions (pH 8.5). *A*: protein (40 μg) from H9c2 cells incubated without (Con) or with Tris-Tyrode buffer pH 8.5 for the times indicated was assessed by immunoblot analysis using an antibody specific for the phosphorylated form of MSK1 at Thr581 (*top*). Actin protein levels were detected in identical samples as a control for loading (*bottom*). *C*: MSK1 phosphorylation induced by exposing H9c2 cells to alkaline conditions (pH 8.5) for 5 min was abolished in the presence of either 10 μM SB-203580 or 25 μM PD-98059 (*top*). Equal protein loading was assessed by probing identical samples with an anti-actin antibody (*bottom*). *B* and *D*: densitometric analysis of phospho-MSK1 bands by laser scanning. Values are means ± SE of at least 3 independent experiments. $*P < 0.05$, $**P < 0.01$ vs. control values.

muscle cells (41). However, the activation patterns of MAPKs in those studies were not identical to those we detected, and this can be attributed to the distinct approaches of alkalosis used and to differences existing among cell types. This is also evident by the fact that although in the work of Susa and Wakabayashi (41) ERK activation by alkalosis was through the NADPH oxidase-mediated formation of reactive oxygen species, in our model, apocynin, a NADPH oxidase inhibitor, did not attenuate the alkalosis-induced MAPK activation (data not shown).

On the contrary, activation of the three MAPKs was blocked by the NHE1 inhibitor HOE-642 (Fig. 4). NHE1 is one of the most important regulators of pH_i in cardiac cells. It exchanges one H^+ from the inner of the cell with one Na^+ from the extracellular space (35), thus causing intracellular alkalinization when it is active. Consequently, inhibition of this activity would lead to a local intracellular increase of $[H^+]$, which counteracts any increase in cellular pH. Indeed, cytosolic alkalinization of H9c2 cells caused by incubating them with the alkaline Tris-Tyrode buffer (pH 8.5) was prevented by HOE-642 (Table 1), a result consistent with the observed decrease in

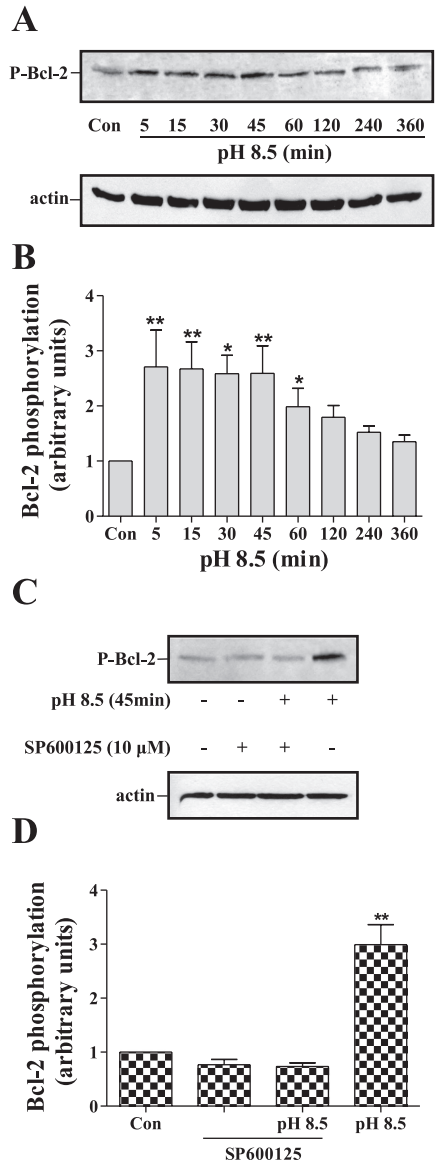

Fig. 7. Effect of alkalosis on Bcl-2 phosphorylation. *A*: phosphorylation of Bcl-2 at Ser70 was detected in extracts (30 μg of protein) from control H9c2 cells (Con) or cells incubated with Tris-Tyrode buffer pH 8.5 for the times indicated (*top*). Equal loading was assessed in identical samples with an antibody against actin (*bottom*). *C*: Ser70 Bcl-2 phosphorylation (*top*) or actin protein levels (*bottom*) were also assessed in extracts from cells treated under alkaline conditions (pH 8.5, 45 min) in the absence or presence of 10 μM SP-600125. *B* and *D*: densitometric analysis of phospho-Bcl-2 bands by laser scanning. Values are means ± SE of at least 3 independent experiments. $*P < 0.05$, $**P < 0.01$ vs. control values.

