A peptide that inhibits the mitogenic stimulation of Swiss 3T3 cells by bombesin or vasopressin

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The synthetic peptide [D-Arg¹,D-Pro²,D-Trp^{7,9},Leu¹¹]substance P inhibits the stimulation of DNA synthesis induced in Swiss 3T3 cells by bombesin or vasopressin, but not that induced by a wide range of other growth factors and mitogens. The stimulation induced by 10 pM–3 nM-bombesin is inhibited by 1–30 μM-antagonist in a manner consistent with competition at the bombesin receptor. The inhibition by the antagonist of the stimulation induced by vasopressin suggests a previously unrecognized interaction of the antagonist with vasopressin receptors. The antagonist should be useful in studies of cell proliferation both *in vivo* and *in vitro*.

INTRODUCTION

The frog skin-derived tetradecapeptide bombesin, and related peptides present in mammalian tissues including gut (Bloom *et al.*, 1979; Walsh *et al.*, 1979) brain (Brown *et al.*, 1978; Moody & Pert, 1979; Walsh *et al.*, 1979), tumour cells (Moody *et al.*, 1981; Wood *et al.*, 1981; Erisman *et al.*, 1982) and milk (Jahnke & Lazarus, 1984; Ekman *et al.*, 1985), elicit a wide range of cellular responses *in vivo* and *in vitro*, including the stimulation of cell proliferation (Carney *et al.*, 1983; Rozengurt & Sinnett-Smith, 1983; Willey *et al.*, 1984). The investigation of the biochemical mechanisms involved in the stimulation of cell proliferation by bombesin or other growth factors would be aided by the availability of specific antagonists. Recently, a substance P analogue ([D-Arg¹,D-Pro²,D-Trp^{7,9},Leu¹¹]substance P) has been demonstrated to be a competitive antagonist of the binding of bombesin to its receptors on pancreatic acinar cells (Jensen *et al.*, 1984). We now show here that this peptide inhibits the mitogenic stimulation of Swiss 3T3 cells by bombesin. The specificity of this inhibition is indicated by the lack of effect of the peptide on the stimulation induced by a wide range of other growth factors and mitogens. In addition, however, we have found that the peptide inhibits the mitogenic stimulation of Swiss 3T3 cells induced by vasopressin, suggesting a previously unrecognized interaction of the antagonist with vasopressin receptors.

EXPERIMENTAL

Materials

Culture medium, antibiotics, trypsin and newborn-calf serum were obtained from Flow Laboratories, and [methyl-³H]thymidine was from Amersham International. Substance P, [D-Arg¹,D-Pro²,D-Trp^{7,9},Leu¹¹]substance P and vasopressin were from Cambridge Research Biochemicals, bombesin was from Bachem and phorbol 12-myristate 13-acetate was from Sigma Chemical Co. Prostaglandin F_{2α} (Sigma Chemical Co.) was kindly donated by Mr. A. J. Davis. Epidermal growth factor and

platelet-derived growth factor were prepared as described previously (Savage & Cohen, 1972; Brown *et al.*, 1983).

Methods

Stock cultures of Swiss mouse 3T3 cells were maintained and passaged as described previously (Brown & Blakeley, 1983). For experimental use cells were seeded into 24-well cluster trays (Nunc) at 12 500 cells/well and grown for 7 days, by which time they were confluent (at about 60 000 cells/well) and quiescent (as judged by the incorporation of [³H]thymidine). Each well was rinsed with 0.5 ml of serum-free Dulbecco's modified Eagle's medium before adding 0.5 ml of this medium containing [³H]thymidine (1 μCi/ml; 1 μM) either with or without 0.5 μg of insulin/ml. The cells were then incubated for 30 min at 37 °C with the indicated concentration of antagonist or substance P, which were added at 20 μl/well from dilutions prepared in Dulbecco's modified Eagle's medium containing 1 mg of defatted bovine serum albumin/ml; control wells received the same volume addition of this medium. Mitogens were then added from dilutions prepared in the same way. After incubation for 28 h at 37 °C, the trichloroacetic acid-insoluble radioactivity was determined as described previously (Brown & Blakeley, 1983).

