

# Beryllium fluoride-induced cell proliferation: a process requiring P21<sup>ras</sup>-dependent activated signal transduction and NF- $\kappa$ B-dependent gene regulation

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**Abstract:** We studied the effect of beryllium fluoride on murine peritoneal macrophages and determined its effects on signal transduction and genetic regulation. At low concentration (1–5 nM), BeF<sub>2</sub> caused an approximate twofold increase in [<sup>3</sup>H]thymidine uptake and cell number, but above 5 nM, it showed cytotoxic effects. BeF<sub>2</sub> increased cellular inositol (1,4,5)trisphosphate (IP<sub>3</sub>) and [Ca<sup>2+</sup>]<sub>i</sub> about twofold. The rise in [Ca<sup>2+</sup>]<sub>i</sub> occurred consequent to release from IP<sub>3</sub>-sensitive Ca<sup>2+</sup> stores and from influx, mainly via L-type channels. A significant increase in the levels of MEK1, ERK1, p38 MAPK, and JNK phosphorylation was observed in BeF<sub>2</sub>-exposed macrophages. The levels of NF- $\kappa$ B and CREB transcription factors and the proto-oncogenes *c-fos* and *c-myc* were also elevated significantly. Intracellular Ca<sup>2+</sup> chelation blocked the effect of BeF<sub>2</sub>. We conclude that BeF<sub>2</sub> at low concentration exerts its mitogenic effects in peritoneal macrophages by elevating [Ca<sup>2+</sup>]<sub>i</sub>, which triggers the activation of p21<sup>ras</sup>-dependent MAPK signaling cascades. *J. Leukoc. Biol.* 71: 487–494; 2002.

**Key Words:** *mitogenesis · MAPK regulation · AlF<sub>n</sub> · CREB*

## INTRODUCTION

Beryllium metal and its salts are widely used in industrial processes, ranging from electronics and telecommunication to nuclear reactors and weapons [1]. The metal and its salts are carcinogenic in experimental animals and induce cell transformation in mammalian cell culture [1–6]. At the mechanistic level, beryllium salts bind to nucleoproteins and inhibit enzymes involved in DNA synthesis and also induce gene mutations in cultured cells [1–4, 7–10]. Not all beryllium salts induce the same effects, and beryllium fluorides (BeF<sub>n</sub>) represent a unique situation. BeF<sub>n</sub>, like aluminum fluorides (AlF<sub>n</sub>), have protean effects on signal transduction and energy metabolism; for example, BeF<sub>n</sub> inhibit ATPases [11–13]. BeF<sub>n</sub> and AlF<sub>n</sub> affect G-protein-dependent processes. The  $\alpha$  subunit of heterotrimeric G proteins in the guanosine 5'-diphosphate (GDP)-bound form can be activated by AlF<sub>n</sub> or BeF<sub>n</sub> [14–18]. It is thought that the metal ion-fluoride complexes mimic the terminal phosphate of guanosine 5'-triphosphate (GTP), such

that the structure of the G $\alpha$ -GDP-AlF<sub>n</sub> (or BeF<sub>n</sub>) complexes resembles that of the GTP-bound form of the protein [14–18]. Activation of purified G proteins by metal ion-fluoride complexes results in a change in the conformation of the G $\alpha$  subunit and release of G $\beta\gamma$ , which promotes association of the G $\alpha$  subunits with effector molecules downstream of the G protein [14–18]. The lifetime of the interaction of these metal ion-fluoride complexes with G proteins, and hence of the activated G proteins, is influenced by several effectors including GTPase-activating proteins (GAPs) [16–18].

Mitogen activated protein kinases (MAPKs) are a family of Ser/Thr kinases that mediate intracellular signal transduction in response to various stimuli. MAPKs are involved in cellular stress responses and apoptosis [19–23] and are themselves activated by phosphorylation [19–22]. Three major MAPK families have been identified: the extracellular signal-regulated kinases (ERK1/2), the stress-activated protein kinases known as *c-jun* NH<sub>2</sub>-terminal kinases (JNK), and the p38 MAPKs [19–22]. Ras-dependent activation of MAPK kinases that activate ERK, (MEK)1 and 2, leads to activation of ERK1/2 [19–22]. This pathway provides a common route by which signals from different growth-factor receptors converge to activate major transcription factors such as activated protein-1 (AP-1) [19–23]. Ras also activates JNK, which plays a role in regulating AP-1 activity [24]. Variations in [Ca<sup>2+</sup>]<sub>i</sub> are a major determinant in the regulation of the Ras/MAPK pathway [19–23]. G-protein-dependent activation of phospholipase C results in hydrolysis of membrane phosphoinositides generating inositol (1,4,5)trisphosphate (IP<sub>3</sub>) and diacylglycerol (DAG) [25, 26]. IP<sub>3</sub> raises [Ca<sup>2+</sup>]<sub>i</sub> by binding to specific receptors on the endoplasmic reticulum (ER), thus mobilizing membrane-sequestered Ca<sup>2+</sup> [25, 26]. DAG is required for activating protein kinase C (PKC) [25, 26].

Recently, we have observed that exposure of murine peritoneal macrophages to beryllium chloride (BeCl<sub>2</sub>) increased [Ca<sup>2+</sup>]<sub>i</sub> levels in an IP<sub>3</sub>-independent manner and enhanced [<sup>3</sup>H]thymidine uptake in a calcium-dependent manner. In this study, we demonstrate that exposure of macrophages to BeF<sub>n</sub> alters calcium homeostasis and mitogenesis in a manner sig-

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Received August 29, 2001; revised August 29, 2001; accepted October 11, 2001.

nificantly different than that observed with  $\text{BeCl}_2$  treatment [27]. We show that in macrophages,  $\text{BeF}_n$  at low concentration ( $<10$  nM) increase [ $^3\text{H}$ ]thymidine uptake and cell number about twofold. Concomitantly, we observed a similar increase in the levels of phosphorylated MEK1, ERK1, p38 MAPK, and JNK. Inhibition of these kinases by specific inhibitors significantly attenuated the  $\text{BeF}_n$ -induced effects described above.  $\text{BeF}_n$  also affected the levels of the transcription factors, nuclear factor- $\kappa\text{B}$  (NF- $\kappa\text{B}$ ) and cyclic AMP response element-binding (CREB) protein. The effects of  $\text{BeF}_n$  are dependent on  $[\text{Ca};2^+]_i$  levels, which at least in part are a consequence of  $\text{IP}_3$  generation.  $\text{BeF}_n$  also triggered  $\text{Ca}^{2+}$  uptake by L-type channels.

