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Research Article

The Adjuvant Effect of Bacitracin on Nasal Absorption of Gonadorelin and Buserelin in Rats

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Nasal absorption of gonadorelin (luteinizing hormone-releasing hormone; LH-RH) and buserelin, an LH-RH agonist, was studied in anesthetized rats. Administration of peptides was by nasal instillation of aqueous peptide/buffer solutions. Peptide absorption was monitored using different techniques: (a) by specific radioimmunoassays for serum levels of lutropin (LH), (b) by the cumulative urinary excretion of buserelin, and (c) by the ovulatory activity after nasal LH-RH and buserelin, respectively. Without adjuvant the nasal absorption of LH-RH and buserelin was relatively poor compared to subcutaneous or intravenous injection. Using absorption adjuvants of different types, e.g., sodium taurodihydrofusidate (STDHF) and bacitracin, marked increases in nasal absorption and, therefore, significant nasal adjuvant activity were found, as demonstrated by an increase in the biological response after nasal administration of the peptides. The mucosal compatibility of bacitracin at the concentrations used for enhancement of absorption was confirmed by an *in vitro* investigation using isolated gastric mucosa of guinea pigs as a test model.

KEY WORDS: nasal absorption; absorption adjuvants; bacitracin; sodium taurodihydrofusidate; absorption enhancers; gonadorelin; buserelin; mucosal tolerance.

INTRODUCTION

Among the problems presently associated with the delivery of peptides and proteins, the search for nonparenteral absorption routes plays an important role. Because of the digestive proteinase and peptidase activity of the gut, gastrointestinal absorption of many peptides is severely limited. Therefore, other mucosal absorption sites are currently being evaluated for peptide absorption, e.g., the rectal, vaginal, buccal, and even the ocular mucosa. The mucosa of the nasal cavity attracted great interest, mainly because of its relatively high permeability for peptides, and the nasal pathway is currently the route of choice for nonparenteral administration of peptides (1). A large number of peptides have been shown to pass the mucosa of the nasal cavity efficiently, e.g., enkephalin analogues (2), calcitonin (3), adrenal corticotropic hormone (ACTH) (4), and even polypeptides such as insulin (5) and proteins such as interferon (6).

Nevertheless, new problems arise from using this transmucosal route of administration. The low permeability of peptide permeation and marked mucosal peptidase activity require suitable absorption adjuvants of low local toxicity in order to improve the systemic uptake. In the literature various mucosal adjuvants have been reported (7–11). The mechanisms of enhancement suggested for these compounds are quite variable. Drug absorption can be influenced by opening aqueous pores by calcium ion chelation or by increasing the fluidity of the natural lipid bilayer membrane (12). Another class of enhancers such as oleic acid, linoleic acid, and Azone causes rapid and transient changes of the permeability of the mucosa. Cellular SH proteins may be involved in this change (13). More recently, special interest has been focused on inhibitors of peptidase activity, which is located in the mucus itself or on/within the mucosal cells (14). Finally, multiple mechanisms may operate simultaneously.

The focus of our studies is on gonadorelin (luteinizing hormone-releasing hormone; LH-RH) and buserelin, a highly active agonist analogue of gonadorelin. The nasal absorption of both peptides was studied in the presence or absence of bacitracin. Bacitracin is an antibiotic polypeptide complex produced by *Bacillus subtilis* and *B. licheniformis*. Commercial bacitracin is a mixture of at least nine bacitracin-type compounds (15). Bacitracin A—the main component—has a partly cyclic structure of alternating D and L amino acids. It is frequently used as a local antibiotic, with a therapeutic concentration of 0.5% (3.5×10^{-3} *M*). Furthermore, it is known to inhibit amino peptidase activity *in vitro* at the even lower concentration of 0.04% (2.9×10^{-4} *M*) (16). Its well-established local tolerance made it an excellent candidate as an absorption adjuvant. For comparison, sodium taurodihydrofusidate (STDHF) was used, which was previously shown to be a potent adjuvant (17–19).

