Inhibition of mitogen-induced DNA synthesis by bafilomycin A_1 in Swiss 3T3 fibroblasts

Andrew J. SAURIN,* Jane HAMLETT,* Michael J. CLAGUE† and Stephen R. PENNINGTON*‡

*Department of Human Anatomy and Cell Biology, and †Physiological Laboratory, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, U.K.

Quiescent cells (in G_0) can be stimulated to enter the cell cycle and proceed to DNA synthesis in S-phase by a wide range of growth factors and mitogens. Activation of cell-surface growth factor receptors with intrinsic protein tyrosine kinase activity initiates autophosphorylation of the receptors and subsequent activation of signal transduction cascades. After activation the receptors undergo ligand-induced internalization to endosomes, which become acidified by the action of a vacuolar H^+-ATPase (V-ATPase). The extent to which vesicular acidification plays a role in mitogenic signalling by receptors with intrinsic tyrosine kinase activity remains unknown. Here we have shown that bafilomycin A_1, a specific inhibitor of V-ATPase, inhibits endosome acidification and mitogen-induced DNA synthesis in Swiss 3T3 fibroblasts. Addition of bafilomycin A_1 at successively later times during G_1 progressively decreased the inhibition of DNA synthesis such that no inhibition was observed when bafilomycin A_1 was added at the onset of S-phase. Bafilomycin A_1 also induced a dramatic but reversible change in the morphology of Swiss 3T3 cells. However, the rapid activation of c-*fos* mRNA accumulation by epidermal growth factor and insulin was unaffected by bafilomycin A_1. Together, the results suggest that activation of the V-ATPase plays an important role in the mitogenic signalling pathways that occur during the G_1 phase of the cell cycle but is not required for the initial epidermal growth factor and insulin-evoked signalling events that lead to c-*fos* mRNA expression.

INTRODUCTION

The addition of appropriate combinations of growth factors and mitogens to quiescent murine fibroblasts (in G_0) initiates entry into the cell cycle and much is known about the cellular events that follow growth factor receptor activation (reviewed in [1,2]). Considerable attention has been focused on the elucidation of signal transduction pathways used by receptors with intrinsic protein tyrosine kinase activity and in defining the cellular responses that occur during G_1 and contribute to cell cycle progression. It is now well established that autophosphorylation of the receptors on tyrosine residues provides a number of sites for interaction with SH2 domain-containing proteins (reviewed in [3,4]). Proteins that have been shown to interact directly with receptor tyrosine kinases in this way include: phospholipase Cγ, phosphatidylinositol 3-kinase, c-*src* , Grb-2 and the ras GTPase-activating protein [4–6].

The binding of polypeptide growth factors to their high-affinity plasma membrane receptors causes the receptors to cluster within clathrin-coated pits, which then invaginate to form endocytic clathrin-coated vesicles. Successive endocytic compartments become progressively more acidic owing to proton transport into the vesicle by the activation of a vacuolar H^+-ATPase (V-ATPase) [7,8]. This acidification plays an important role in the intracellular trafficking and biochemical processing of internalized receptors, bound ligands, and fluid-phase solutes [9,10]. The acidification of the endosomes to a pH of approx. 5.5 promotes dissociation of many growth factors, including epidermal growth factor (EGF) and insulin, from their receptors. The receptors may then be returned to the plasma membrane or alternatively may remain in the endosome, enter the lysosomal pathway and be degraded. The molecular mechanism by which receptors are selected for recycling or degradation remains unknown. Although ligand-mediated internalization and degradation of receptors provides an efficient mechanism for receptor down-regulation, it remains possible that this pathway serves additional roles. In particular, studies into the fate of internalized polypeptide growth factors have shown that fragments of platelet-derived growth factor, basic fibroblast growth factor, Schwannoma-derived growth factor, insulin, nerve growth factor and EGF can be found in the nucleus [11–13]. This has led to the suggestion, albeit very speculative, that these fragments may play a role in the activation of gene expression. These and other observations suggest the processes associated with receptor internalization are important in mitogenic signalling by receptor protein tyrosine kinases.

