A somatostatin analogue inhibits MAP kinase activation and cell proliferation in human neuroblastoma and in human small cell lung carcinoma cell lines

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Received 1 August 1996

Abstract Somatostatin possesses antisecretory and antiproliferative activity on some human tumors. We herein report that, in a human neuroblastoma cell line, the somatostatin analogue BIM 23014 inhibited mitogen-activated protein (MAP) kinase activity stimulated by either insulin-like growth factor-1, whose receptor bears a tyrosine kinase, or carbachol, which acts at a G-protein coupled receptor. In a human small cell lung carcinoma line BIM inhibited serum-stimulated MAP kinase activation. These inhibitory actions occur in a dose range quite similar to that observed for suppression of proliferation induced by the analogue in the same cell lines. The decrease in cAMP elicited by the analogue in the two cell lines is not responsible for its inhibitory action on MAP kinase and cell growth. Moreover, the analogue did not modify intracellular [Ca^{2+}] and pH. An involvement of a phosphatase activity is suggested.

Key words: Somatostatin; Proliferation; MAP kinase

1. Introduction

Somatostatin (SST) is a peptide hormone which plays an important role in controlling the endocrine, paracrine and autocrine secretion of several anterior pituitary and gastrointestinal hormones [1,2].

SST has been shown also to possess an antiproliferative effect in some human cancers including breast cancer, pancreatic cancer, small cell lung carcinoma and neuroblastoma [3]. The biological activity of the peptide is dependent on its interaction with specific receptors, the structures of which have recently been elucidated from cloning experiments [4]. Five distinct SST receptor genes have been cloned and the corresponding receptors are all members of a G-protein coupled, seven transmembrane domain family. Some of these receptors (if not all) are coupled to adenylate cyclase in an inhibitory fashion [4].

Both human small cell lung carcinoma (SCLC) and neuroblastoma (NB) are tumors characterized by a poor prognosis and unsuccessful pharmacological treatment. To find a new pharmacological approach to control malignant cell proliferation would be of great value. The recent availability of a potent and long lasting SST analogue (BIM 23014, also known as lanreotide [5]) has allowed us to further investigate the antiproliferative effect of this SST-like agent in SCLC and NB and to attempt to understand its cellular mechanism(s) of action. We found that BIM inhibited MAP kinase activity stimulated by different types of mitogens in a dose range similar to that necessary for inhibition of cell proliferation. A decrease in cAMP or modification of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and pH were not involved in the analogue's antimitogenic action.

2. Materials and methods

2.1. Cell lines

Small cell lung carcinoma cell lines (GLC-8, NCI-N-592 and H-69) and neuroblastoma cell line (SY5Y) were routinely grown in RPMI-1640 medium (Sigma Chemicals, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, Grand Island, NY, USA). All the cell lines were kept at 37°C in a humidified atmosphere of 5% CO_2 in air.

2.2. Mitogenicity assay

SCLC cells were plated in RPMI-1640 medium supplemented with 10% FBS in 96-well microtiter plates, at a density of 10^5 cells/well, treated with the indicated concentrations of BIM and incubated for 48 h. Quiescent SY5Y cells, plated at a density of 2×10^4 cells/well, were stimulated with serum or IGF-1, in the presence or in the absence of BIM, for 48 h. BIM was added twice a day during the 48 h of incubation. [^{3}H]Thymidine (1 μCi/well; specific activity 2 Ci/mmol, Amersham, UK) was added during the last 6 h of incubation. SCLC cells were then collected on filters with an automatic cell harvester (Titerteck, Flow Laboratories, Rockville, MD, USA) and the filters were counted using standard procedures. SY5Y cells were extracted in 10% TCA and the radioactivity incorporated into TCA insoluble material was evaluated after solubilization in NaOH 0.5 M.

