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A SUBSTANCE P ANTAGONIST ALSO INHIBITS SPECIFIC BINDING AND MITOGENIC EFFECTS OF VASOPRESSIN AND BOMBESIN-RELATED PEPTIDES IN SWISS 3T3 CELLS

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SUMMARY: While vasopressin and peptides of the bombesin family bind to different receptors in quiescent Swiss 3T3 cells, the antagonist [D-Arg^{1},D-Pro^{2},D-Trp^{7,9},Leu^{11}] substance P blocks the specific binding of both (^{3}H) vasopressin and ^{125}I-gastrin-releasing peptide to these cells. In addition, the antagonist inhibits the mobilization of Ca^{2+} and induction of DNA synthesis by vasopressin. These results indicate that [D-Arg^{1},D-Pro^{2},D-Trp^{7,9},Leu^{11}] substance P has the ability to interact with the receptors for three structurally unrelated peptide hormones.

The amphibian tetradecapeptide bombesin (1) is a potent mitogen for Swiss 3T3 cells (2). Mammalian peptides structurally related to bombesin including gastrin-releasing peptide (GRP) and the neuromedins (3-7), also stimulate DNA synthesis in these cells. These peptides interact with high-affinity receptors in Swiss 3T3 cells which are distinct from those of other mitogens for these cells (8). Following binding the peptides of the bombesin family elicit a complex array of biological effects, including stimulation of monovalent and divalent ionic fluxes (9), activation of protein kinase C and inhibition of ^{125}I-epidermal growth factor (^{125}I-EGF) binding (10). Both the binding of peptides of the bombesin family to their cellular receptors and all the biological effects elicited by these peptides are blocked by the novel bombesin antagonist, [D-Arg^{1},D-Pro^{2},D-Trp^{7,9},Leu^{11}] substance P (8-11). Since this is

Abbreviations: GRP, gastrin releasing peptide; SP, Substance P; EGF, epidermal growth factor; pmp^{1},O-Me-Tyr^{2},-[Arg^{8}]-vasopressin, [1-(γ-Mercapto-γ,γ-cyclopentamethylene propionic acid), 2-(O-methyl) tyrosine]-Arg^{8}-vasopressin.

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the only known antagonist for bombesin it may be useful for elucidating the physiological role of bombesin-related peptides. During the course of studies designed to examine the selectivity of its effects in Swiss 3T3 cells, we found that this antagonist also inhibits the mobilization of Ca^{2+} and induction of DNA synthesis by the neurohypophyseal hormone vasopressin. Our findings demonstrate that these effects are most likely due to the inhibition of binding of vasopressin to its receptor in Swiss 3T3 cells. Since vasopressin interacts with specific high-affinity sites (12) which are distinguishable from those for peptides of the bombesin family, it appears that the peptide [D-Arg^1,D-Pro^2,D-$Trp^{7,9}$,Leu^{11}] substance P can interact with various, independent, cellular receptors thereby preventing their biological effects.

MATERIALS AND METHODS

Cell culture procedures (13,14), assays of DNA synthesis by (^{3}H)-thymidine incorporation (14), measurements of ^{125}I-GRP (8) and (^{3}H)-vasopressin binding (12) and cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) using the fluorescent Ca^{2+} indicator quin-2 (15) were as described.

Materials: Arginine vasopressin (367 units/ml) and bovine serum albumin (essentially fatty acid and globulin free) were obtained from the Sigma Chemical Co. (St. Louis, Mo). GRP and [D-Arg^1, D-Pro^2, D-$Trp^{7,9}$,Leu^{11}]substance P were obtained from Bachem Fine Chemicals (Saffron Walden, Essex, U.K.). [1-(γ-Mercapto-γ,γ-cyclo-pentamethylene propionic acid), 2-(O-methyl) tyrosine]-Arg^8-vasopressin (Pmp^1, O-Me-Tyr^2-[Arg^8]-vasopressin) was from Peninsula Laboratories Inc., Belmont CA. Quin 2 and Quin 2-AME were obtained from Lancaster Synthesis (Morcambe, Lancs, U.K.). (^{3}H) vasopressin (50 Ci/mmol; 1Ci = 37 GBq) was from New England Nuclear (Boston, MA). ^{125}I-GRP (2000Ci/mmol) and Ca^{2+} were from the Radiochemical Centre (Amersham, U.K.).