To determine the importance of lysosomal processing in the mitogenic activity of polypeptide growth factors, both primary and tertiary alkylamines have been used to raise intra-lysosomal pH and thus prevent ligand dissociation and processing [14,15]. These alkylamines become protonated in the acidic environment of lysosomes and so raise the vesicular pH. Studies with these reagents have shown that they are capable of inhibiting EGF- and insulin-induced DNA synthesis [16]. However, the relatively high concentrations required and the non-specific nature of such lipophilic compounds has limited the interpretation of these experiments [7].

Bafilomycin A_1, a macrolide antibiotic isolated from *Streptomyces griseus* [17], is a highly specific and potent inhibitor of V-ATPases [18]. When added to intact cells, bafilomycin A_1 prevents acidification of endosomes and protein degradation in lysosomes [19]. Here we show that the addition of bafilomycin A_1 to

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; FCS, foetal calf serum; MAP, mitogen-activated protein; pH_i, cytosolic pH; PMA, phorbol 12-myristate 13-acetate, S-F, serum-free; RT, reverse transcriptase; V-ATPase, vacuolar H^+-ATPase.

‡ To whom correspondence should be addressed.

quiescent murine Swiss 3T3 fibroblasts in G_0 inhibited the stimulation of DNA synthesis by purified growth factors and the phorbol ester phorbol 12-myristate 13-acetate (PMA). The inhibitory effect of bafilomycin A_1 was reversible and correlated with its ability to inhibit endosomal acidification.

EXPERIMENTAL

Cell culture and measurement of DNA synthesis

Swiss 3T3 fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (w/v) heat-inactivated foetal calf serum (FCS), 44 mM $NaHCO_3$, 25 mM glucose, 35 units/ml penicillin G, 80 m-units/ml streptomycin, pH 7.2, in a humidified atmosphere of 10% CO_2 in air at 37 °C. Cells (approximately 4×10^4/ml) were seeded into 96-well plates or 35 mm Petri dishes and incubated until confluent and quiescent (5 days) before use.

To examine the effects of bafilomycin A_1 on mitogen-stimulated DNA synthesis, cells were washed twice with serum-free DMEM (S-F DMEM; 200 μl) and then incubated for 1 h in S-F DMEM (200 μl) containing 1 μCi/ml [6-^{3}H]thymidine and the indicated concentrations of bafilomycin A_1. After the addition of the indicated mitogens, the incubations were continued for a further 24 h in a humidified atmosphere of 10% CO_2 in air at 37 °C. In some experiments bafilomycin A_1 was added to the cells either 1 h before or at the indicated times after the addition of EGF and insulin. Incubations were stopped by washing the cells twice with 200 μl of ice-cold PBS and [^{3}H]thymidine incorporation into DNA was measured by harvesting the cells from each well on to glass-fibre filters. After air-drying the radioactivity on the filters was measured by liquid-scintillation counting.

Analysis of vacuolar acidification

To detect vacuolar acidification, cells were stained with acridine orange by the method of Geisow et al. [20]. Cells, grown to confluence on glass coverslips, were incubated in 10% CO_2 in air at 37 °C for 1 h with S-F DMEM containing the indicated concentrations of bafilomycin A_1. The cells were then incubated at 37 °C for a further 10 min in the presence of 5 μg/ml acridine orange. After washing four times with ice-cold PBS, the coverslips were mounted onto slides with PBS and immediately examined by fluorescence microscopy (Leitz). Fluorescence micrographs were taken with the same shutter speed throughout, using Ilford Pan F film (50 ASA).