2.3. Determination of MAP kinase activity

H-69 and SY5Y cells were serum-deprived for 48 h in RPMI alone or in 0.1% FBS in RPMI, respectively. The cells ($1.5–2.0\times10^6$) were preincubated with BIM or forskolin for 10 min before treatment with the various stimuli. After 5 min, the medium was removed and the cells were washed and resuspended (H-69 cells) or scraped (SY5Y cells) in 0.5 ml of homogenization buffer, according to Seger et al. [6]. After sonication (2×7 s) and centrifugation ($15000\times g$ for 10 min at 4°C), the supernatants were fractionated on DEAE-cellulose minicolumns. MAP kinase activity was determined by phosphate incorporation into myelin basic protein (MBP, Sigma) in the presence of [γ-^{32}P]ATP (2 μCi/sample; specific activity 3000 Ci/mmol; Amersham, UK), also according to Seger et al. [6].

2.4. Determination of adenylate cyclase activity

Adenylyl cyclase assays were performed with membrane preparations stimulated by forskolin, as previously described [7,8].

2.5. Ca^{2+} measurements with fura-2

The cells were washed in Krebs-Ringer-HEPES medium (KRH) containing (in mM): 125 NaCl; 5 KCl; 1 KH_2PO_4; 2 $MgSO_4$; 0.5 $CaCl_2$; 25 HEPES-NaOH, pH 7.4; 6 glucose and 1 mg/ml bovine serum albumin. The cells were then incubated for 30 min, at 37°C, with the Ca^{2+}-sensitive dye fura-2 (Calbiochem, San Diego, CA, USA), added as the acetoxymethylester, at a final concentration of 2 μM. After rinsing once with KRH medium, aliquots (5×10^6 cells for H-69 and 1.5×10^6 for SY5Y) of the cell suspension were transferred to a thermostated cuvette (34°C) in a volume of 1.5 ml, main-