## MATERIALS AND METHODS

### Materials

The sources of thioglycollate, cell culture materials, [ $^3\text{H}$ ]thymidine, [ $^3\text{H}$ ]myo-inositol, verapamil, econazole, 1,2-bis(*O*-aminophenyl-ethane-ethane)-*N,N,N',N'*-tetraacetic acid-acetoxymethyl ester (BAPTA/AM), genestein, staurosporin, chelerythrin, manumycin A, SB 203580, PD98059, Fura-2/AM, U73122, and U0126 have been described previously [28–30].  $\text{BeF}_2$  and  $\text{BeCl}_2$  were purchased from Alfa Aesar (Ward Hill, MA). Antibodies against phosphorylated MEK1, ERK1, p38 MAPK, and JNK were purchased from New England BioLabs (Mississauga, Ontario, Canada). Antibodies against *c-fos* protein, *c-myc* protein, CREB protein, and NF- $\kappa\text{B}$  protein were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Other chemicals used were procured locally and were of the highest available purity.

### Determination of macrophage cell number after exposure to $\text{BeF}_n$

Because increased DNA synthesis is normally associated with an increase in total cellularity, the number of macrophages before and after overnight exposure to varying concentrations of  $\text{BeF}_2$  was determined. Peritoneal macrophages were harvested from C57BL/6 mice three days after intraperitoneal injection of thioglycollate as described previously [29] and were allowed to adhere in six-well plates in RPMI-1640 medium containing 5% fetal bovine serum (FBS) for 2 h. The adhered cells were scraped carefully, centrifuged at 1200 rpm for 5 min, and suspended in 1.5 ml RPMI-1640 medium containing 0.2% bovine serum albumin (BSA) and 0.5 ml aliquots ( $2 \times 10^5$  cells) pipetted into 15 ml siliconized polypropylene tubes. To the respective tubes, a specified concentration of  $\text{BeF}_2$  was added, the contents mixed gently, and the tubes incubated overnight as above. After overnight incubation, 10  $\mu\text{l}$  trypan blue solution was added to each tube, the tubes were gently shaken during incubation for 2 min, and a 10  $\mu\text{l}$  aliquot was used for counting the number of cells by hemocytometers. In experiments where the modulation in cell numbers of  $\text{BeF}_2$ -exposed macrophages by  $[\text{Ca}^{2+}]_i$ , MAPKs, or Ras was examined, the macrophages were treated with the intracellular calcium chelator BAPTA/AM (10  $\mu\text{M}/30$  min); U0126 (1  $\mu\text{M}/10$  min), a specific inhibitor of MEK1; PD98059 (50  $\mu\text{M}/90$  min), a specific inhibitor of ERK; or SB203580 (25  $\mu\text{M}/30$  min), a specific inhibitor of p38 MAPK, or manumycin A (25  $\mu\text{M}/60$  min), a specific inhibitor of farnesyltransferase [28]. The latter enzyme is required for activation of p21<sup>ras</sup> [28]. These agents were added to their respective tubes, which were incubated for the specified time before adding  $\text{BeF}_2$  (2 nM). Cell numbers were determined as described above.

### [ $^3\text{H}$ ]Thymidine uptake by $\text{BeF}_n$ -exposed macrophages

Murine peritoneal macrophages ( $4 \times 10^5$  cells/well in 48-well plates) harvested as above were allowed to adhere for 2 h in RPMI-1640 medium containing 5% FBS, penicillin, streptomycin, and glutamine at 37°C in a humidified  $\text{CO}_2$  (5%) incubator. The monolayers were washed twice with Hanks' balanced salt solution containing 10 mM HEPES, 3.5 mM  $\text{NaHCO}_3$ , pH 7.2 (HHBSS). A volume of the above RPMI medium was added, except that sera was replaced

with 0.2% BSA. [ $^3\text{H}$ ]Thymidine was then added, followed by varying concentrations of  $\text{BeF}_2$  or NaF. The cells were incubated overnight in a humidified  $\text{CO}_2$  (5%) incubator. The incubations were terminated by aspirating the medium and washing macrophages twice, first with 5% trichloroacetic acid (15 min/4°C) and then thrice with HHBSS. The monolayers were lysed with 1 N NaOH, and an aliquot was used for liquid scintillation counting and protein estimation [30].

### The role of $[\text{Ca}^{2+}]_i$ levels and the activity of PKC, phosphatidylinositol-specific phospholipase C (PI-PLC), tyrosine kinases, and MAPKs in $\text{BeF}_n$ -induced [ $^3\text{H}$ ]thymidine uptake

In experiments that examined effects of modulating  $[\text{Ca}^{2+}]_i$  levels in  $\text{BeF}_n$ -induced [ $^3\text{H}$ ]thymidine uptake,  $[\text{Ca}^{2+}]_i$  levels were modulated with BAPTA/AM (10  $\mu\text{M}/30$  min) or nifedipine (5  $\mu\text{M}/10$  min). These agents were added to individual wells, and the cells were incubated for the specified time period before adding  $\text{BeF}_2$ . In studies on modulation of [ $^3\text{H}$ ]thymidine uptake, the respective monolayers were preincubated with U73122 (4  $\mu\text{M}/10$  min), an inhibitor of PI-PLC; chelerythrin (200 nM/10 min), an inhibitor of PKC; genestein (20  $\mu\text{M}/16$  h), an inhibitor of tyrosine kinases, U0126 (2  $\mu\text{M}/10$  min), an inhibitor of MEK1; PD 98059 (50  $\mu\text{M}/90$  min), an inhibitor of ERK; SB203580 (15  $\mu\text{M}/30$  min), an inhibitor of p38 MAPK; or manumycin A (10  $\mu\text{M}/1$  h), a farnesyltransferase inhibitor, before adding  $\text{BeF}_2$  and [ $^3\text{H}$ ]thymidine. Details of incubations and determination of cell lysate radioactivity were as described above.

### Measurement of $\text{IP}_3$

Murine peritoneal macrophages were obtained as described above. The cells were centrifuged, suspended in RPMI-1640 medium containing 12.5 units of penicillin/ml, 6.5  $\mu\text{g}/\text{ml}$  streptomycin, 2 mM glutamine, and 5% FBS, plated in six-well plates ( $4 \times 10^6$  cells/well in six-well plates), and allowed to adhere for 2 h at 37°C in a humidified 5%  $\text{CO}_2$  incubator. Monolayers were washed with HHBSS, a volume of above-mentioned RPMI, except that serum was substituted with 0.2% fatty acid-free BSA added, followed by the addition of [ $^3\text{H}$ ]myo-inositol (8  $\mu\text{Ci}/\text{well}$ ), and the monolayers were incubated overnight as described above. The monolayers were washed with chilled HHBSS containing 10 mM LiCl, 1 mM  $\text{CaCl}_2$ , and 1 mM  $\text{MgCl}_2$  thrice, a volume of wash buffer was added, and monolayers were preincubated for 3 min at 37°C as above, before stimulating with  $\text{BeF}_2$  (2 nM) or with aluminum chloride (10  $\mu\text{M}$ )-sodium fluoride (50 mM). Other details of fractionation and quantification of radioactivity of  $\text{IP}_3$  were performed as described previously [29].