RESULTS AND DISCUSSION

In common with other mitogens that stimulate the proliferation of Swiss 3T3 cells, bombesin has limited mitogenic activity when added alone, but produces a marked synergistic stimulation with insulin or insulin-like growth factors (Rozengurt, 1980; Jimenez de Asua *et al.*, 1981; Rozengurt & Sinnett-Smith, 1983). Thus, in the presence of insulin, significant stimulation of [³H]thymidine incorporation by Swiss 3T3 cells was induced by 10–30 pM-bombesin and maximal stimulation by bombesin concentrations of 1 nM and above (Fig. 1a). In the presence of [D-Arg¹,D-Pro²,D-Trp^{7,9},Leu¹¹]substance P (designated antagonist) the mitogenic dose-response curve for bombesin was shifted to the

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Fig. 1. Inhibition of bombesin-stimulated DNA synthesis in Swiss 3T3 cells

(a) Dose-response curve for bombesin (+0.5 μg of insulin/ml) in the presence ($\blacktriangle$) or in the absence ($\triangle$) of 10 μM -antagonist; $\times$, no added mitogen. (b) Effect of antagonist or substance P on the stimulation by 0.5 μg of insulin/ml with or without 100 pM-bombesin: $\circ$, insulin + antagonist; $\square$, insulin + substance P; $\bullet$, insulin + bombesin + antagonist; $\blacksquare$, insulin + bombesin + substance P; $\times$, no added mitogen. The results shown are the means $\pm$ S.E.M. for triplicate samples, and (a) and (b) are taken from different experiments in which the incorporation of [^3H]thymidine by cultures stimulated by 10% serum was 130536 ± 8083 and 154105 ± 6172 c.p.m./well respectively.

Table 1. Specificity of antagonist effects on DNA synthesis in Swiss 3T3 cells

All methods were as described in the text, and the results shown are the means $\pm$ S.E.M. for the numbers of samples given in parentheses; * indicates significant inhibition ($P < 0.01$, by Student's t test); similar results were obtained in two to five experiments with each mitogen. The concentrations of mitogens were chosen to give stimulations in the same range as that induced by 100 pM-bombesin. Abbreviations: EGF, epidermal growth factor; PMA, phorbol 12-myristate 13-acetate; PDGF, platelet-derived growth factor; PGF_{2 α} , prostaglandin F_{2 α} .

Mitogen		[^3H]Thymidine incorporation (c.p.m./well)	
		Control	+ Antagonist (30 μM)
Expt. 1	None	2844 $\pm$ 416 (3)	—
	Serum	181045 $\pm$ 3001 (3)	—
	Insulin (0.5 $\mu\text{g}/\text{ml}$)	10381 $\pm$ 1661 (3)	—
	Insulin + bombesin (100 pM)	42933 $\pm$ 5017 (4)	16276 $\pm$ 2165 (3)*
	Insulin + EGF (300 pg/ml)	49596 $\pm$ 3925 (4)	48132 $\pm$ 2231 (3)
	Insulin + PMA (10 nM)	37838 $\pm$ 2788 (3)	36151 $\pm$ 1376 (3)
	Insulin + vasopressin (1 nM)	28031 $\pm$ 1758 (4)	9236 $\pm$ 918 (3)*
	Insulin + PDGF (2 $\mu\text{g}/\text{ml}$)	60853 $\pm$ 5439 (4)	64861 $\pm$ 4392 (3)
	Insulin + PGF _{2α} (80 nM)	31469 $\pm$ 317 (3)	34129 $\pm$ 2481 (3)
Expt. 2	None	1353 $\pm$ 105 (3)	—
	Serum	173037 $\pm$ 4986 (3)	—
	Insulin (0.5 $\mu\text{g}/\text{ml}$)	7291 $\pm$ 1272 (3)	—
	Insulin + bombesin (100 pM)	26818 $\pm$ 4425 (3)	10408 $\pm$ 2402 (3)*
	Insulin + substance P (30 μM) + bombesin (100 pM)	31390 $\pm$ 2472 (3)	17053 $\pm$ 2152 (3)*

right, although the maximum response was unaltered (Fig. 1a). The effectiveness of the antagonist was dose-dependent: in five separate experiments the stimulation induced by 100 pM-bombesin was half-maximally inhibited by between 3 and 10 μM -antagonist, and 30 μM -antagonist gave 82–90% inhibition (Fig. 1b). This pattern of antagonism is similar to that previously demonstrated against bombesin-induced amylase secretion from pan-

creatic acini (Jensen *et al.*, 1984). The antagonist itself had no mitogenic activity when added alone or in combination with insulin, and it did not inhibit the small stimulation of [^3H]thymidine incorporation induced by insulin alone (Fig. 1b).