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PII S0014-5793(96)01159-3

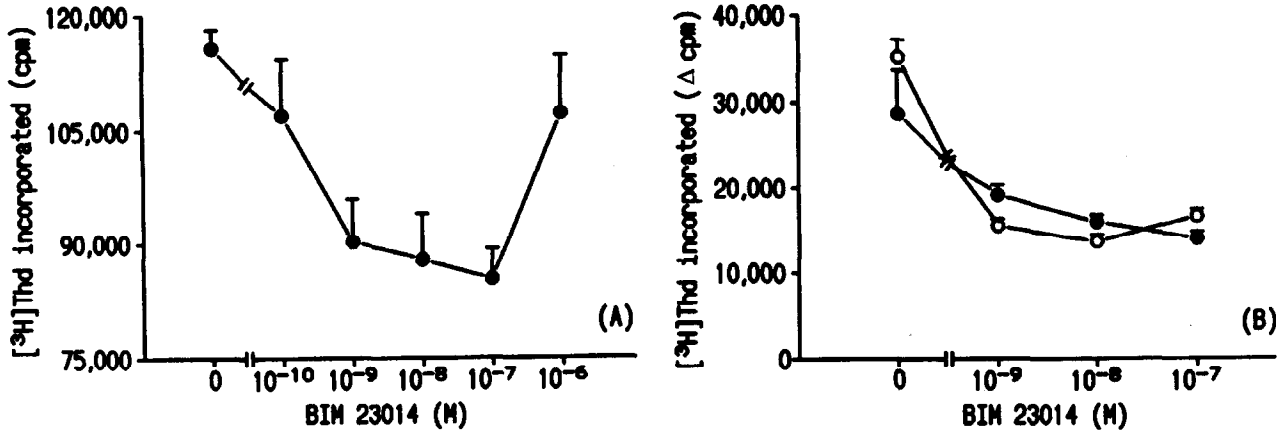

Fig. 1. BIM 23014 inhibition of serum-stimulated DNA synthesis in H-69 cells (A) and of serum- and IGF-1-induced DNA synthesis in SY5Y cells (B). A: H-69 cells incubated in 10% FBS; B: quiescent SY5Y cells incubated in RPMI containing 10% FBS (●) or IGF-1 100 ng/ml (○). The cells were treated for 10 min with increasing concentrations of BIM 23014 before the addition of mitogens. The experiments were then performed as described in Section 2. Data are expressed: in (A) as cpm of [^{3}H]thymidine ([^{3}H]Thd) incorporated by the cells; in (B) as differences (Δcpm) of the [^{3}H]Thd incorporated by the stimulated cells over the [^{3}H]Thd incorporated by control cells (RPMI alone, 26381 ± 4316 cpm; $n = 3$) and are the mean ± S.D. of triplicate samples from a representative experiment which was repeated twice with similar results.

tained under continuous stirring and analyzed in a Perkin Elmer LS/5B fluorometer. Calibrations for [Ca^{2+}] were carried out as previously described [9].

3. Results

Fig. 1A shows that the SST analogue BIM 23014 inhibited [^{3}H]thymidine ([^{3}H]Thd) incorporation in H-69 cells in the presence of 10% FBS in a concentration-dependent manner. Maximum inhibition (about 25%) was obtained at 100 nM. The IC_{50}, based upon the maximum reduction in [^{3}H]Thd incorporation, was estimated to be approximately 350 pM. At the highest concentration tested (1 μM) the analogue lost its action and the [^{3}H]Thd incorporation returned to normal levels. In similar experiments performed in two other SCLC cell lines (GLC-8 and NCI-N-592) BIM, at the same concentrations, was unable to modify DNA synthesis (data not shown).

A substantially greater decrease of [^{3}H]Thd incorporation following BIM administration was observed also in the neuroblastoma cell line SY5Y (Fig. 1B). The cells were made quiescent by serum deprivation for 24 h and then stimulated with serum or IGF-1 in the presence or in the absence of increasing concentrations of BIM. The maximal inhibition against both stimuli was about 50% and the IC_{50}s, calculated as described above, were estimated to be approximately 500 pM.

Having established the antiproliferative role for the SST analogue in the two cell lines, we next examined the possible cellular mechanisms that might be responsible for this action. Since serum- and IGF-1-stimulated DNA synthesis is measured many hours after the application of the analogue, we decided to investigate the effect of BIM on a much earlier event elicited by mitogens (i.e. MAP kinase activation). Fig. 2A,B shows that BIM was capable also of completely inhibiting serum-stimulated MAP kinase activity in the two cell lines examined. BIM was administered 10 min before the stimuli, which were applied to the cells for an additional 5 min. In