### Measurements of changes in intracellular calcium levels in $\text{BeF}_n$ -exposed macrophages

Changes in  $[\text{Ca}^{2+}]_i$  levels in Fura-2/AM-loaded macrophages on exposure to  $\text{BeF}_2$  were measured by digital imaging microscopy essentially as described previously [29]. Briefly, freshly harvested murine peritoneal macrophages were suspended in RPMI-1640 medium containing 5% FBS, penicillin, streptomycin, and glutamine. They were then allowed to adhere on glass coverslips and kept in 35-mm petri dishes at 37°C for 2 h in a humidified  $\text{CO}_2$  (5%) incubator. The nonadherent cells were aspirated, and the monolayer was washed with HHBSS, a volume of RPMI medium was added, and cells were incubated overnight as above. Macrophages incubated overnight were loaded with Fura-2/AM (4  $\mu\text{M}/30$  min at 25°C in dark) and washed thrice with HHBSS, and a volume of HHBSS was added. The cells on coverslips were exposed to  $\text{BeF}_2$ , and changes in  $[\text{Ca}^{2+}]_i$  in single cells were quantified as described previously [29]. The origin of elevated levels of  $[\text{Ca}^{2+}]_i$  in  $\text{BeF}_2$ -exposed cells could result from its elevated release from  $\text{IP}_3$ -sensitive  $\text{Ca}^{2+}$  stores, influx of extracellular  $\text{Ca}^{2+}$  via specific channels,  $\text{IP}_3$ -independent release from ER  $\text{Ca}^{2+}$  stores, and/or inhibition of  $[\text{Ca}^{2+}]_i$  extrusion. The contribution of  $\text{IP}_3$ -dependent release of  $\text{Ca}^{2+}$  from ER calcium stores to the total  $[\text{Ca}^{2+}]_i$  pool in  $\text{BeF}_2$ -exposed macrophages was analyzed by treating Fura-2/AM-loaded cells with PI-PLC inhibitor U73122 (4  $\mu\text{M}/10$  min) before adding  $\text{BeF}_2$  and measuring changes in Fura-2 fluorescence [31]. The contribution of  $\text{BeF}_2$ -induced influx of  $\text{Ca}^{2+}$  to the total  $[\text{Ca}^{2+}]_i$  pool in  $\text{BeF}_2$ -exposed cells was assessed by treating the cells with nifedipine (5  $\mu\text{M}/10$  min), specific blockers of L-type  $\text{Ca}^{2+}$  channel [32], or flunarizine  $\cdot$  HCl (5  $\mu\text{M}/10$  min), a selective blocker of

low-threshold T-type channel, before exposure to  $\text{BeF}_2$  and measurement of changes in Fura-2 fluorescence.

## Western blotting of phosphorylated MEK1, ERK1, p38 MAPK, and JNK in macrophages exposed to $\text{BeF}_n$

Freshly harvested peritoneal macrophages in RPMI-1640 medium containing penicillin, streptomycin, glutamine, and 5% FBS were allowed to adhere in six-well plates ( $3 \times 10^6$  cells/well) for 2 h as above. The monolayers were washed twice with HHBSS, and a volume of above-mentioned RPMI-1640 medium was added, except that the serum was substituted with 0.2% BSA, and plates were incubated overnight as above. The monolayers were washed twice, and a volume of RPMI medium containing 0.2% BSA was added. The cells were then exposed to buffer or  $\text{BeF}_2$  (2 nM/20 min) after adding the specific inhibitors/modulators of MAPKs. The concentrations of these inhibitors and the incubation time periods were as described above. The incubations were terminated by aspirating the medium. The lysis of cells, their electrophoresis, and Western immunoblotting were performed according to the manufacturer's instruction. In each case, equal amounts of protein were used for electrophoresis. The detection of phosphorylated MAPKs by enhanced chemifluorescence (ECF) and quantification of their distribution were performed by Phosphorimager<sup>TM</sup> (Storm 860, Molecular Dynamics, Sunnyvale, CA).

## Western blotting of c-fos, c-myc, CREB, and NF- $\kappa$ B proteins in macrophages exposed to $\text{BeF}_n$

Freshly harvested peritoneal macrophages in RPMI-1640 medium containing penicillin, streptomycin, glutamine, and 5% FBS were allowed to adhere in six-well plates ( $3 \times 10^6$  cells/well) for 2 h as above. The monolayers were washed twice with HHBSS, and a volume of RPMI-1640 medium was added, except that the serum was substituted with 0.2% BSA, and plates were incubated overnight as above. The monolayers were washed, a volume of RPMI medium containing 0.2% BSA was added, and the cells were exposed to buffer or to  $\text{BeF}_2$  (2 nM/20 min). The respective incubations were terminated by aspirating the medium. The lysis of cells, their electrophoresis, and Western immunoblotting were done according to the manufacturer's instruction. In each case, an equal amount of protein was used for electrophoresis. The detection of immunoblots was performed by ECF and quantification of their distribution by Phosphorimager<sup>TM</sup> (Storm 860, Molecular Dynamics).

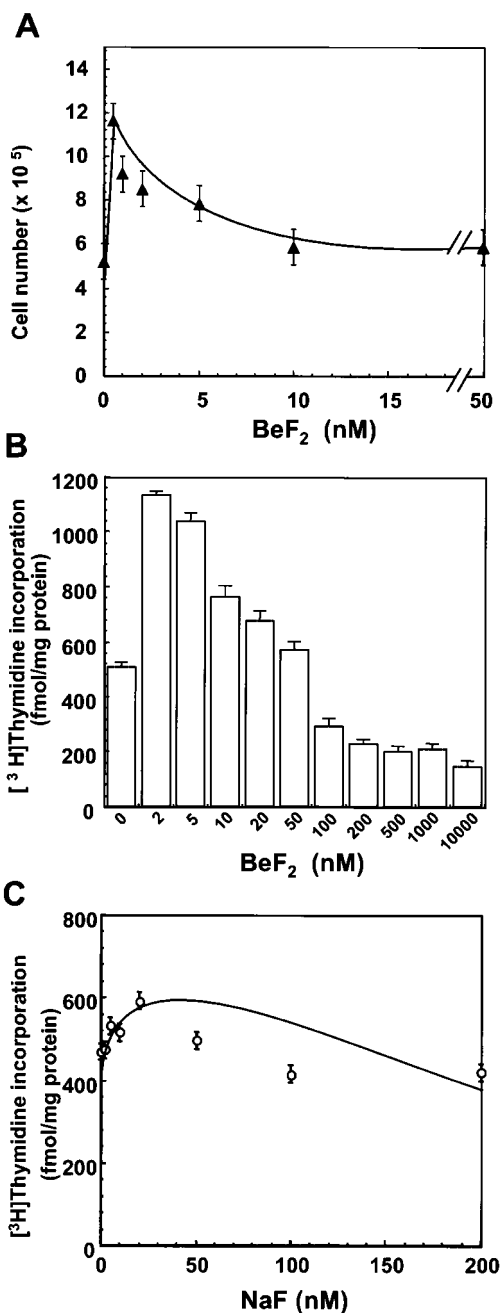
## Measurement of the effect of NF- $\kappa$ B activation on $\text{BeF}_n$ -induced [ $^3\text{H}$ ]thymidine incorporation