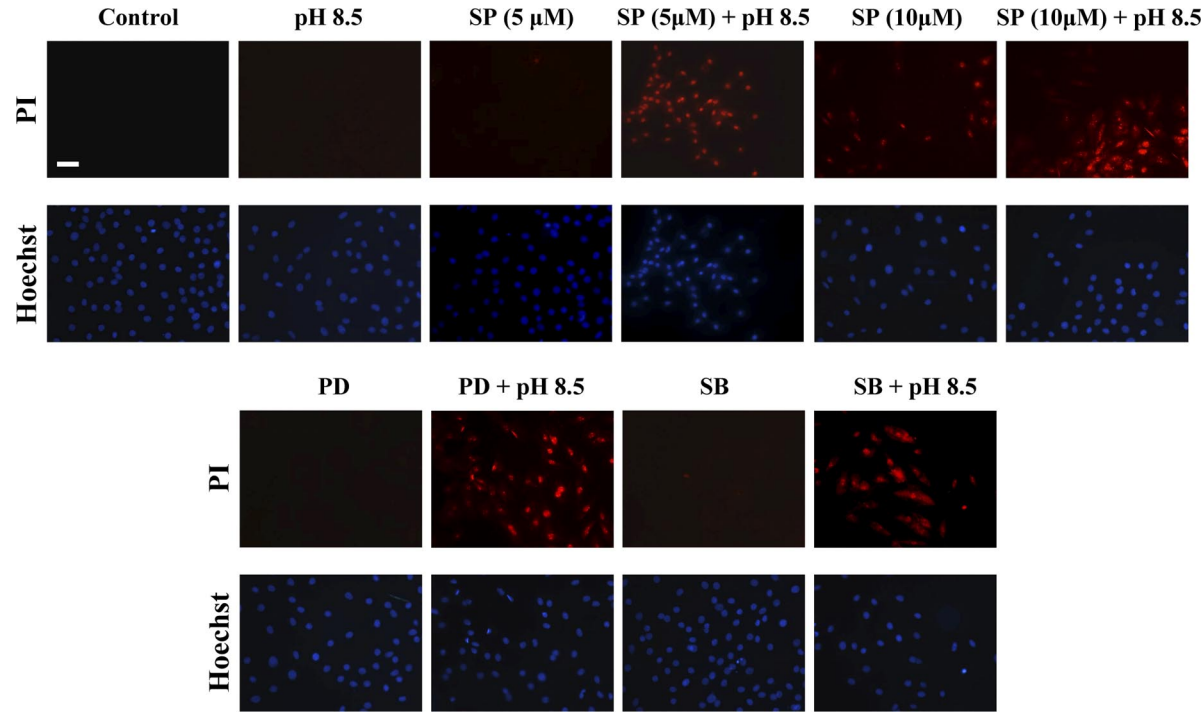

Fig. 8. Viability of cells exposed to control (Con) or alkaline conditions (pH 8.5) for 4 h, in the absence or presence of the JNK inhibitor SP-600125 (5 or 10 μM; SP), the MEK1/2 inhibitor PD-98059 (25 μM; PD), or the p38 MAPK inhibitor SB-203580 (10 μM; SB), was assessed by fluorescence microscopy using propidium iodide (PI) staining of dead cells. Pictures of the respective fields showing cell nuclei stained with Hoechst 33258 are also included. Images are representative of 3 independent experiments. Scale bar, 50 μm.

MAPK phosphorylation levels in the presence of this inhibitor. The possible role of this NHE1 activity could be the equilibration of pH_o to physiological levels, since the extracellular environment is also important for proper cellular function.

In addition, a direct interaction between NHE1 and the MAPK signaling pathways in H9c2 cells cannot be excluded. It was previously demonstrated that MAPKs affect NHE1 activity and vice versa (2, 16, 18, 23, 33, 48). In cardiac myocytes, specifically, this relation is shown by the fact that, in response to an acid load, NHE1 is phosphorylated via the ERK signaling pathway, and inhibition of this by PD-98059 or UO126 attenuates NHE1 activity (16), a role that these kinases could have in our model, too. Conversely, NHE1 participates in ERK and p38 MAPK activation by high-glucose-induced hypertrophy and pacing-induced heart failure, respectively (2, 33, 48). Therefore, it seems that NHE1 participates in the MAPK signaling pathways and, depending on the stimulus, it lies upstream or downstream from these kinases.

The activation of signal transduction pathways usually culminates in the modulation of the activity of various transcription factors, and in the present study JNKs, activated by alkalosis, phosphorylated c-Jun at residue Ser63 (Fig. 5). c-Jun is a component of the AP-1 transcription factor (6), and the phosphorylation at residues Ser63 and Ser73, which lie within the transactivation domain of the protein, increases its transcriptional activity through the loss of repression mediated by an inhibitory complex associated with histone deacetylase 3 (6, 45). c-Jun is also phosphorylated at its COOH terminal, where its DNA binding domain is located. This phosphorylation is catalyzed in vivo by glycogen synthase kinase (GSK)-3 or casein kinase (CK)II and inhibits DNA binding (25). Even though COOH-terminal phosphorylation was not examined in this study, we observed increased binding of nuclear extracts onto oligonucleotides bearing the AP-1 consensus sequence (Fig. 5). This reaction could not be due to a mass action effect since total c-Jun protein levels remained constant under the experimental conditions examined, a result indicating that the proteins that repress this binding were inactivated. Furthermore, AP-1 DNA binding was inhibited by SP-600125, suggesting that JNKs can affect both the transcriptional activity of c-Jun and the ability of AP-1 to attach to the promoters of the target genes, thus regulating gene expression under alkaline conditions.