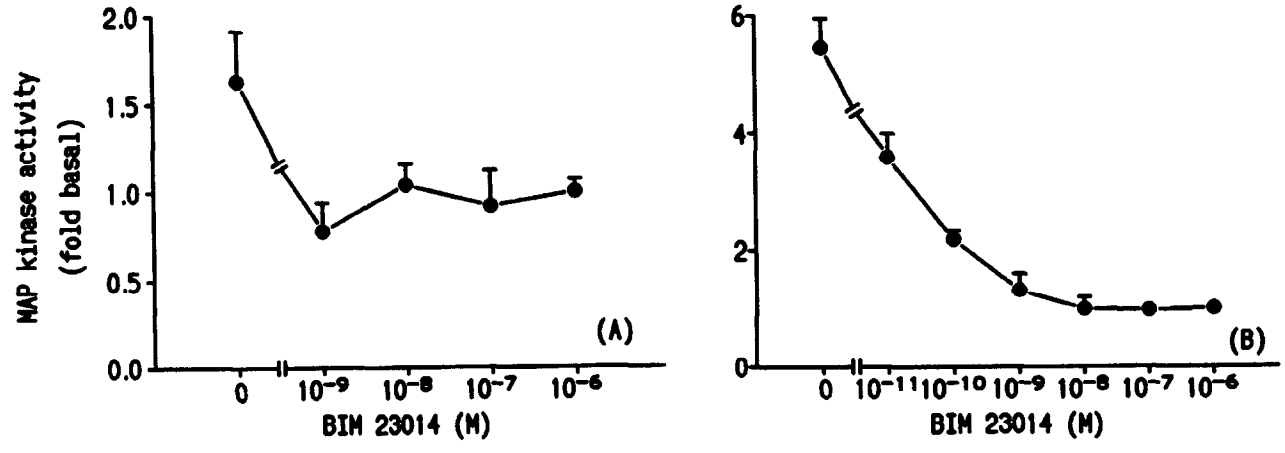

Fig. 2. BIM 23014 inhibition of serum-induced MAP kinase activity in H-69 cells (A) and SY5Y cells (B). Cells were preincubated for 10 min with the indicated concentrations of BIM 23014 and then stimulated with 20% FBS for 5 min. MAP kinase activity was evaluated as described in Section 2. Data are expressed as fold of pmol of ^{32}P incorporated by MBP/min/mg protein under basal conditions (i.e. in the presence of extracts obtained from untreated cells; these values are 6.53 ± 0.87 and 1.76 ± 0.17 pmol/min/mg protein for H-69 and SY5Y cells, respectively; $n = 6$). Each point is the mean ± S.E.M. of three separate experiments, each performed in duplicate.

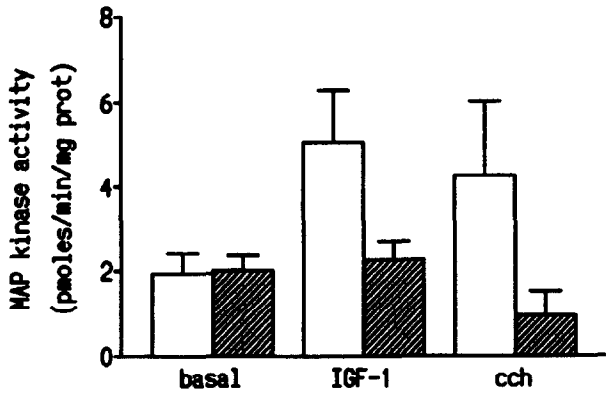

Fig. 3. BIM 23014 inhibition of MAP kinase activity stimulated by IGF-1 and carbachol in SY5Y cells. SY5Y cells were stimulated for 5 min with 100 ng/ml IGF-1 or 1 mM carbachol (cch) after 10 min of preincubation with medium alone (open bars) or 1 nM BIM 23014 (hatched bars). Data are expressed as pmol of ^{32}P incorporated by MBP/min/mg protein and are the mean ± S.E.M. of three separate experiments, each performed in duplicate.

H-69 cells, it appears that 1 nM BIM brought MAP kinase activity back to basal levels while 1 μM was without additional effect upon this enzyme activity. In SY5Y cells the IC_{50} for the inhibitory effect on the kinase activity was estimated to be 100 pM. As illustrated in Fig. 3, BIM, at a concentration of 1 nM, completely inhibited the MAP kinase activation induced by both IGF-1, a tyrosine kinase receptor agonist, and by carbachol, whose receptors belong to the seven transmembrane G-protein coupled family and are reported to be expressed in SY5Y cells [10].

We were interested in understanding the intracellular signal(s) elicited by BIM which lead to inhibition of MAP kinase activity. Five types of SST receptors have recently been cloned [4] and at least some (if not all) of them are negatively coupled to adenylate cyclase (AC). The inhibition of forskolin-induced cAMP formation in H-69 and SY5Y cell membranes by BIM is shown in Table 1. However, as shown in Fig. 4A,B, introduction of the cAMP analogue 8-bromo-adenosine cyclic 3′-5′-monophosphate (8-BrcAMP, 1 mM) inhibited the incorporation of [^{3}H]Thd stimulated by serum (A, H-69 cells) and by serum and IGF-1 (B, SY5Y cells). Moreover, the exposure to forskolin completely inhibited the serum-stimulated MAP kinase activity in both H-69 and SY5Y cell lines (Fig. 4C).

