Dissociation of the Antimicrobial Activity of Bacitracin USP from its Renovascular Effects

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Bacitracin is a nephrotoxic antibiotic that has recently been shown to induce contractile effects in aortas isolated from rabbits by stimulating receptors for 5-hydroxytryptamine (5-HT). The possible renovascular actions of this antibiotic were investigated. Bacitracin USP increased the vascular resistance in a concentration-dependent manner (9 to 175 μg/ml) in rat kidneys perfused with a constant flow of Krebs solution. This was significantly inhibited by 5-HT antagonists, but only partially at the higher bacitracin concentration. An antagonist of the chymotryptic peptide fMet-Leu-Phe failed to influence the pressor effect of bacitracin in rat kidneys. Indomethacin modestly reduced the effect of all potent pressor agents in the rat organ. Bacitracin USP was separated in several fractions by using C18 reverse-phase chromatography. Two distinct fractions were vasoconstrictive when infused in rat kidneys; both fractions were 5-HT mimetics. These peaks were different from the major antibiotic peak, bacitracin A, which was identified by using analytical high-pressure liquid chromatography, mass spectrometry, and inhibition of Micrococcus luteus growth. The less polar vasoactive peak corresponded to at least two minor peptides of the bacitracin family. The most abundant of these vasoactive peptides had no direct contractile effect on an aorta isolated from a rabbit, but a preliminary metabolic study in rat kidneys suggests that it is apparently transformed into a potent 5-HT agonist that is active on the aorta preparation. Bacitracin A, the major constituent of bacitracin with antimicrobial activity, had no vasoconstrictor effect in the test systems that we used; however, we did rule out the possibility that the renovascular stimulants found in the bacitracin mixture do not derive spontaneously or by biotransformation from the antibacterial forms of bacitracin.

Bacitracin is a polypeptide antibiotic that was isolated from Bacillus subtilis cultures in 1943 (20). It is believed that commercial bacitracin is a mixture of 9 to 22 different types of molecules, most of which are uncharacterized (6, 19). Bacitracin A is the chief constituent and the major antibiotic of the mixture. It is inhibitory to many gram-positive and some gram-negative microorganisms, and resistance to its antibacterial activity or allergy to this antibiotic rarely develops. However, bacitracin is rarely used systematically owing to its nephrotoxicity, and it is generally reserved for topical uses. Following intramuscular injection, albuminuria, cellular cylindruria, and azotemia have been reported to occur. Tubular and glomerular necrosis have been documented in patients with bacitracin-induced nephrotoxicity (16, 20). Other side effects include nausea and vomiting, pain at the site of injection, and skin rashes (16, 20).

It is perhaps not surprising that the numerous constituents of bacitracin exhibit a large array of pharmacologic activities. Commercial bacitracin preparations interfere with dihydropyridine-binding sites (24) and with certain types of receptor-mediated events observed in vitro (5, 8, 23). The inhibitory effect of bacitracin on a protease termed insulinase has been assigned to one of the minor components (17). The peptide antibiotic is an agonist of receptors for fMet-Leu-Phe on human neutrophil leukocytes, eliciting a chomotactic response and competing for the binding of a triated form of fMet-Leu-Phe on these cells (1). We have unexpectedly observed that bacitracin USP is a consistent contractile agent in isolated vascular smooth muscle preparations. A pharmacologic analysis of bacitracin-induced contraction of an aorta isolated from a rabbit was undertaken (7) and showed that the antibiotic is a 5-hydroxytryptamine (5-HT; serotonin)-mimetic agent that was competitively antagonized by ketanserin and methysergide. The 5-HT2 type of serotonin receptors, which were present in the aorta preparation, was involved in this action.

The study described here was undertaken to verify the hypothesis that bacitracin can exert a vasoconstrictor effect in kidneys at concentrations that can be theoretically obtained in vivo during systemic use of the drug. Furthermore, we tested whether the renal vasoconstrictor effect of bacitracin could be related to its known capacity to stimulate 5-HT of fMet-Leu-Phe receptors (1, 7). This would be plausible because 5-HT is a well-known renal vasoconstrictor (10, 12), and fMet-Leu-Phe also has the capacity to elicit vasomotor effects in several experimental systems (3, 14, 15). Finally, a fractionation of bacitracin USP was attempted in order to separate candidate vasoactive agents from the antibacterial constituents in the mixture. Ultimately, this line of investigation may shed some light on the mechanisms of the renal nephrotoxicity of bacitracin.