Measurement of c-fos mRNA accumulation by reverse transcriptase (RT)–PCR

Quiescent Swiss 3T3 cells in 35 mm Petri dishes were washed twice with S-F DMEM (1.0 ml) and then incubated for 1 h in S-F DMEM (1.0 ml) either in the presence or absence of 125 nM bafilomycin A_1. After the addition of EGF (10 ng/ml) and insulin (0.2 μg/ml) or no further additions, the incubations were continued for 30 min in a humidified atmosphere of 10% CO_2 in air at 37 °C. To stop the incubations, cell monolayers were washed twice with ice-cold PBS (1.0 ml) and then harvested by direct addition of 4 M guanidinium isothiocyanate solution containing 25 mM sodium acetate, pH 7.0, 100 mM 2-mercaptoethanol and 0.5% *N*-laurylsarkosyl (0.16 ml). Total cellular RNA was then prepared [21]. Heat-denatured (65 °C, 10 min) RNA (0.1 μg) was used as template for cDNA synthesis using random hexanucleotide primers with commercially available reagents (Pharmacia, first-strand cDNA synthesis) in a final reaction volume of 5 μl. Reaction mixes were overlaid with mineral oil (10 μl) and incubated at 37 °C for 1 h and then for 5 min at 90 °C. PCR components were added to cDNA reactions to bring PCR conditions to 23.5 mM Tris (pH 8.3), 70.4 mM KCl, 4.5 mM dithiothreitol, 4.2 mM $MgCl_2$, 740 μM each dNTP, 0.024 mg/ml BSA, 2 μM forward primer, 2 μM reverse primer, 1 μCi [^{32}P]dCTP and 0.5 unit of thermostable DNA polymerase. PCR amplification conditions were: initial denaturation at 96 °C for 5 min, followed by 35 amplification cycles of 94 °C for 1 min; 50 °C for 1 min; 72 °C for 1 min and a final incubation of 72 °C for 10 min. The sequences of the primers used for PCR amplification of c-*fos* cDNA were: forward primer, 5′-AGGGAACGGAATAAGATGGC-3′ and reverse primer, 5′-CCTTCTCTTTCAGCAGATTGG-3′. PCR products were separated by gel electrophoresis on 2% (w/v) agarose gels containing ethidium bromide (0.5 μg/ml final concentration). PCR products were revealed by exposure of gels to a source of ultraviolet irradiation (254 nm). After agarose gel electrophoresis, the gels were fixed in 7% (w/v) TCA, dried under vacuum and exposed to Fuji-RX X-ray film. The autoradiographs were used to locate the ^{32}P-labelled products, which were excised from the gels and subjected to liquid-scintillation counting.

RESULTS AND DISCUSSION

Effect of bafilomycin A_1 on DNA synthesis

The effect of bafilomycin A_1 on mitogen-stimulated DNA synthesis in Swiss 3T3 fibroblasts was measured by the incorporation of [^{3}H]thymidine in response to EGF and insulin or PMA. Figure 1a shows that bafilomycin A_1 inhibited DNA synthesis in response to the optimal mitogenic combination of EGF and insulin. Bafilomycin A_1 at concentrations below 25 nM had no effect on DNA synthesis (Figure 1a), in contrast with the marked inhibition observed at concentrations above 25 nM. In some experiments the mitogenic response to EGF and insulin was inhibited completely by 125 nM bafilomycin, whereas in others this concentration of the V-ATPase inhibitor produced approx. 65% inhibition of the response (Figure 1a). The reason for this variability has not been established but may reflect the insolubility of the antibiotic in aqueous solution (A. J. Saurin, unpublished work). In these experiments, bafilomycin A_1 was diluted with ethanol before addition to the cells and it was demonstrated that ethanol alone had no effect on DNA synthesis (results not shown).