The involvement of the NF- $\kappa$ B transcription factor in  $\text{BeF}_n$ -induced uptake was examined by incubating the cells with two specific inhibitors of NF- $\kappa$ B, namely BAY 11-7082 (10  $\mu\text{M}$ /10 min) [33] and  $\text{PGA}_1$  (25  $\mu\text{M}$ /10 min) [34]. These inhibitors prevent agonist-induced phosphorylation and degradation of I $\kappa$ B, resulting in the inactivation of NF- $\kappa$ B activation, and hence, inhibit its translocation to nuclei [35–37]. These inhibitors were added to respective monolayers and incubated for the specified time before adding  $\text{BeF}_2$ . Details of incubation and determination of cell lysate radioactivity were the same as described above. Statistical analysis of the data was performed by Student's *t*-test, and *P* values  $\leq 0.05$  are considered significant.

## RESULTS

### The effect of $\text{BeF}_n$ exposure on cell division and [ $^3\text{H}$ ]thymidine uptake in peritoneal macrophages

Over the concentration range of 1–5 nM,  $\text{BeF}_2$  caused an increase in cell numbers, which at 2 nM, was about twofold (Fig. 1A). This was paralleled at this concentration by a similar increase in [ $^3\text{H}$ ]thymidine uptake (Fig. 1B). Above this range, there was a decline in cell numbers and [ $^3\text{H}$ ]thymidine uptake. The latter results suggest that cell cytotoxicity occurs above a  $\text{BeF}_2$  concentration of 5 nM. Because fluoride itself is toxic, we examined the effect of adding NaF to the macro-



**Fig. 1.**  $\text{BeF}_2$ -induced cell division and [ $^3\text{H}$ ]thymidine incorporation into peritoneal macrophages. (A) Effect of increasing concentration of  $\text{BeF}_2$  on murine peritoneal macrophage cell number after 24 h incubation. The cell numbers are mean  $\pm$  SE from two independent experiments counted in quadruplicate. (B) Effect of increasing concentrations of  $\text{BeF}_2$  on [ $^3\text{H}$ ]thymidine incorporation into macrophages. Values are mean  $\pm$  SE from two experiments performed in quadruplicate and are expressed as fmol [ $^3\text{H}$ ]thymidine incorporated/mg cellular protein. The experimental data were significantly different at  $P < 0.005$  compared with buffer-treated controls. (C) Effect of increasing concentrations of NaF on [ $^3\text{H}$ ]thymidine incorporation in macrophages. Values are mean  $\pm$  SE from two experiments performed in quadruplicate and are expressed as fmols [ $^3\text{H}$ ]thymidine incorporation/mg cellular protein.

phages. Below a concentration of 100 nM, NaF did not affect [ $^3\text{H}$ ]thymidine uptake (Fig. 1C). Above this concentration, some reduction in uptake of [ $^3\text{H}$ ]thymidine did occur. Trypan blue uptake by macrophages also increased at higher concen-

trations of BeF<sub>2</sub> (unpublished results). We conclude that the effects of BeF<sub>n</sub> species at higher concentration on cell division are most likely the effect of beryllium-induced toxicity.

### The effect of BeF<sub>n</sub> exposure of macrophages on phosphoinositide hydrolysis

Cleavage of membrane phosphoinositides by PI-PLC results in a transient increase in IP<sub>3</sub>, which interacts with specific ER receptors to cause an increase in cytosolic-free Ca<sup>2+</sup> [25, 26]. Cytosolic-free Ca<sup>2+</sup> is involved in the regulation of specific cell-cycle events and DNA synthesis [38–40]. BeF<sub>n</sub>- or AlF<sub>n</sub>-exposed macrophages demonstrated a transient increase in IP<sub>3</sub> of 25 ± 5% and 50 ± 10%, respectively, within 5–10 s of treatment. The results with BeF<sub>n</sub> are significant in two respects: The concentration of BeF<sub>n</sub> (2 nM) used was far less than AlF<sub>n</sub> (10 μM). Moreover, BeCl<sub>2</sub>-treated macrophages do not show an effect on IP<sub>3</sub> synthesis even at much higher concentrations [27].

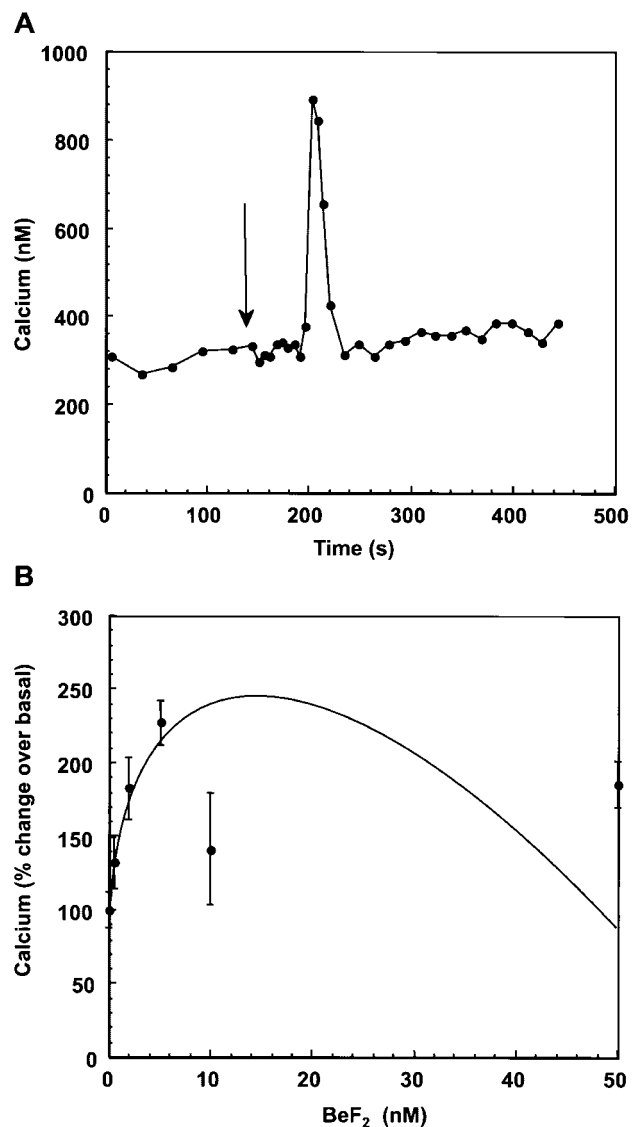
### The effect of BeF<sub>n</sub> exposure of macrophages on [Ca<sup>2+</sup>]<sub>i</sub>