From these results it appears that the inhibition of MAP kinase activity and of cell proliferation by BIM are mediated by an intracellular signal distinct from cAMP.

It has been reported that SST inhibits the voltage-dependent Ca^{2+} channels (VDCCs) and as a consequence the depolarization-induced $[Ca^{2+}]_i$ increase and hormone release from pituitary cells [11,12]. Both H-69 and SY5Y cells are known to possess VDCCs [13,14] and to contain peptides which can be released and possibly act as autocrine growth factors [15,16]. Since MAP kinase activity has been shown to be stimulated by calcium in some types of cells [17], measurements of $[Ca^{2+}]_i$ were carried out by the fura-2 technique in both cell lines and the effect of BIM tested. H-69 and SY5Y cells were found to have a resting $[Ca^{2+}]_i$ of 35 ± 10 and 75 ± 11 nM, respectively ($n = 7$). After depolarization with 50 mM KCl, $[Ca^{2+}]_i$ went to 70 ± 15 and 148 ± 14 nM, respectively ($n = 5$). Pretreatment of the cells with BIM (100 nM) had no effect, either on the resting $[Ca^{2+}]_i$ (42 ± 7 and 60 ± 16 nM for H-69 and SY5Y cells respectively; $n = 5$) nor on the KCl-stimulated $[Ca^{2+}]_i$ rise (75 ± 10 and 135 ± 20 nM, $n = 5$).

It has been reported that in enteric endocrine cells SST causes an acidification of intracellular pH via inhibition of Na^+/H^+ exchange [18]. In both cell lines the administration of BIM did not induce any variation in the cytoplasmic pH, as measured by the fluorescent probe BCECF (data not shown).

4. Discussion

We show here that the SST analogue BIM inhibited [^{3}H]Thd incorporation in one out of three small cell carcinoma cell lines tested in accordance with the data in the literature, where only a percentage of the SCLC express somatostatin receptors [19]. By inference, the resistant SCLC lines used in our study were likewise devoid of SST receptors, although this remains to be establish as the cause of their resistant nature. The biphasic concentration-response curve most likely derives from a desensitization of the receptors at the higher concentrations used (1 μM). The data suggest the existence of a precise concentration range to be used to obtain the antiproliferative effect in these cells. BIM was also capable of inhibiting [^{3}H]Thd incorporation in human neuroblastoma cells used (SY5Y) to a greater extent than H-69 cells.

Having established the antiproliferative effect of BIM in the two cell lines, we then attempted to elucidate the cellular mechanism(s) affected by the analogue which might be responsible for its action. First of all, since [^{3}H]Thd incorporation is tested many hours after both drug and mitogen exposure, we measured a much earlier event stimulated by growth factors (i.e. the activation of MAP kinase). MAP kinases belong to a family of phosphorylating enzymes which integrate and amplify messages from different growth factors and are considered crucial for the immediate transmission of the mitogenic signal from the membrane to the nucleus [20]. Our finding that BIM inhibited MAP kinase activity suggests that its site of action is close to sites responsible for cellular events triggered immediately after the growth factor-receptor interaction. Since the analogue was capable of inhibiting the MAP kinase activity stimulated by both IGF-1, which possess a tyrosine kinase receptor, and by carbachol, whose receptor is G protein-coupled, its site of action might presumably be on some step common to the two pathways leading to MAP kinase activation. BIM was able to block the MAP kinase

Table 1
Adenylate cyclase activity in membranes of H-69 and SY5Y cells

Cell line	Basal	Forskolin 50 μM	Forskolin 50 μM+BIM 23014 1 μM	Inhibition (%)
H-69	27.3 ± 1.4	465.2 ± 22.0	292.9 ± 33.1	37.0
SY5Y	35.1 ± 2.0	382.8 ± 20.2	301.7 ± 5.9	21.0

The assays were performed as described in Section 2. Results are expressed as pmol cAMP/min/mg protein and are the mean ± S.D. of a representative experiment that was repeated once with similar results.