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MATERIALS AND METHODS

Materials

Buserelin [D-Ser(Bu^t)^6-LH-RH(1-9)-nonapeptide-ethylamide; Hoe 766] and gonadorelin (LH-RH) were obtained from Hoechst AG (D-Frankfurt). Bacitracin (73,000 U/g) was from Sigma (D-München), and sodium taurodihydrofusidate was from California Biotechnology (US-Mountain View, Calif.).

Plasma Profiles of Lutropin (LH)

Male rats (Wistar) of 90–100 g were anesthetized by fractionated s.c. injections of urethane (ethylcarbamate, 0.7 ml/100 g, 25% solution). The animals were randomly assigned to groups of six to eight rats. Supplemental urethane was administered as necessary throughout the study.

For administration 2 μl of the aqueous peptide/buffer solution was instilled into the nose (1 μl into each nostril) using glass constriction pipettes inserted at about 6 mm into the nostril and emptied by gentle blowing. During this procedure, the rats were held by their neck, the floor of the nasal cavity being approximately horizontal. The volume administrated was small enough to exclude significant leakage into the GI tract. Even under the theoretical assumption of full leakage and GI absorption, the dose administered would have been too small to stimulate detectable LH release.

Blood (0.5 ml) was sampled periodically from the jugular vein. The loss of intravasal volume was compensated at appropriate intervals by the i.p. injection of isotonic saline (0.5 ml every hour).

The whole blood was centrifuged at 3000 rpm for 4 min; the plasma samples were stored at −20°C until assay. Plasma LH was determined by a rat LH radioimmunoassay (RIA) using reagents kindly provided by the National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases (NIADDK), Rat Pituitary Program (20).

Evaluation of Ovulation-Inducing Activity

Female rats (Wistar) of 50–60 g were primed with 10 IU pregnant mare serum gonadotropin (PMSG) s.c. on the first day of the study at 0900 hr. Following this priming, spontaneous ovulation would occur on day 3 between 1400 and 1600 hr due to estrogen priming of LH release. The rats were administered phenobarbital (4 mg/kg i.p.) on day 3 at 1300 hr to block this spontaneous ovulation (20). One hour later the test peptide, e.g., LH-RH or buserelin, dissolved in 1% gelatin-saline with or without bacitracin, was administrated nasally. This pretreatment restricts ovulatory stimulation to nasally administered LH-RH or buserelin, respectively.

On the next morning between 0900 and 1000 hr (day 4) the animals were sacrificed. The isolated oviducts were stained with patent blue and extended horizontally between two sheets of slide glass to observe the ova microscopically. The presence of more than one ovum with attached granulosa cells in the ampulla of the oviduct was regarded as a positive reaction.

Urinary Excretion of Buserelin

Male rats (Wistar) of 90–100 g were treated with 20 μg buserelin by nasal administration of 2 × 10 μl solution in each nostril using a 50-μl syringe connected to a special nasal cannula. The cannula consisted of a No. 18 needle (0.6-mm o.d.) shortened to a length of 6 mm and sheated with polyethylene tubing PE 50 to avoid trauma to the nasal mucosa. For determination of urinary excretion, the rats were placed in metabolism cages for 24 hr. Urine was sampled during the whole period. Excreted buserelin was measured by high-performance liquid chromatography (HPLC) and radioimmunoassay. The separation method and the assay were described earlier by Sandow *et al.* (21,22). The HPLC/RIA assay measures only intact buserelin (1–9) without interference from any metabolites.

Mucosal Compatibility Evaluated by Patent Blue Permeation Assay

The mucosal compatibility of bacitracin, the amphiphilic compounds sodium taurodihydrofusidate and sodium fusidate, and the bile salts sodium deoxycholate and sodium taurocholate was compared employing a method previously described by Wirth *et al.* (23), using isolated guinea pig gastric mucosa. For the test the mucosa was incubated for 90 min with a luminal solution (phosphate buffer, pH 7) containing the test compound or buffer alone (control). After a subsequent 1-hr luminal incubation of the mucosa with patent blue (300 μg/ml) in phosphate buffer, pH 7), the serosal patent blue concentration was determined. An increase in dye permeation indicates a loss of mucosal integrity due to loosening of epithelial tight junctions or damage to cells or cell membranes.