It has previously been demonstrated that bafilomycin A_1 inhibits the increase in cell numbers in asynchronously growing cultures of cells maintained in serum-containing media [22,23]. The data reported here indicate that bafilomycin A_1 inhibits DNA synthesis in response to the addition of purified growth factors to quiescent cells. We also investigated the effect of destruxin B, a structurally unrelated inhibitor of the V-ATPase [24], on EGF-and insulin-induced DNA synthesis. Destruxin B caused a dose-dependent inhibition of DNA synthesis in response to EGF and insulin; thus 10, 25 and 50 μM destruxin B inhibited DNA synthesis by 39%, 63% and 72% respectively. These data support the idea that the inhibitory action of bafilomycin A_1 and destruxin B is mediated specifically by inhibition of the V-ATPase.

The activation of protein kinase C by phorbol esters has been shown to increase endosomal acidification in 3T3 cells [25] and stimulate V-ATPase in neutrophils [26]. We therefore sought to determine the effect of bafilomycin A_1 on PMA-induced DNA synthesis. Bafilomycin A_1 consistently inhibited DNA synthesis in response to PMA (Figure 1b). It is noteworthy that the dose–response curve for bafilomycin A_1 inhibition of PMA-induced DNA synthesis was shifted to lower concentrations

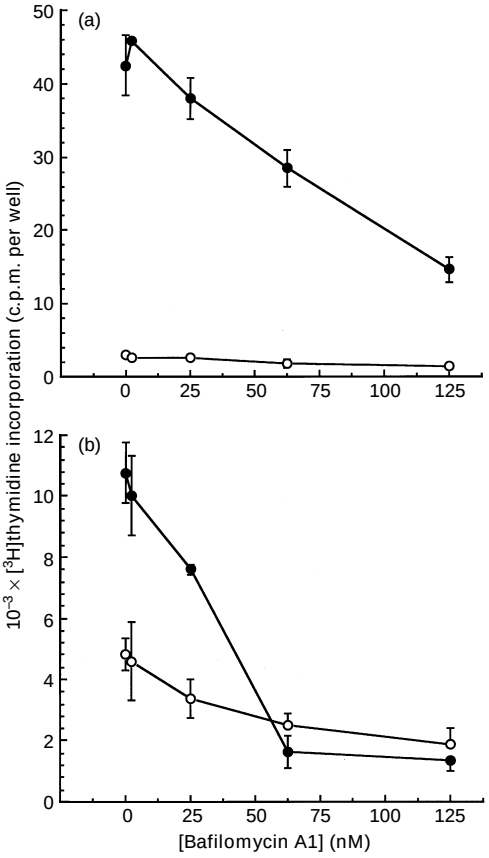

Figure 1 Effect of bafilomycin A_1 on DNA synthesis

Quiescent 3T3 cells were incubated in SF-DMEM containing 1 μCi/ml [^{3}H]thymidine for 1 h with the indicated concentrations of bafilomycin A_1. The cells were then incubated for a further 24 h with either no additions (○) or 10 ng/ml EGF and 0.2 μg/ml insulin (**a**, ●) or 10 nM PMA (**b**, ●). Results are expressed as the means ± S.D. for triplicate incubations. Similar results were obtained in a further four experiments.

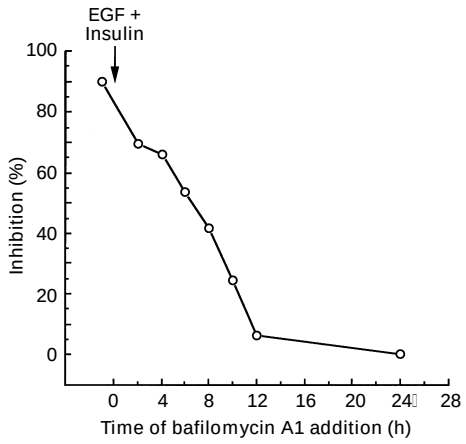

Figure 2 Effect of delayed addition of bafilomycin A_1 on DNA synthesis

Quiescent 3T3 cells were incubated in SF-DMEM containing 1 μCi/ml [^{3}H]thymidine for 1 h before the addition of EGF (10 ng/ml) and insulin (0.2 μg/ml) or no further additions (control). The cells were then incubated for a further 24 h. Bafilomycin A_1 (125 nM) was added at the indicated times either before or after the addition of EGF and insulin. Results are expressed as the inhibition of EGF- and insulin-induced DNA synthesis (over control). Similar results were obtained in a further three experiments.

compared with its effect on the response to EGF and insulin (Figure 1). These data indicate that bafilomycin A_1 inhibits DNA synthesis in response to mitogens that act independently of the activation of cell-surface receptors.