Exposure of macrophages to BeF<sub>n</sub> species increased [Ca<sup>2+</sup>]<sub>i</sub> levels by two- to threefold (Fig. 2A). The increase was largely in single, large spikes, sometimes accompanied by smaller spikes. The effect of BeF<sub>2</sub> is concentration-dependent, and a maximal effect is seen at 2–5 nM. Thereafter, responses declined and were essentially absent above 50 nM concentrations of BeF<sub>2</sub> (Fig. 2B). By contrast, macrophages exposed to BeCl<sub>2</sub> show an increase in [Ca<sup>2+</sup>]<sub>i</sub>, which reaches a plateau at 100 nM, and the response is sustained thereafter, up to a concentration of at least 500 nM BeCl<sub>2</sub> in the absence of IP<sub>3</sub> synthesis [27].

To further assess the contribution of IP<sub>3</sub>-dependent mechanisms in raising [Ca<sup>2+</sup>]<sub>i</sub>, we used U73122, an inhibitor of PI-PLC. Cells pretreated with U73122 prior to addition of BeF<sub>2</sub> showed a 30–40% decrease in the [Ca<sup>2+</sup>]<sub>i</sub> response compared with cells treated only with BeF<sub>2</sub> (Fig. 3, A and B). These results are consistent with the hypothesis that BeF<sub>n</sub> treatment triggers IP<sub>3</sub>-dependent increases in [Ca<sup>2+</sup>]<sub>i</sub>. However, IP<sub>3</sub>-independent mechanisms for mobilizing Ca<sup>2+</sup> are also activated by BeF<sub>n</sub> and appear to account for 60–70% of the increase in free cytosolic Ca<sup>2+</sup>. Therefore, we used Ca<sup>2+</sup> channel blockers to confirm this hypothesis. Nifedipine (5 μM), which blocks L-type channels, blunted the rise in [Ca<sup>2+</sup>]<sub>i</sub>, and flunarizine • HCl, which blocks T-type channels, had no effect (Fig. 3, C and D). We conclude from these studies that BeF<sub>n</sub> increase [Ca<sup>2+</sup>]<sub>i</sub> by IP<sub>3</sub>-dependent and -independent mechanisms, the latter of which involves L-type Ca<sup>2+</sup> channels.

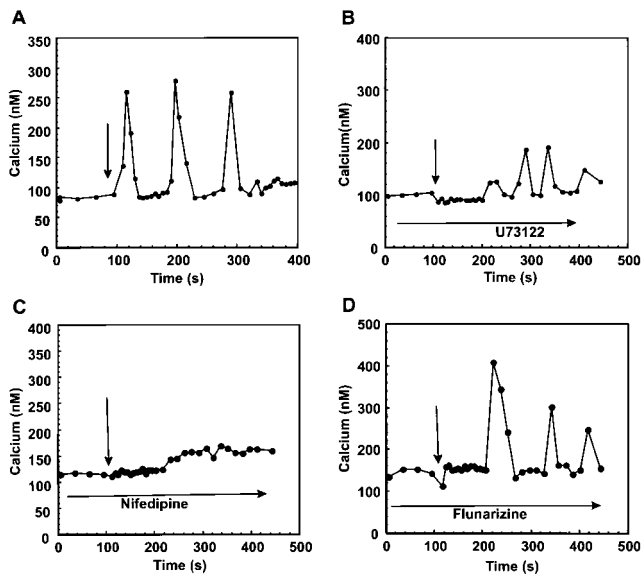
### Elevated cytosolic Ca<sup>2+</sup> and BeF<sub>n</sub>-induced macrophage proliferation

Because cytosolic-free Ca<sup>2+</sup> regulates specific cell-cycle events and DNA synthesis [37, 38], we next studied the effect of modulating [Ca<sup>2+</sup>]<sub>i</sub> on BeF<sub>n</sub>-induced increases in cell number and [<sup>3</sup>H]thymidine uptake (Fig. 4). Modulation of Ca<sup>2+</sup> in BeF<sub>2</sub>-treated macrophages with BAPTA/AM or nifedipine decreased cell numbers significantly and inhibited [<sup>3</sup>H]thymidine incorporation (Fig. 4, A and B). Likewise, limiting the availability of IP<sub>3</sub> by inhibiting PI-PLC with U73122 also resulted



**Fig. 2.** Changes in [Ca<sup>2+</sup>]<sub>i</sub> in Fura-2/AM-loaded macrophages exposed to BeF<sub>2</sub>. (A) A representative cell from 100–150 cells from five individual experiments each using 25–30 cells showing an increase in [Ca<sup>2+</sup>]<sub>i</sub> on exposure to BeF<sub>2</sub> (2 nM). In these studies, 75–85% of cells showed a positive response. The increase occurred as a single spike, multiple spikes, or as a sustained response. (B) Effect of increasing concentrations of BeF<sub>2</sub> on changes in [Ca<sup>2+</sup>]<sub>i</sub> in Fura-2/AM-loaded macrophages. The values are mean ± SE of changes in individual cells [Ca<sup>2+</sup>]<sub>i</sub> at 2 min after addition of BeF<sub>2</sub> from two experiments each using 25–30 cells. The arrow in (A) indicates the time of addition of BeF<sub>2</sub>.

in a 30–40% reduction in [<sup>3</sup>H]thymidine uptake by BeF<sub>2</sub>-treated cells (Fig. 4C). We conclude that BeF<sub>n</sub>-induced cell proliferation is dependent on the availability of cytosolic Ca<sup>2+</sup>. Generally, elevations of [Ca<sup>2+</sup>]<sub>i</sub> cause membrane localization and activation of PKC, which then initiate protein phosphorylation-dependent cellular events [26]. Therefore, studies were performed to understand the role of PKC in BeF<sub>2</sub>-treated macrophages and cell proliferation. Chelerythrin and staurosporin treatment significantly decreased the effect of BeF<sub>2</sub> exposure on macrophage uptake of [<sup>3</sup>H]thymidine (Fig. 4C). These studies demonstrate that PKC activity is important in the mechanism of BeF<sub>n</sub>-induced macrophage proliferation.