RESULTS

Bacitracin had significant adjuvant activity on the absorption of buserelin and LH-RH (Fig. 1), as demonstrated by the significant increase in serum LH in the animals at a dose which has only a slight effect without bacitracin when compared to the control level of untreated animals. Moreover, these results were supported by data showing increased ovulatory activity of a standard dose of LH-RH in

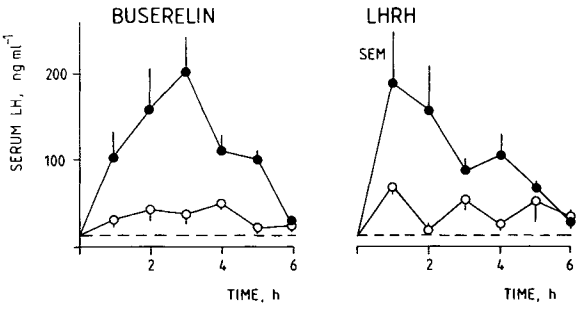

Fig. 1. Adjuvant effect of bacitracin on nasal absorption of buserelin and LH-RH in rats. Buserelin, 10 ng/rat i.n.; LH-RH, 800 ng/rat i.n. (○) No bacitracin; (●) 10^{-2} *M* bacitracin; (– – –) control. Each point represents the mean ± SE of six animals.

Table I. Ovulation-Inducing Activity of Nasal LH-RH: Adjuvant Effect of Bacitracin

Group No.	Treatment nasal	Dose per rat (μg)[a]	No. of rats ovulating	Serum LH (ng ml^{-1})	
				Mean	SE
1	Control	—	0/6	152.5	8.1
2	LH-RH	2	2/6	225.4	22.4
3	LH-RH	4	3/6	331.6	57.2
4	LH-RH	8	6/6	757.0	93.0
5	+ Bacitracin[b]	0.5	1/6	372.6	60.5
6	+ Bacitracin[b]	1	3/6	372.0	53.9
7	+ Bacitracin[b]	2	5/6	714.4	168.1

[a] Fifty- to sixty-gram rats primed with 10 IU PMSG.
[b] Bacitracin, 10^{-2} *M* (14 mg ml^{-1}); application volume, 2 μl.

juvenile rats, when bacitracin was added to the nasal solution (Table I). The data show that with a dose of 2 μg LH-RH per rat without bacitracin, only two of six animals ovulated, whereas the same dose of LH-RH with bacitracin induced ovulation in five of six animals. Without bacitracin, a dose of 4 to 8 μg LH-RH per rat was needed to achieve the same rate of ovulation. To verify these data, indicating increased absorption, blood samples were taken 1 hr after nasal administration of the LH-RH solution. The mean serum LH level of the animals treated with 2 μg LH-RH without adjuvant was 225.4 ± 22.4 ng/ml. In contrast, animals treated with the same dose in 10^{-2} *M* bacitracin solution exhibited a significantly higher serum LH level, 714 ± 168.1 ng/ml.

Additional support for increased nasal absorption in the presence of bacitracin was obtained by monitoring the urinary excretion of intact buserelin within 24 hr of nasal treatment. The urinary buserelin excretion of the animals treated with a dose of 20 μg buserelin without adjuvant was 453.27 ± 198.1 ng/24 hr, whereas the addition of bacitracin to the nasal solution led to a buserelin excretion of 1046.7 ± 35.1 ng/24 hr.

Further enhancement of nasal absorption is achieved by increasing the concentration of bacitracin added to the peptide. The results (Fig. 2) demonstrate that the step-by-step increase in the bacitracin concentration from zero to 10^{-3}, 10^{-2}, and 5×10^{-2} *M* resulted in an impressive enhancement of serum LH in the animals, by more than one order of magnitude.