The biochemical signalling mechanisms by which bombesin stimulates DNA synthesis and proliferation in Swiss 3T3 cells may involve a stimulation of membrane

Fig. 2. Inhibition of vasopressin-stimulated DNA synthesis in Swiss 3T3 cells

Dose-response curve for vasopressin (+0.5 μ g of insulin/ml) in the presence (▲) or in the absence (Δ) of 10 μ M-antagonist; x, no mitogen added. The results shown are the means $\pm$ S.E.M. for triplicate samples. The incorporation of [3 H]thymidine by cultures stimulated by 10% serum was $130\,536 \pm 8083$ c.p.m./well.

polyphosphoinositide turnover (Brown *et al.*, 1984). The exposure of Swiss 3T3 cells to bombesin results in a rapid increase in the breakdown of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate and diacylglycerol (Brown *et al.*, 1984), both of which are potential second messengers (Berridge, 1984; Berridge & Irvine, 1984; Irvine *et al.*, 1984; Nishizuka, 1984): inositol 1,4,5-trisphosphate induces the release of Ca^{2+} from intracellular stores (Berridge & Irvine, 1984; Irvine *et al.*, 1984), and diacylglycerol is a potent activator of protein kinase C (Nishizuka, 1984). Several other stimulators of the proliferation of Swiss 3T3 cells (vasopressin, platelet-derived growth factor and prostaglandin $\text{F}_{2\alpha}$) have also been found to stimulate phosphatidylinositol 4,5-bisphosphate breakdown and raise the intracellular free $[\text{Ca}^{2+}]$ in these cells (Berridge *et al.*, 1984; Brown *et al.*, 1984; Moolenaar *et al.*, 1984; Morris *et al.*, 1984). The specificity of the antagonist was therefore tested by using these and other mitogens at concentrations that, in the presence of insulin, induced stimulations similar to that obtained with 100 pM-bombesin (Table 1). The antagonist (30 μ M) had no effect on the stimulation induced by epidermal growth factor, platelet-derived growth factor or prostaglandin $\text{F}_{2\alpha}$, or by phorbol 12-myristate 13-acetate (a potent activator of protein kinase C; Nishizuka, 1984). These results indicate that the inhibition of bombesin-induced stimulation is unlikely to be due either to a general metabolic inhibition of the cells or to an effect on post-receptor mechanisms such as phosphatidylinositol 4,5-bisphosphate breakdown, intracellular free $[\text{Ca}^{2+}]$ modulation and/or

protein kinase C activation. It is also unlikely that the effects of the antagonist on these cells are mediated through substance P receptors (the other class of receptor with which the antagonist is known to interact; Jensen *et al.*, 1984). Substance P (1 nM–30 μ M) had no mitogenic activity on these cells (Fig. 1b), did not affect the stimulation of DNA synthesis by any combination of mitogens tested (Fig. 1b, and other results not shown), and did not prevent the inhibitory action of the antagonist against bombesin-induced stimulation (Table 1).

While examining the specificity of the antagonist, we noted that it consistently inhibited the stimulation of Swiss 3T3 cells by vasopressin (Table 1). Again, this inhibition is unlikely to be due to non-specific post-receptor effects of the antagonist, since the stimulations induced by platelet-derived growth factor and prostaglandin $\text{F}_{2\alpha}$ were not inhibited (Table 1). Rather, the antagonist appears to act in a competitive manner against the stimulation induced by vasopressin, as illustrated by a shift in the vasopressin dose-response curve (Fig. 2). The structural basis of this antagonism is not obvious from the sequences of the peptides: it would be interesting both to examine the effects of the antagonist on other vasopressin-induced cellular responses and to compare the actions of this antagonist with those of other vasopressin antagonists described previously (Jard, 1983; Manning & Sawyer, 1983).

The demonstration that [D-Arg¹,D-Pro²,D-Trp^{7,9},Leu¹¹]substance P acts as an antagonist of the bombesin-induced stimulation of DNA synthesis in Swiss 3T3 cells, as well as antagonizing bombesin-mediated amylase secretion from pancreatic acini (Jensen *et al.*, 1984), confirms its potential for use in studies of the interaction of bombesin with its receptors, and of the signalling mechanisms controlling the consequent intracellular responses. This antagonist will be especially useful in studies of cell-growth control, since very few specific antagonists of peptides promoting cell proliferation have been described, and bombesin is a very potent stimulator of the proliferation of Swiss 3T3 cells (Rozenfurt & Sinnott-Smith, 1983). A further use for the antagonist may be in the investigation of bombesin- or vasopressin-like bioactivity in tissue extracts and body fluids. Finally, bombesin stimulates the proliferation of various cell types other than Swiss 3T3 cells (Carney *et al.*, 1983; Willey *et al.*, 1984), and it has been suggested that bombesin-related peptides may function as autocrine factors in stimulating the growth of a variety of tumours that contain and may secrete these peptides (Moody *et al.*, 1983; Price *et al.*, 1985; Sporn & Roberts, 1985). It should be interesting to determine whether the growth of such tumours can be inhibited by exposure to the bombesin antagonist.

We thank Miss Diane Blakeley for valuable technical assistance, Dr. Brian Heap and Dr. Tony Flint for helpful comments, and Mrs. Janet Tickner for typing the manuscript. This work was supported by The Joint Research Board of St. Bartholomew's Hospital, London.

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Received 12 August 1985; accepted 6 September 1985