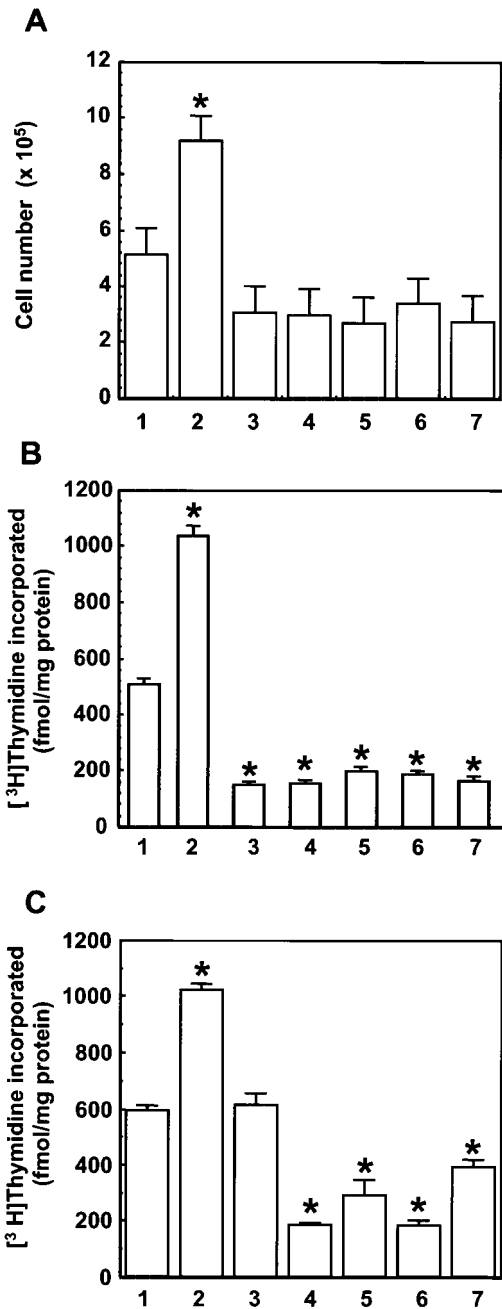
**Fig. 3.** Modulations of BeF<sub>2</sub>-induced increase in [Ca<sup>2+</sup>]<sub>i</sub> in Fura-2/AM-loaded macrophages. (A) Oscillatory increases in [Ca<sup>2+</sup>]<sub>i</sub> in macrophages exposed to BeF<sub>2</sub> (2 nM). (B) Effect of PI-PLC inhibitor U73122 (4 μM/10 min) on BeF<sub>2</sub>-induced increase in [Ca<sup>2+</sup>]<sub>i</sub>. (C) Effect of L-type Ca<sup>2+</sup> channel-blocker nifedipine (5 μM) on BeF<sub>2</sub>-induced increase in [Ca<sup>2+</sup>]<sub>i</sub>. (D) Effect of T-type Ca<sup>2+</sup> channel-blocker flunarizine • HCl (5 μM) on BeF<sub>2</sub>-induced increase in [Ca<sup>2+</sup>]<sub>i</sub>. The inhibitors were present during Ca<sup>2+</sup> measurements. The data shown in each case are representative of two to three individual experiments, each using 20–25 cells.

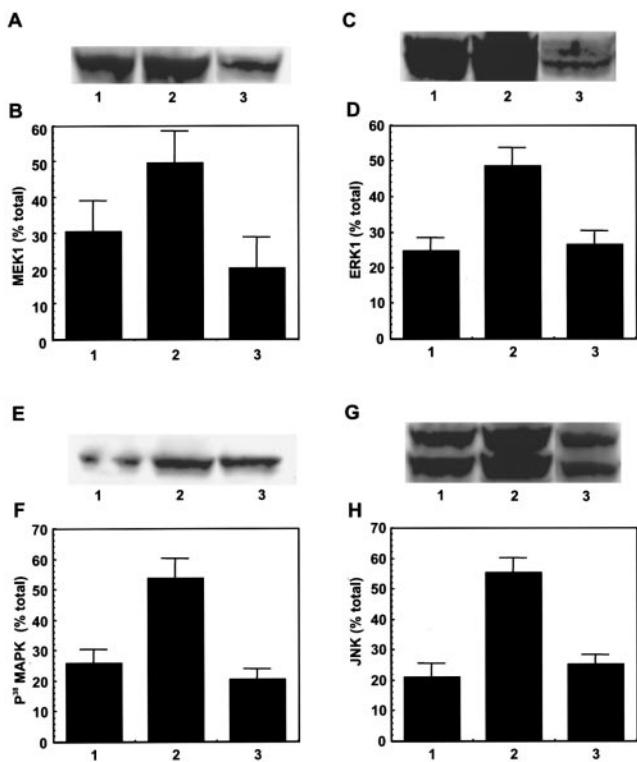
### Signal-transduction mechanisms of BeF<sub>n</sub>-induced macrophage proliferation

Distinct signaling pathways mediate mitogenic signals induced by a variety of hormones, growth factors, or heavy metals