To establish when cells are responsive to the inhibitory action of bafilomycin A_1 the antibiotic was added to cells at various times after they had been treated with EGF and insulin (Figure 2). The data show that, as before, addition of 125 nM bafilomycin A_1 1 h before EGF and insulin almost completely inhibited DNA synthesis in response to the mitogens. Delaying the addition of bafilomycin reduced its ability to inhibit DNA synthesis (Figure 2). Interestingly, addition of bafilomycin A_1 12 h after the addition of EGF and insulin had no effect on DNA synthesis (Figure 2). In Swiss 3T3 cells, entry into S-phase and hence DNA synthesis begins 10–12 h after the addition of mitogens to quiescent cells [27]. The data therefore show that bafilomycin A_1 does not inhibit DNA synthesis when added to cells that are entering S-phase. Significantly, the data also show that cells in S-phase can be incubated with bafilomycin A_1 for 12 h without any deleterious effect (Figure 2). This provides evidence that the inhibitory action of bafilomycin A_1 on cell proliferation is exerted during the G_1 phase of the cell cycle and that the antibiotic does not directly inhibit the process of DNA synthesis.

Inhibition of endosomal acidification by bafilomycin A_1

The effect of bafilomycin A_1 on endosomal acidification was studied by vital fluorescence staining with acridine orange, an 'acidotropic' weak base that is taken up by living cells and accumulates in acidified compartments such as lysosomes [28,29]. The fluorescence of acridine orange is green at low concentrations and orange at high concentrations [28].

In untreated, quiescent 3T3 cells, strong granular fluorescence with an orange colour was observed in the cytoplasm (Figure 3a), with diffuse green fluorescence in the nuclei and punctate staining of the nucleoli. This distribution suggests that the granular fluorescence is due to acidified lysosomes. The orange granular fluorescence was diminished in the cytoplasm of cells that had been preincubated with 25 nM (Figure 3b) and 62.5 nM (Figure 3c) bafilomycin A_1 and was not observed in cells that had been preincubated with 125 nM bafilomycin A_1 (Figure 3d). Only diffuse green fluorescence in the nucleus and cytoplasm was seen in cells that had been incubated with 125 nM bafilomycin A_1 (Figure 3d). These data indicate that bafilomycin A_1 inhibits vacuolar acidification in 3T3 fibroblasts.

The complete loss of the orange granular fluorescence in the cytoplasm caused by 125 nM bafilomycin A_1 was reversed by washing the cells and incubating at 37 °C for 1 h in DMEM containing 10 % (w/v) FCS (Figure 3e). In these cells the orange cytoplasmic fluorescence was as intense as in control (untreated) cells (see Figure 3a).

Interestingly, at lower concentrations of bafilomycin A_1 (i.e. 2.5 nM) there was no apparent effect on vacuolar acidification as revealed by acridine orange fluorescence but there was a subtle change in the size and localization of the vacuoles. Thus in the presence of 2.5 nM bafilomycin A_1 the cytoplasmic vacuoles were slightly larger and changed from a dispersed localization to one that was predominantly perinuclear (results not shown).