MSK1 was another protein whose phosphorylation was induced by alkalosis in H9c2 cells (Fig. 6). MSK1 phosphorylation is catalyzed by both ERKs and p38 MAPK (22), and this was also demonstrated in our study. By contrast, a previous study on H9c2 cells in our laboratory (1) showed that oxidative stress-induced MSK1 phosphorylation was mediated only by p38 MAPK and not ERKs, although the latter were also activated by that type of stress. Therefore, it is evident that the stimulating factor determines which kinases phosphorylate MSK1 and, in the case of alkalosis, MSK1 might be a convergent point for the ERK and p38 MAPK signaling pathways.

MSK1 has diverse physiological functions; it has been shown to regulate gene expression by modulating transcription factors (1, 15) and the chromatin structure, the latter through phosphorylation of histone H3 (39). What is more, cytosolic MSK1 can enhance protein synthesis by phosphorylating, and thus inhibiting, eIF4E-binding protein 1 (4E-BP1), a protein that blocks the initiation of translation (28). Since protein synthesis rate is increased in cardiac cells subjected to alkalosis

(with the same media used in this study) (10, 12), this may also occur in H9c2 cells and be regulated by the p38 MAPK and ERK signaling pathways through the aforementioned action of MSK1 on 4E-BP1.

Bcl-2 was also phosphorylated in response to alkalosis in H9c2 cells (Fig. 7). The phosphorylation examined was that at Ser70, and this was JNK dependent, in agreement with previous studies (29, 47). This specific modification has been correlated with both induction and prevention of the protective activity of Bcl-2 (19, 36, 47), but its actual role has not been delineated yet. This is further complicated by the fact that Bcl-2 prosurvival activity is also depressed by phosphorylation of other residues of the Bcl-2 molecule (36, 43), whose effect on protein function might prevail over that of Ser70 phosphorylation.

In our study, inhibition of Ser70 phosphorylation by 10 μM SP-600125 correlated with diminished cell viability induced by the same compound under alkaline conditions (Fig. 8), possibly indicating that this modification might act protectively. It must be noted that the PI staining technique used in this study to assess cell death identifies necrotic cells; nevertheless, this dye can also enter into late apoptotic cells (14), and this could further substantiate a role for Bcl-2 (an antiapoptotic protein) in the survival of H9c2 cells during alkalosis.

The fact that some death was also observed in control cells incubated with 10 μM SP-600125 could not compromise the importance of JNKs for H9c2 cell survival under alkaline conditions, since a vast number of H9c2 cells also died when they were incubated with the alkaline Tris-Tyrode buffer, but not with the normal HEPES-Tyrode buffer, in the presence of this inhibitor at 5 μM. These two concentrations of SP-600125 (5 and 10 μM) are at or below the in vivo IC_{50} for JNK and below the IC_{50} for ERKs and p38 MAPK (4), indicating that the effects of SP-600125 in this study are specifically due to inhibition of JNKs. This specificity is also shown by the fact that the same inhibitor (at 10 μM) induced either survival or death in H9c2 cells when they were subjected to sustained or transient oxidative stress, respectively (32).

A protective role for JNKs in cardiac myocytes has also been documented by other groups (8, 9, 37). The targets that determine the effects of JNKs on cell survival are still not clear in this cell type. Besides the possible involvement of Bcl-2, the protective role of JNKs could be exerted by modification of other prosurvival proteins. Such a candidate is Akt, which can be phosphorylated by JNKs on Thr450, a modification that has been recently proposed to prime this protein for subsequent phosphorylation by 3-phosphoinositide-dependent protein kinase (37). An additional possibility would be the inactivation of proapoptotic proteins by JNKs, and such an example could be BAD, which is inactivated upon phosphorylation by JNKs on Thr201 (50).

Furthermore, our results reveal that the ERK and p38 MAPK pathways are also important in protecting H9c2 cells from death under alkalosis (Fig. 8). A similar beneficial involvement of ERKs has been also reported in cardiomyocytes recovered from simulated ischemia (34), and a protective role of p38 MAPK against doxorubicin-induced toxicity has been demonstrated in H9c2 cells (44). However, the involvement of p38 MAPK and JNKs in cardiomyocyte survival is ambiguous since they both promote it or reduce it depending on the stimulus (5).

In conclusion, our results demonstrate, for the first time, that alkalosis differentially induces all three MAPK signaling pathways in H9c2 cardiac myoblasts, with NHE1 playing a key role. The activated MAPKs regulate both cytosolic and nuclear proteins; p38 MAPK and ERK signaling pathways converge at the level of MSK1, indicating that these two pathways may control the same physiological response. On the other hand, JNKs regulate the phosphorylation of c-Jun and AP-1 DNA binding activity. The three MAPKs examined seem to be necessary for H9c2 cell survival under alkaline conditions, and JNKs could exert this effect possibly through Bcl-2. Overall, our results associate the MAPKs with the modulation of gene expression and cell survival mechanisms, and an interesting future direction would be the investigation of possible gene targets and further protective mechanisms regulated by these kinases in H9c2 cells under alkaline conditions.

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