RESULTS

Vasopressin binds to specific, high-affinity binding sites in Swiss 3T3 cells (12). The specific binding of [^{3}H] vasopressin to quiescent Swiss 3T3 cells was inhibited by unlabelled vasopressin in a concentration-dependent manner (Fig. 1A); the apparent Kd for ^{3}H-vasopressin binding was 18 nM. In contrast GRP had no effect on (^{3}H)-vasopressin binding at concentrations up to 1 μg/ml (360 nM). The reciprocal experiment showed that the specific binding of ^{125}I-GRP was inhibited by unlabelled GRP, but not by either [Arg^8] vasopressin at

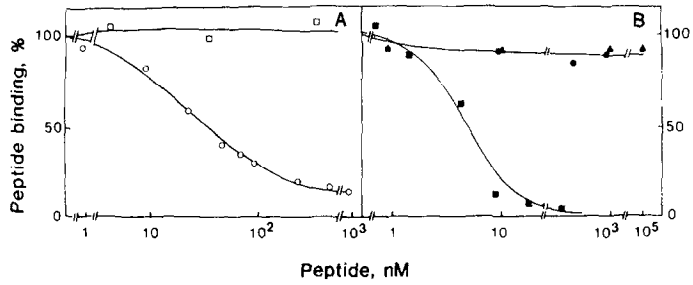

Figure 1 A. Effect of GRP and vasopressin on the specific binding of (^{3}H) vasopressin to Swiss 3T3 cells. Confluent and quiescent cultures were washed twice with Dulbecco's modified Eagle's (DME) medium and then incubated for 30 min at 37°C in the presence of (^{3}H) vasopressin (12nM) and in the presence of either GRP (□) or vasopressin (○) at the concentrations indicated. Each point represents the mean of four determinations (SEM < 20%). B. Effect of vasopressin, Pmp^1, O-Me-Tyr^2-[Arg^8]-vasopressin, and GRP on the specific binding of ^{125}I-GRP to Swiss 3T3 cells. Confluent and quiescent cultures of 3T3 cells were washed twice with DME medium and then incubated for 30 min at 37°C in medium containing ^{125}I-GRP (0.05 nM) and various concentrations of either vasopressin (●), Pmp^1, O-Me-Tyr^2-[Arg^8] vasopressin (▲), or GRP (■). Values shown represent the mean of duplicate determinations. All measurements shown in this and subsequent vasopressin and GRP binding experiments represent the percentage of the specific binding obtained in control cultures. Other experimental details in this and the subsequent figures were as described in Materials and Methods.

concentrations up to 0.92 μM, or by the specific vasopressin antagonist, Pmp^1, O-Me-Tyr^2-[Arg^8]-vasopressin, at concentrations up to 100 μM (Fig. 1B).

While the results shown in Figure 1 strongly suggest that vasopressin and peptides of the bombesin family bind to different receptors in Swiss 3T3 cells, the substance P (SP) analogue [D-Arg^1, D-Pro^2, D-Trp^{7,9},Leu^{11}]SP, (16) blocked the specific binding of both ^{125}I-GRP and (^{3}H) vasopressin to these cells. Fig. 2A shows that the antagonist markedly decreased the specific binding of a fully mitogenic concentration of (^{3}H) vasopressin (11nM) to quiescent 3T3 cells. The effect is concentration dependent, with half-maximal inhibition of binding occurring at 4.2μM. At the highest concentration of antagonist used (100 μM), (^{3}H) vasopressin binding was reduced to 11% of the control value. [D-Arg^1, D-Pro^2, D-Trp^{7,9},Leu^{11}]SP also inhibited ^{125}I-GRP binding within a similar concentration range (Fig.2B and ref.8). The K_i values for the inhibition of (^{3}H) vasopressin and ^{125}I-GRP binding by the antagonist were 2.6 μM and 2 μM respectively. Although Swiss 3T3 cells do not have