Changes in cell morphology induced by bafilomycin A_1

After incubation of 3T3 fibroblasts with 125 nM bafilomycin A_1 for 24 h the V-ATPase inhibitor evoked a dramatic change in the

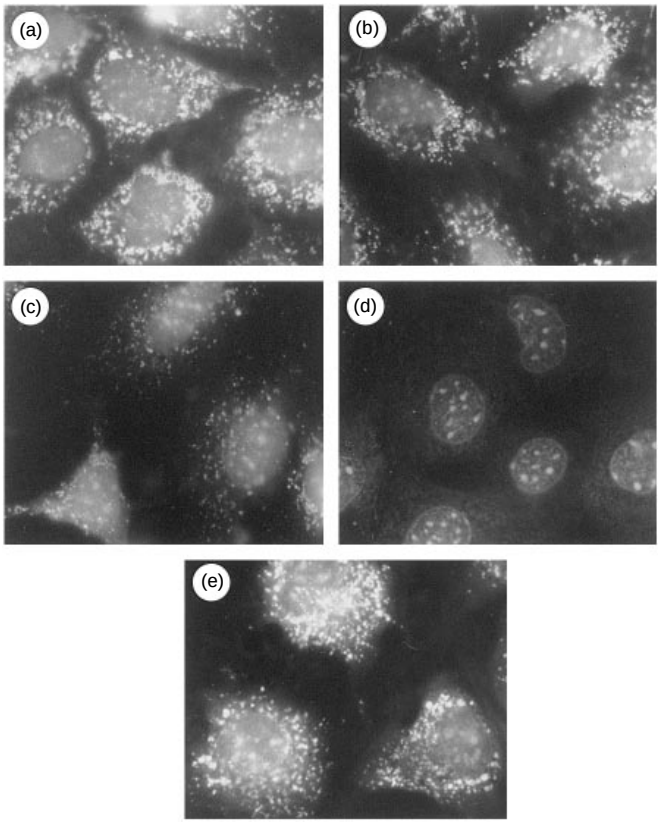

Figure 3 Inhibition of vacuolar acidification by bafilomycin A_1

Quiescent Swiss 3T3 fibroblasts in SF-DMEM were incubated for 1 h at 37 °C with no additions (**a**), 25 nM (**b**), 62.5 nM (**c**) or 125 nM (**d**,**e**) bafilomycin A_1. The cells were then either stained with acridine orange immediately (**a**–**d**) or washed twice with SF-DMEM and incubated in DMEM containing 10% (w/v) FCS for 1 h (**e**). Acridine orange staining was achieved by incubating the cells with the dye for 10 min at 37 °C. After extensive washing the cells were mounted in PBS and rapidly examined under a fluorescence microscope.

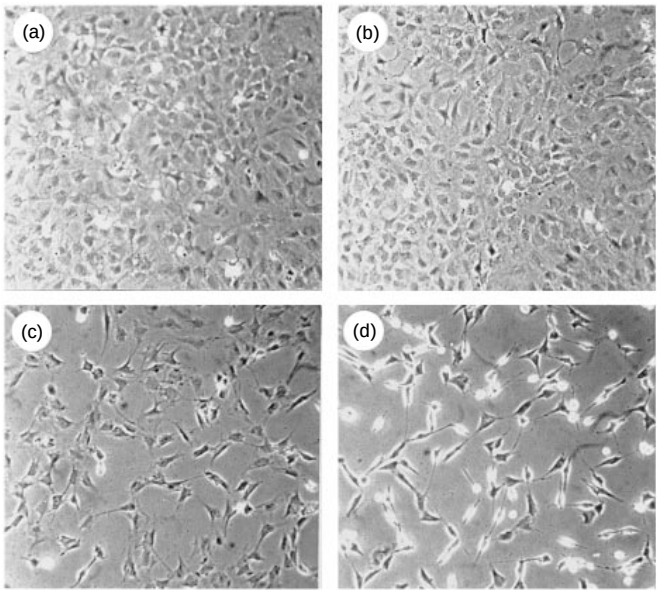

Figure 4 Effect of bafilomycin A_1 on cell morphology

Quiescent Swiss 3T3 fibroblasts were incubated for 24 h at 37 °C in serum-free DMEM containing no additions (**a**), 25 nM (**b**), 62.5 nM (**c**) or 125 nM (**d**) bafilomycin A_1.