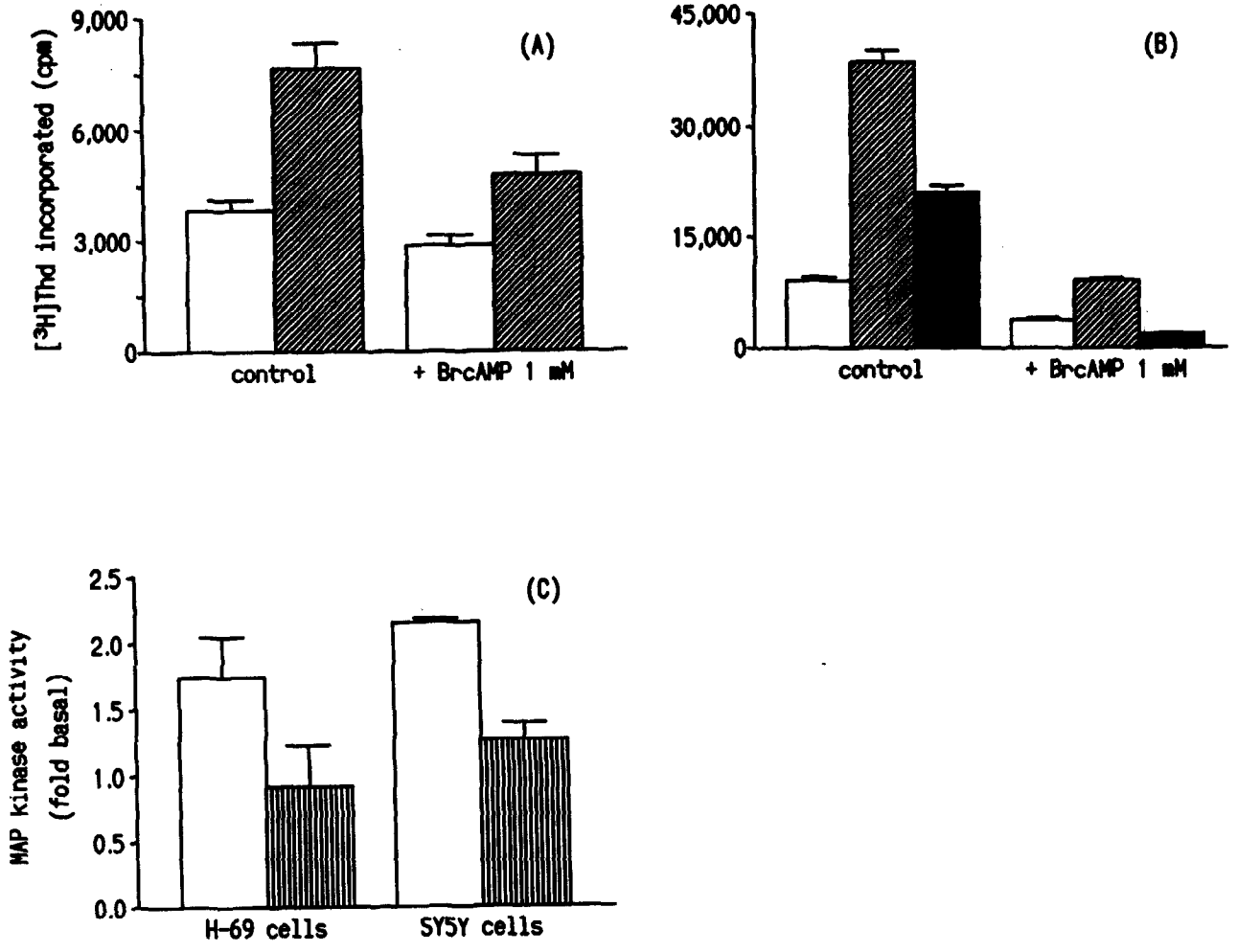

Fig. 4. Elevation of intracellular cAMP inhibited serum- and IGF-1-induced DNA synthesis and MAP kinase activity in H-69 and SY5Y cells. A: H-69 cells; B: SY5Y cells. Cells were incubated in RPMI alone (open bars) or containing 10% FBS (hatched bars) or IGF-1 100 ng/ml (solid bars). When indicated, 1 mM BrcAMP was added 10 min before mitogens. The experiments were then performed as described in Section 2. Data are expressed as in Fig. 1A. C: H-69 and SY5Y cells were treated for 10 min with medium alone (open bars) or forskolin 50 μM (vertical bars) and then stimulated for 5 min with 20% FBS. Data are expressed as in Fig. 2. Basal values of pmol of ^{32}P incorporated by MBP/min/mg protein are 7.15 ± 0.62 and 2.2 ± 0.52 for H-69 and SY5Y cells, respectively ($n = 6$).

activity to a greater extent than DNA synthesis, whose inhibition was only partial. This might be due to activation of alternative pathways for DNA synthesis after the MAP kinase blockage, to a partial desensitization or to metabolic degradation of the analogue, since DNA synthesis and MAP kinase activity were measured 48 h and 15 min after BIM treatment, respectively.

It is well known that in most of the cell types tested, SST receptors are negatively coupled to AC and therefore, they decrease intracellular cAMP formation [4]. Although in the two cell lines tested BIM is capable of inhibiting forskolin-stimulated AC, it is unlikely that this is responsible for inhibition of proliferation since cAMP is a growth-suppressant in both cell lines. In fact, elevation of intracellular cAMP levels either by 8-BrcAMP or forskolin prevented both DNA synthesis and MAP kinase activation.

Both H-69 and SY5Y cells possess VDCCs on their plasmalemma [13,14]. We have previously shown that, in SCLC cells, proliferation depends, at least in part, upon the presence of extracellular Ca^{2+} and that in these cells Ca^{2+} antagonists are capable of inhibiting DNA synthesis [21]. Since in excitable cells SST has been shown to inhibit VDCCs function [11,12], and thereby hormone release, we