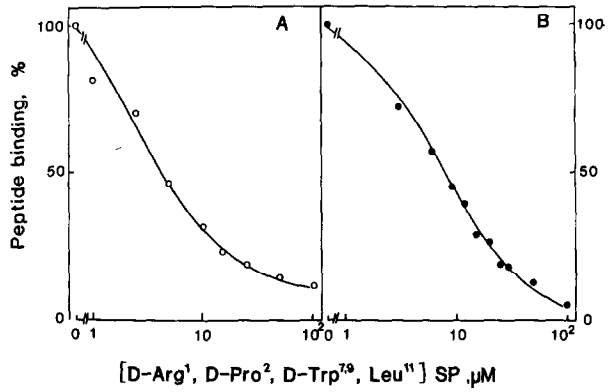

Figure 2 Inhibition of specific (^{3}H) vasopressin (panel A) and ^{125}I-GRP (panel B) binding to Swiss 3T3 cells by [D-Arg^{1},D-Pro^{2},D-Trp^{7,9},Leu^{11}]SP. Quiescent 3T3 cells were washed twice and then incubated at 37°C for 30 min in the presence of either (^{3}H) vasopressin (11nM; open circles) or ^{125}I-GRP (1.5nM; closed circles) in the presence of the concentrations of antagonist indicated.

receptors for SP (2,8) the antagonist has a somewhat higher (4-fold) affinity for SP receptors than for the bombesin receptors in pancreatic acinar cells (11).

Vasopressin and bombesin both cause the rapid mobilization of Ca^{2+} from an intracellular pool in quiescent Swiss 3T3 cells, as measured by $^{45}Ca^{2+}$ efflux and measurements of changes in cytosolic free calcium concentration ($[Ca^{2+}]_i$) (9,15,17,18). Figure 3 shows that bombesin (upper panel) and vasopressin (lower panel) caused an increase in $[Ca^{2+}]_i$ which occurred within 5 sec after the addition of peptides reaching a maximum in 15-30 sec. The effects were transient with $[Ca^{2+}]_i$ usually returning to control levels in 3-5 minutes. The stimulation of $[Ca^{2+}]_i$ by bombesin and vasopressin was markedly inhibited by [D-Arg^{1}, D-Pro^{2}, D-Trp^{7,9},Leu^{11}]SP (Fig. 3, right traces). The effect of the antagonist on bombesin and vasopressin-induced changes in $[Ca^{2+}]_i$ was selective, as similar changes produced by platelet-derived growth factor (PDGF) were not affected by [D-Arg^{1}, D-Pro^{2}, D-Trp^{7,9},Leu^{11}]SP at a concentration of 100 μM (9 and results not shown).

The mitogenic effect of vasopressin was strikingly inhibited by the addition of [D-Arg^{1},D-Pro^{2},D-Trp^{7,9},Leu^{11}]SP (Fig.4). The

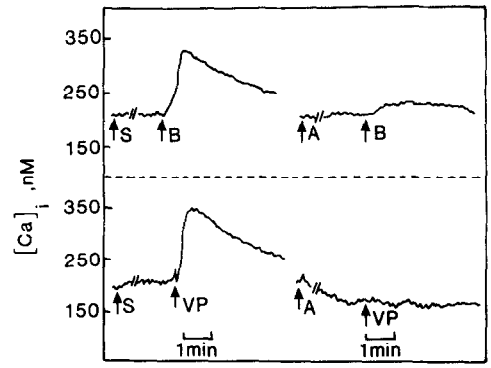

Figure 3 Effect of [D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]SP on the change in $[Ca^{2+}]_i$ caused by either bombesin (upper) or vasopressin (lower). Quiescent Swiss 3T3 cells on Cytodex 2 beads were washed and incubated with fluorescent indicator quin 2, as described in Materials and Methods. They were then suspended in 2 ml of an electrolyte solution, placed in the fluorimeter and stirred at 37°C. Fluorescence was recorded continuously. After a suitable control period, 75 μM [D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]SP (A) or an equivalent volume of solvent (S) were added. Three minutes later, either 3.1 nM bombesin (B) or 18.4 nM vasopressin (VP) were added and the fluorescence followed until it returned near control levels. In all cases, the measurements of $[Ca^{2+}]_i$ were performed after sequential addition of Triton X-100 and EGTA as described in Materials and Methods.