morphology of the cells (Figure 4d). This change in morphology involved an unambiguous rounding up and elongation of the cell, producing a long spindle-shaped structure. In contrast, no change in cell morphology was detected when cells were incubated with 25 nM bafilomycin A_1 (Figure 4b). However, 62.5 nM bafilomycin A_1 induced a distinct change in the morphology of a high proportion of the fibroblasts (Figure 4c). Although 125 nM bafilomycin A_1 seemed to cause a reduction in the number of cells (compare Figures 4d and 4a), much of the effect was caused by the elongation and rounding-up of the bafilomycin A_1-treated cells. Analysis of cell numbers revealed that there was less than a 20% reduction in cell number in response to incubation for 24 h with 125 nM bafilomycin A_1 (results not shown). The mechanism by which bafilomycin A_1 evokes the observed change in cell morphology remains unknown.

The bafilomycin A_1-induced alteration in the morphology of the cells was reversed by incubating them at 37 °C for 24–48 h in DMEM containing 10% (w/v) FCS. This treatment was sufficient to allow the cells to regain normal morphology (results not shown). These recovered cells showed acidified vacuoles as revealed by vital fluorescence microscopy (Figure 3e) and were capable of serum-stimulated DNA synthesis measured by [^{3}H]thymidine incorporation (results not shown).

Effect of bafilomycin A_1 on c-*fos* mRNA accumulation

The activation of cell-surface receptors that possess intrinsic protein tyrosine kinase activity initiates a complex array of intracellular signalling events including the activation of the mitogen-activated protein (MAP) kinase cascade [30,31]. These growth-factor-activated signal transduction cascades lead to the rapid and transient transcriptional activation of a large number of genes including the c-*fos* proto-oncogene. We therefore sought to establish the extent to which bafilomycin A_1 influences growth-factor-stimulated c-*fos* expression. Measurement of c-*fos* mRNA accumulation was undertaken with RT–PCR and in a series of control experiments it was demonstrated that Northern blot and RT–PCR analysis of c-*fos* gene expression gave quantitatively similar results (results not shown). Figure 5 shows that, as expected, EGF and insulin caused a dramatic increase in c-*fos* mRNA accumulation. Bafilomycin A_1 increased c-*fos* mRNA accumulation and had no effect on the increase in c-*fos* mRNA evoked by EGF and insulin. Thus the increase in c-*fos* mRNA in response to EGF and insulin as revealed by quantitation of PCR product formation after RT–PCR (Figure 5b) was very similar in the presence (4983 c.p.m. above control) and absence (4776 c.p.m. above control) of bafilomycin A_1. These data show that inhibition of the V-ATPase has no effect on EGF and insulin-stimulated c-*fos* gene expression. This suggests that activation of the signal transduction pathways that lead to c-*fos* gene expression in response to EGF and insulin is not dependent on vacuolar acidification.

Together, the data presented here show that bafilomycin A_1 inhibits endosomal acidification in Swiss 3T3 fibroblasts and that inhibition of the V-ATPase attenuates protein kinase C- and growth-factor-induced DNA synthesis (Figure 1). Importantly, bafilomycin A_1 had no effect on DNA synthesis when added to cells at a time close to the onset of DNA synthesis (Figure 2), which shows that the antibiotic does not directly inhibit DNA synthesis. The ability of bafilomycin A_1 to inhibit DNA synthesis