For comparison, the enhancement by another potent absorption adjuvant (sodium taurodihydrofusidate) was also investigated (17–19). It was confirmed that the fusidate is indeed an efficient absorption adjuvant for buserelin and LH-RH as demonstrated by the significant elevation of the serum LH levels in contrast to the profiles without the adjuvant (Fig. 3).

In order to compare the enhancement of buserelin activity exerted by bacitracin and sodium taurodihydrofusidate, the pharmacodynamic profiles of LH release were evaluated by calculating the area under the curves (AUC) with and without adjuvant. The ratios of the AUCs with and without adjuvant are indicative of the adjuvant capacity. The effect of bacitracin ranged from a 1.8-fold pharmacodynamic increase with 10^{-3} *M* bacitracin to 2.8-fold with 10^{-2} *M* bacitracin and 9.2-fold with 5×10^{-2} *M* bacitracin. The addition of 1% fusidate to a nasal solution containing buserelin re-

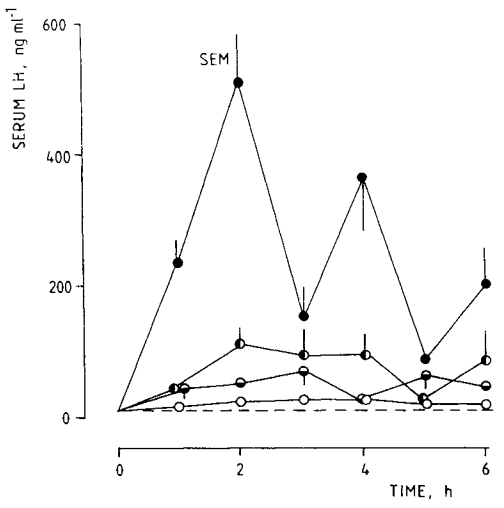

Fig. 2. Dose-dependent adjuvant effect of bacitracin on nasal absorption of buserelin in rats. Buserelin dose was 10 ng/rat i.n. (○) No bacitracin; (◒) 10^{-3} *M* bacitracin; (◐) 10^{-2} *M* bacitracin; (●) 5×10^{-2} *M* bacitracin; (– – –) control. Each point represents the mean ± SE of six animals.

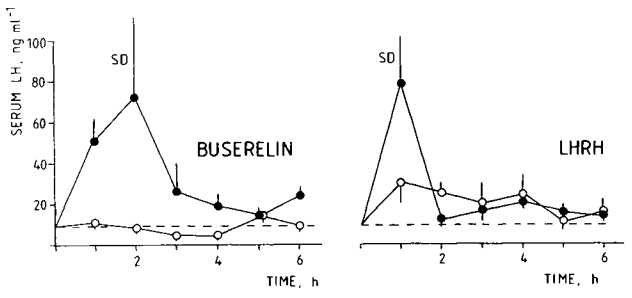

Fig. 3. Adjuvant effect of sodium taurodihydrofusidate (STDHF) on nasal absorption of buserelin and LH-RH in rats. Buserelin, 10 ng/rat i.n.; LH-RH, 800 ng/rat, (○) No adjuvant; (●) + STDHF, 1%; (– – –) control. Each point represents the mean ± SD of six animals.

Table II. Mucosal Compatibility of Test Compounds as Indicated by Patent Blue Permeation Through Isolated Guinea Pig Gastric Mucosa

Compound	Concentration (mg ml^{-1})	Serosal patent blue concentration (μg ml^{-1})	SD	N	Increase vs control
Control	—	0.79	±0.2	25	—
STDHF	3	7.66	±2.77	20	+865%
Fusidic acid[a]	3	10.59	±3.57	12	+1235%
Taurocholic acid[a]	3	2.36	±0.89	10	+198%
Deoxycholic acid[a]	3	11.28	±3.04	24	+1322%
Bacitracin	10	0.75	±0.20	18	0%

[a] Sodium salt.

sulted in a 4.7-fold increase in the biological activity of buserelin.