antagonist at a concentration of 100 μM markedly increased the concentration of vasopressin required to produce half-maximal stimulation of DNA synthesis (Fig.4 panel A). The fact that inhibition was completely reversed at higher concentrations of vasopressin demonstrates that the antagonist is not toxic to the cells. Fig. 4B shows the effect of increasing concentrations of antagonist on vasopressin-induced mitogenesis. At a concentration of 100 μM the mitogenic efect of a saturating concentration of vasopressin was reduced to only 5% of the control value.

In conclusion, the results presented here demonstrate that the tachykinin-related peptide, [D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]SP is a potent inhibitor of both early and late biological responses evoked by vasopressin in Swiss 3T3 cells. While this manuscript was in preparation Corps et al. reported that [D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]SP inhibited bombesin and vasopressin-induced mitogenesis (19), but did not investigate the mechanism of action of the antagonist. Our results demonstrate that these effects are most likely due to the interaction of the antagonist with specific receptors for vasopressin in 3T3 cells. Thus,

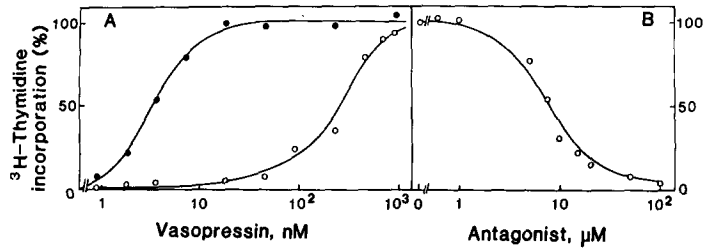

Figure 4 Inhibition of vasopressin-induced DNA synthesis in Swiss 3T3 cells by [D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]SP. Left: Quiescent cultures of 3T3 cells were incubated at 37°C in the presence of various concentrations of vasopressin and 1μg/ml insulin in either the absence (●) or presence (○) of 100 μM antagonist. Right: Quiescent 3T3 cells were incubated at 37°C in the presence of 18.4nM vasopressin, 1μg/ml insulin and the concentrations of antagonist shown. In both experiments, values are expressed as the percentage of the [^{3}H] thymidine incorporation obtained in the absence of [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}]SP. Each point represents the mean of duplicate determinations.

in addition to recognizing the receptors for both SP (16,20) and peptides of the bombesin family (8-11), [D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]SP is also an antagonist for vasopressin. Furthermore, this does not appear to be a property peculiar to this peptide since [D-Pro^4, Lys^6, D-Trp^{7,9,10}, Phe^{11}]SP another SP antagonist (21) also inhibited the binding and mitogenic activity of both bombesin and vasopressin (results not shown). The antagonist acts in a selective fashion as shown by the lack of effect on DNA synthesis and other early responses induced by other mitogens for Swiss 3T3 cells including PDGF, fibroblast-derived growth factor, EGF, insulin; phorbol-12,13 dibutyrate, and prostaglandin E_1 (8-10). This novel and interesting finding has several implications. Firstly, the fact that a peptide is recognized by three independent receptors suggests that significant similarities may exist between their binding sites. Secondly, both bombesin-related peptides and vasopressin are present in high levels in small cell carcinoma of the lung (SCCL) (21-27). Furthermore, recent findings suggest that bombesin-like peptides can act as autocrine growth factors for these cells (28,29), and ectopic production of vasopressin by SCCL is thought to be responsible for a number of clinical syndromes often associated with this important tumour (27). Thus, the identification of an antagonist which recognizes the receptors

for both peptides may be of interest in future clinical approaches to the treatment of SCCL.

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