hypothesized that BIM could interfere with the release of some autocrine growth factor by inhibiting Ca^{2+} influx. Moreover, it has been shown that in some types of cells, like PC12 cells, MAP kinase activity is stimulated by influx of Ca^{2+} through VDCCs [17]. However, in fura-2 loaded cells we could not demonstrate any effect of the analogue on depolarization-induced Ca^{2+} rise. It should be said however, that this technique is not sensitive enough to detect small effects, such as a 10–20% decrease.

Growth factors, by activating the Na^+/H^+ exchange, induce an increase in intracellular pH and this cytoplasmic alkalinization is considered a permissive event for DNA synthesis to occur [22]. It has been reported that in enteric endocrine cells SST causes an acidification of intracellular pH, dependent on extracellular Na^+ and inhibited by amiloride [18]. If BIM inhibited the Na^+/H^+ exchange in our cells, this event would be a good candidate responsible for the analogue's antiproliferative action. But we verified that in both cell lines the analogue did not have any effect on intracellular pH.

In conclusion, to our knowledge this is the first evidence

that stimulation of SST receptors inhibits MAP kinase activity which parallels inhibition of cell proliferation. Our data, showing that BIM inhibits MAP kinase activity stimulated by both IGF-1 and carbachol, indicate that BIM acts at a very early post-receptor level and at some step common to the signalling cascade initiated by both tyrosine kinase and G-protein coupled receptors leading to MAP kinase activation. Having excluded an involvement of cAMP, Ca^{2+} and pH, a tyrosine phosphatase activity, which has recently been found to be associated with SST receptors [23,24], might be suggested as responsible for the analogue's action.

Acknowledgements: We wish to thank Dr. S.B. Sparber for reading the manuscript. We also thank F. D'Atri and I. Forza for technical assistance. This work was supported by an A.I.R.C. (Associazione Italiana Ricerca Cancro) grant to L.M.V.

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