**Fig. 4.** Modulations of BeF<sub>2</sub>-induced increase in macrophage cell number and [<sup>3</sup>H]thymidine incorporation. (A) Effect of MAPK inhibitors and [Ca<sup>2+</sup>]<sub>i</sub> chelators on BeF<sub>2</sub>-induced increase in macrophage cell number. The respective inhibitors were added to the cultures, and the cells were incubated for the indicated time before adding BeF<sub>2</sub>, which was present during the overnight incubation. The bars are: (1) buffer, (2) BeF<sub>2</sub> (2 nM), (3) U0126 (1 μM/10 min) + BeF<sub>2</sub>, (4) PD98059 (50 μM/90 min) + BeF<sub>2</sub>, (5) SB203580 (15 μM/30 min) + BeF<sub>2</sub>, (6) manumycin A (10 μM/60 min) + BeF<sub>2</sub>, and (7) BAPTA/AM (10 μM/30 min) + BeF<sub>2</sub>. The data presented are from two experiments performed in quadruplicate and are expressed as mean ± SE. (B) Effect of Ca<sup>2+</sup> chelators, PKC, tyrosine kinases, and MAPK inhibitors on BeF<sub>2</sub>-induced increase in [<sup>3</sup>H]thymidine incorporation into macrophages. The respective inhibitors were added to monolayers, and the cells were incubated for the indicated time before adding BeF<sub>2</sub>, which was present during the overnight incubation. The bars are: (1) buffer, (2) BeF<sub>2</sub> (2 nM), (3) BAPTA/AM (10 μM/30 min) + BeF<sub>2</sub>, (4) chelerythrin (200 nM/10 min) + BeF<sub>2</sub>, (5) genestein (20 μM/16 h) + BeF<sub>2</sub>, (6) PD98059 (50 μM/90 min) + BeF<sub>2</sub>, and (7) SB 203580 (15 μM/30 min) + BeF<sub>2</sub>. The results are the mean ± SE from two to three experiments performed in quadruplicate. (C) Effect of inhibition of IP<sub>3</sub> generation, L-type Ca<sup>2+</sup> channels, ERK1, and NF-κB activation on BeF<sub>2</sub>-induced increase in [<sup>3</sup>H]thymidine incorporation. The bars are: (1) buffer, (2) BeF<sub>2</sub> (2 nM), (3) U73122 (4 μM/10 min) + BeF<sub>2</sub>, (4) BAY 11-7082 (25 μM/10 min) + BeF<sub>2</sub>, (5) PGA<sub>1</sub> (25 μM/10 min) + BeF<sub>2</sub>, (6) nifedipine (5 μM/10 min) + BeF<sub>2</sub>, and (7) U0126 (1 μM/10 min) + BeF<sub>2</sub>. The results are mean ± SE from two experiments performed in quadruplicate and are expressed as [<sup>3</sup>H]thymidine incorporated/mg cellular protein. [<sup>3</sup>H]Thymidine incorporation into cells treated with various inhibitors used above in the absence of BeF<sub>2</sub> was comparable with or only slightly lower than the buffer-treated cells. The data are significantly different at the 5% level compared with vehicle-treated controls. (\*P < 0.05).

[19–23]. Significant convergence of signals from receptor tyrosine kinases and G-protein-coupled receptors occurs at the level of MAPKs [19–23]. Therefore, we next studied the involvement of p21<sup>ras</sup>-dependent MAPKs in BeF<sub>n</sub>-induced cell proliferation (Fig. 4C). The involvement of protein phosphorylation was evaluated by use of genestein, and manumycin A was used to inhibit farnesyltransferase. The latter is required for p21<sup>ras</sup> activation [41]. Both inhibitors nearly abolished the effect of BeF<sub>2</sub> on macrophage proliferation. Ras mediates its effects on cellular proliferation in part by activation of a cascade of protein kinases [19–23]. Therefore, we probed such events by using a variety of inhibitors including U0126 (MEK1), PD98059 (ERK1/2), and SB203580 (p38 MAPK). All three compounds decreased BeF<sub>2</sub>-induced macrophage proliferation significantly (Fig. 4C).





**Fig. 5.** Activation of MAPKs in macrophages by BeF<sub>2</sub> and the role of [Ca<sup>2+</sup>]<sub>i</sub>. The activation of MEK1 (A and B), ERK1 (C and D), p38 MAPK (E and F), and JNK (G and H) is presented as immunoblots (A, C, E, and G), and their quantitative distribution is as measured by Phosphorimager™ (B, D, F, and H). Immunoblots are representative of at least three separate experiments. (A and B) Activation of MEK1. The bars are: (1) buffer, (2) BeF<sub>2</sub> (2 nM), and (3) BAPTA/AM + BeF<sub>2</sub>. (C and D) Activation of ERK1. The bars are: (1) buffer, (2) BeF<sub>2</sub>, and (3) BAPTA/AM + BeF<sub>2</sub>. (E and F) Activation of p38 MAPK. The bars are: (1) buffer, (2) BeF<sub>2</sub>, and (3) BAPTA/AM + BeF<sub>2</sub>. (G and H) Activation of JNK. The bars are: (1) buffer, (2) BeF<sub>2</sub>, and (3) BAPTA/AM + BeF<sub>2</sub>.

The next series of studies directly examined the effects of BeF<sub>2</sub> on the phosphorylation of several of the components of the p21<sup>ras</sup>-dependent MAPK pathway (Fig. 5). Specifically, MEK1 (Fig. 5, A and B), ERK1 (Fig. 5, C and D), p38 MAPK (Fig. 5, E and F), and JNK (Fig. 5, G and H) phosphorylation was examined. In each case, BeF<sub>2</sub>-exposed macrophages demonstrated an approximate twofold increase in phosphorylation of these proteins. Pretreatment with BAPTA/AM abolished the action of BeF<sub>2</sub> on up-regulating protein phosphorylation.

### BeF<sub>n</sub> exposure and NF-κB regulation in macrophages

In response to a variety of stimuli, including viral and bacterial pathogens, cytokines, and stress-activating agents, the latent cytoplasmic NF-κB/IκB complex is activated by phosphorylation of IκB [35–37]. This targets IκB for ubiquitination and proteosomal degradation releasing NF-κB [35, 36]. Thus, NF-κB is activated and able to translocate to the nucleus where it stimulates gene transcription [35–37]. Therefore, we examined the effect of BeF<sub>n</sub> on macrophages with respect to NF-κB activation. BeF<sub>2</sub> addition caused about a twofold increase in NF-κB activation, as determined by quantifying Western blots

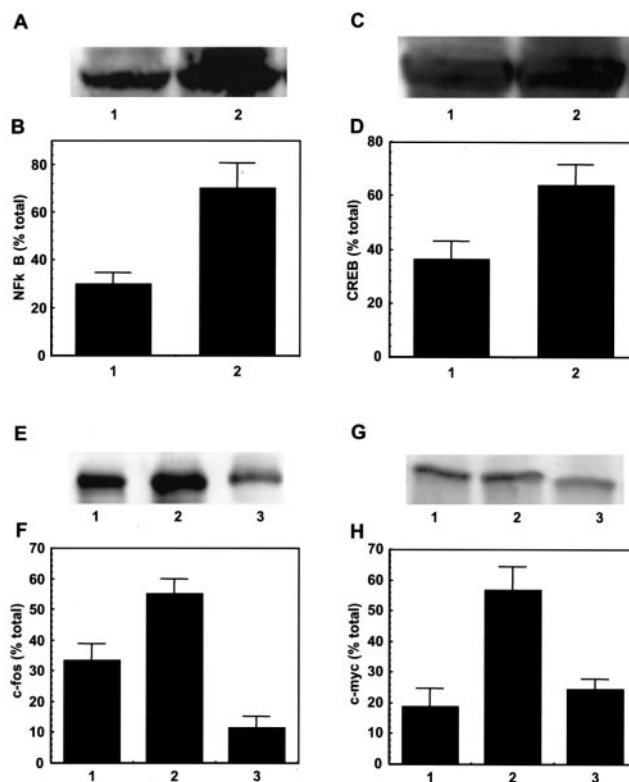
of macrophage extracts (Fig. 6, A and B). Next, we pretreated BeF<sub>2</sub>-exposed macrophages to two specific inhibitors of IκB phosphorylation, BAY 11-7082 and PGA<sub>1</sub>. Both agents also significantly decreased the effect of BeF<sub>2</sub> on increasing [<sup>3</sup>H]thymidine uptake (Fig. 4B).