The mucosal compatibility of bacitracin was compared to those of amphiphilic compounds in the isolated guinea pig gastric mucosa (Table II). Sodium taurodihydrofusidate, sodium fusidate, and sodium deoxycholate caused a marked increase in patent blue permeation at a concentration of 3 mg/ml, indicating loss of mucosal integrity. The effect of sodium taurochloate was much weaker. Bacitracin, 10 mg/ml, equivalent to 7×10^{-3} *M*, however, had no effect on patent blue permeation, indicating preservation of the mucosal structure.

DISCUSSION

In order to improve the nasal absorption of peptides, various adjuvants have previously been tested and reported to be effective. One of the main screening criteria for adjuvants is the mucosal compatibility of such agents.

Recently Longenecker *et al.* (17–19) were able to demonstrate the potent adjuvant effect of sodium taurodihydrofusidate (STDHF). In spite of its marked chemical similarity to bile salts, this adjuvant was shown to be much more biocompatible than bile salts. Several hypothetical mechanisms for its adjuvant activity were proposed: (a) its tensioactive behavior, (b) the formation of a peptide/adjuvant complex, and (c) the inhibition of local aminopeptidase activity on and/or in the nasal tissue. Our comparison of STDHF and bacitracin showed that the adjuvant activities of both compounds in rats were roughly the same. It is noteworthy that, after simultaneous administration of both adjuvant and peptide, the maximum adjuvant effect of STDHF on the absorption of (a) LH-RH was already reached after 1 hr and that on (b) buserelin after 2 hr. Immediately after the peak value there was a steep decrease to normal LH levels. In contrast, the adjuvant effect of bacitracin with both LH-RH and buserelin was sustained for about 5 hr.

Our data demonstrate that the adjuvant effect of bacitracin is dose dependent. Concentrations as low as 10^{-3} *M* bacitracin showed marked nasal adjuvant activity compared to a treatment without bacitracin. Increasing concentrations, i.e., 10^{-2} and, finally, 5×10^{-2} *M* bacitracin, further enhanced the plasma LH levels. The practical limits for the adjuvant effect of this compound will be certainly set by its aqueous solubility and local compatibility.

Complete preservation of the mucosal structure after incubation with bacitracin was shown in the patent blue permeation assay. In contrast, the amphiphilic compounds sodium taurodihydrofusidate, sodium fusidate, and deoxycholate caused a marked increase in patent blue permeation, indicating a loss of mucosal integrity that could be due to loosening of epithelial tight junctions or damage to cells or cell membranes. Thus, the adjuvant effect of the latter compounds could be attributed at least partially to an impairment of the mucosal barrier. Since the mucosal structure is preserved after bacitracin, a more specific mode of adjuvant action can be postulated for this compound. Similar to the erythrocyte hemolysis test (18), the patent blue permeation assay using gastric mucosa is an attempt to develop meaningful tests as a prerequisite to get unequivocal evidence for nasal mucosal compatibility of absorption adjuvants. A full mechanistic interpretation of the adjuvant activity of bacitracin cannot yet be given.

Bacitracin is commonly administered as a local antibiotic, e.g., in eye drops, ointments, medicinal powders, and sprays for surgical use. Consequently there is a large body of practical experience about its mucosal toxicity and compatibility. When applied topically, sensitivity reactions to the antibiotic are rare even following prolonged administrations (15). At a concentration of about 10^{-1} *M*, bacitracin was found to interfere with the ciliary action of chicken embryo tracheas (24). However, this concentration was 1–2 orders of magnitude higher than the concentration applied here.

Bacitracin is poorly absorbed from mucous membranes. Application of bacitracin to intact skin or mucosae produces no systemic reactions because of an almost-complete lack of absorption of the antibiotic from these sites. It is recommended that a daily local dose of 100,000 IU, i.e., 1.4 g bacitracin, should not be exceeded, because higher doses could lead to undesired absorption (25). There are no human studies as yet on the absorption rate after nasal administration of bacitracin doses in the range of this arbitrary limit of 1.4 g, and the extent of absorption from other mucosal sites has not been investigated.

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