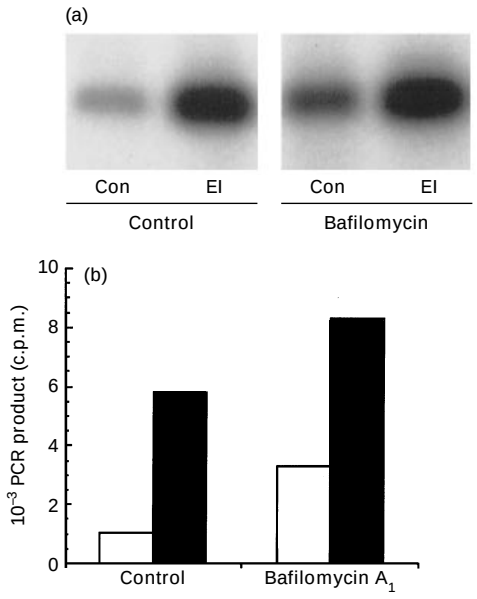

Figure 5 Effect of bafilomycin A_1 on c-*fos* mRNA accumulation

Quiescent 3T3 cells were incubated in SF-DMEM for 1 h either with or without bafilomycin A_1 (125 nM). The cells were then incubated for a further 30 min in either the presence (EI, ■) or the absence (Con, □) of 10 ng/ml EGF and 0.2 μg/ml insulin. After RT–PCR amplification of c-*fos* mRNA in the presence of [^{32}P]dCTP, PCR products, resolved on 2% (w/v) agarose gels, were subjected to autoradiography (**a**) and quantitated by liquid-scintillation counting of the excised bands (**b**).

when added to cells during G_1 suggests that the V-ATPase plays an important role in mitogenic signal transduction pathways that operate during G_1. Furthermore the inhibition of PMA-evoked DNA synthesis implies that the mechanism by which bafilomycin A_1 inhibits cell cycle progression is not restricted to its effects on the endocytic processing of cell-surface receptors and their ligands [19].

It has been suggested that elevation of cytosolic pH (pH_i), which may occur in response to growth factors and activation of protein kinase C, plays an important regulatory role in mitogenic signalling [32]. It is possible that acidification of endocytic vesicles by the V-ATPase or activation of plasma membrane-localized V-ATPase may lead to a concomitant increase in pH_i; bafilomycin A_1 would inhibit such changes in pH_i. However, recent studies have indicated that bafilomycin A_1 does not affect pH_i in NIH3T3 cells [33], suggesting that the activity of the V-ATPase does not influence pH_i and that this does not account for the ability of bafilomycin A_1 to inhibit DNA synthesis.

Bafilomycin A_1 had no effect on EGF- and insulin-induced c-*fos* mRNA accumulation (Figure 5), suggesting that the early receptor-activated signal transduction pathways are independent of vacuolar acidification. This implies that interaction of the receptors with proteins such as phosphatidylinositol 3-kinase, c-*src* , Grb-2 and the insulin receptor substrates, and the activation of, for example, the MAP kinase cascade, occur independently of receptor and ligand processing in the endocytic pathway.

There are a number of alternative mechanisms by which bafilomycin A_1 may inhibit other cellular responses in G_1 that are necessary for cell cycle progression. For example, the availability of intracellular iron is an essential requirement for cell division, and the acidification of endosomes is required for the dissociation of Fe^{3+} from transferrin [34]. Furthermore, inhibition of V-ATPase that has been reconstituted into liposomes attenuates the export of Fe^{2+} from vesicles, which suggests that the V-ATPase may itself function as the Fe^{2+} channel [35]. It is therefore possible that inhibition of DNA synthesis by bafilomycin A_1 is mediated by restriction of the transport of iron. Alternatively, it has been demonstrated that bafilomycin A_1 and structurally related compounds inhibit protein trafficking between the trans-Golgi network and the plasma membrane [10] as well as the processing and secretion of proteins, including hormones and growth factors, via the constitutive secretory pathway [36–38]. Inhibition of protein secretion (possibly of autocrine growth factors) could account for the effects of bafilomycin A_1 on DNA synthesis. Further investigation will be needed to elucidate the mechanism by which inactivation of V-ATPase by bafilomycin A_1 blocks mitogen-induced progression through G_1 and entry into S-phase DNA.

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