### BeF<sub>n</sub> exposure and transcription-factor CREB regulation in macrophages

Many signaling pathways stimulate transcription of target genes through nuclear factors whose activities are regulated primarily by phosphorylation [23]. Cyclic AMP regulates the expression of numerous genes through protein kinase A-dependent phosphorylation of transcription factor CREB [42, 43]. Exposure of macrophages to BeF<sub>2</sub> increased CREB protein levels about twofold (Fig. 6, C and D). These results suggest the involvement of cAMP signaling in BeF<sub>n</sub>-induced mitogenesis in macrophages.

### BeF<sub>n</sub> exposure and regulation of *c-fos* and *c-myc* proteins in macrophages

When quiescent cells enter the cell cycle, expression of early response genes is necessary for progression through G1 and



**Fig. 6.** Activation of transcription factors and expression of early genes in macrophages exposed to BeF<sub>2</sub>. The activation of NF-κB (A and B) and CREB (C and D) is presented as immunoblots (A and C) and their quantitative distribution (B and D). The expression of early genes *c-fos* (E and F) and *c-myc* (G and H) is presented as immunoblots (E and G) and their quantitative distribution (F and H). Immunoblots are representative of three separate experiments. (A and B) Activation of NF-κB. The bars are: (1) buffer and (2) BeF<sub>2</sub>. (C and D) Activation of CREB. The bars are: (1) buffer and (2) BeF<sub>2</sub>. (E and F) Induction of *c-fos* expression. The bars are: (1) buffer, (2) BeF<sub>2</sub>, and (3) BAPTA/AM + BeF<sub>2</sub>. (G and H) Induction of *c-myc* expression. The bars are: (1) buffer, (2) BeF<sub>2</sub>, and (3) BAPTA/AM + BeF<sub>2</sub>.

subsequent proliferation [44]. Many of these are proto-oncogenes that encode nuclear transcription factors, which determine subsequent expression of other genes [45]. Expression of *c-fos* is part of a mitogenic response that is required for cell proliferation [45]. Transcription of the *c-fos* gene is regulated in part by cAMP-response element and serum-response element [42, 43]. Increased  $[Ca^{2+}]_i$  can activate *c-fos* transcription through the latter mechanism, and a rise in nuclear  $Ca^{2+}$  can activate *c-fos* transcription through CREB phosphorylation [42, 43]. *c-myc* is effective in transcriptional activation and cell-cycle progression [46]. In view of the effects of  $BeF_2$  treatment of macrophages described above, we examined the effects of this agent on the expression of *c-fos* and *c-myc* genes. Exposure of peritoneal macrophages to  $BeF_2$  (2 nM) caused a 1.5- to twofold increase in the expression of *c-fos* and *c-myc* proteins (Fig. 6, A–D).  $Ca^{2+}$  chelation by BAPTA/AM abolished this effect.

## DISCUSSION

In this study, we have demonstrated that low concentrations of  $BeF_n$  species (1–5 nM) are mitogenic, and higher concentrations appear to be cytotoxic to macrophages. The studies support the hypothesis that these effects are mediated through the ability of  $BeF_n$  to increase cytosolic-free  $Ca^{2+}$  by  $IP_3$ -dependent and -independent mechanisms. The latter effects are mediated by L-type  $Ca^{2+}$  channels. We propose that the effect of  $BeF_n$  on  $IP_3$  synthesis is mediated by the ability of  $BeF_n$  to bind to and affect heterotrimeric G proteins. As noted above, like  $AlF_n$ ,  $BeF_n$  can mimic the terminal phosphate of GTP in the  $G\alpha$ -GDP- $BeF_n$  complex [14–18]. The effects demonstrated here contrast to those seen in  $BeCl_2$ -exposed macrophages [27]. This agent can also cause an increase in  $[Ca^{2+}]_i$  in macrophages; however, the effect is exclusively the result of entry of extracellular  $Ca^{2+}$  into the cells [27].  $BeCl_2$ -exposed macrophages show no increase in  $IP_3$  levels at concentrations equal to or far higher than the concentrations of  $BeF_2$  studied in the current analysis [27]. Moreover,  $BeCl_2$  treatment does not in itself increase PKC activity [27]. The data suggest that by activating G proteins,  $BeF_n$  trigger a series of events not unlike ligands, which via receptor-binding, activate G proteins [14–18]. Subsequently, phosphoinositide degradation produces  $IP_3$  and DAG [25, 26]. The former mobilizes  $Ca^{2+}$  from the ER via direct interactions with its receptors, which contain a  $Ca^{2+}$  channel [25, 26]. DAG activates PKC in the presence of  $Ca^{2+}$  [25, 26]. The data further indicate that these events activate p21<sup>ras</sup>-dependent MAPK pathways and promote translocation of critical signaling components to the nucleus, which in turn, promote early gene activation in macrophages, thus accounting for the proproliferative effect of  $BeF_n$  species on macrophages. These effects occur at very low concentrations of  $BeF_n$ . The majority of the effects shown here was seen at concentrations of only 2 nM  $BeF_2$ . This contrasts to much higher levels of  $AlF_n$  ( $\mu$ M) required to affect  $IP_3$  synthesis and subsequent effects. At higher concentrations, however,  $BeF_2$  exerts its cytotoxic and genotoxic effects. The mechanism(s) of  $BeF_2$  cytotoxicity and genotoxicity may be many but may

include its direct binding to the macromolecules as well as modulation of signal-transduction cascades.

It is unclear whether  $BeF_n$  species are likely to be present in the environment. However, fluoride is widely available in the environment because it is added to water to harden tooth enamel. Given the low concentrations of  $BeF_n$ , which induce the changes seen here, further studies might be warranted to determine the potential for environmental generation of  $BeF_n$  species. This may be relevant particularly at this time, because increased amounts of beryllium are entering the environment as a result of extensive use in fiber optics, cellular communication network systems, and semiconductors [1].

## ACKNOWLEDGMENT

This work was supported by research grant HL 24066 from the National Heart, Lung, and Blood Institute.

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