MATERIALS AND METHODS

Perfused kidneys. The function of renal resistance vessels (arterioles) has been evaluated in a simple model of perfusion with constant flow (2). The organs were obtained from male Sprague-Dawley rats (weight, 400 to 600 g). The perfusion fluid was warmed (37°C) and oxygenated (95% O2, 5% CO2) Krebs solution (containing the following, in millimolar concentrations: NaCl, 117.5; KCl, 4.7; KH2PO4, 1.2; MgSO4, 1.18; CaCl2, 2.5; NaHCO3, 25.0; glucose, 5.5). Rat kidneys were perfused in situ through the renal artery with...
0.9% saline containing 100 U of heparin per ml in animals that were killed by decapitation immediately before the perfusion. The kidneys were then isolated by cutting the aorta, the renal vein, and the ureter. The organs were placed in a humidified chamber and perfused at a constant flow (4 ml/min) with a roller pump (Piper). The perfusion pressure was recorded on a Harvard polygraph with a pressure transducer (model EM751; Elcromatic Ltd., Glasgow, United Kingdom) from a side arm placed between the pump and the organ.

The perfusion pressure of the preparations was allowed to equilibrate for 15 min. Agonist drugs, which were dissolved in saline, were added at a rate of 0.07 ml/min to the perfusion fluid, close to the kidney, with a Sage syringe pump (model 341A). The duration of the infusion was determined by the time course of the response, but it was usually 10 min or less. A 5- to 10-min washout period before the next challenge was then allowed to permit the return of the perfusion pressure to the baseline level.

Concentration-effect curves for the effects of bacitracin, 5-HT, and fMet-Leu-Phe on the perfusion pressure were constructed by perfusing three increasing concentrations of each agent in different groups of organs. In other experiments, an inhibitory drug was present in the Krebs solution for the duration of the experiment, in order to dissect out the mechanism of bacitracin-induced renovascular responses. In all experiments, the last drug perfused was norepinephrine (1.04 μM), a potent vasoconstrictor that served as a control pharmacologic response and an internal standard for the viability of each preparation.

Fractionation of bacitracin USP and bioassays of fractions. A preparative high-pressure liquid chromatography of bacitracin USP (400 mg) was performed by using a Michel-Miller column (24.5 by 300 mm) filled with Vydac 15-20 μ C18 reverse-phase resin. The mobile phase was a 5 to 45% gradient of acetonitrile in water containing 0.05% trifluoroacetic acid. The flow rate was 6 ml/min, and the antibiotic mixture was eluted in 800 ml of mobile phase that was divided into 10-ml fractions. The fractions were subsequently pooled by groups of five and were lyophilized. Comparative chromatograms were obtained by analytical high-pressure liquid chromatography (HPLC) by using a Waters system consisting of a model M600 controller, two model 510 pumps, a model 486 absorbance detector set at 210 nm, and a model 746 data module. The separation was achieved on a Vydac reverse-phase C18 column (3.9 by 300 mm) with a linear gradient of 2 ml of 5 to 65% solvent B per min in 20 min (solvent A, 0.05% trifluoroacetic acid and water; solvent B, acetonitrile with 0.05% trifluoroacetic acid). Samples of bacitracin USP or of some of the pools were analyzed by HPLC. Purifications of fractions of interest were also performed for chemical and biological characterization.

Most of the pools were redissolved at a concentration of either 10 or 1 mg of solid powder per ml of saline. For fraction numbers under 26 and above 60, for which there was a very low amount of solid residue, 1 ml of saline was used to dissolve the material in each pool of five fractions. The dissolved, pooled fractions were applied to three types of bioassay: the perfused rat kidney (as described above), the aorta isolated from a rabbit (7), and an antibiogram based on a susceptible microorganism, M. luteus.

Strips of rabbit aortas were prepared and incubated in Krebs solution as described previously (7). A 100-μl aliquot of the pooled fractions (10 mg/ml or less) was simply injected into the 5-ml tissue baths (final concentration, 200 μg/ml for fractions 26 to 60; the final concentrations were less for the other pools). Contraction tensions were recorded and are expressed in grams. Each pool was tested in at least two different tissues shown to be responsive to crude bacitracin USP (200 μg/ml) (7). Rat kidneys were perfused with the fraction pools (1 mg/ml or less; final concentration, 17.5 μg/ml or less) as described above. Duplicate determinations were obtained for different organs that were otherwise shown to be responsive to norepinephrine. A locally isolated strain of M. luteus was obtained from a hospital laboratory. The antibiogram was performed by the disk diffusion technique (18) on Mueller-Hinton medium (Difco, Detroit, Mich.). Ten microliters of fraction pools (1 mg/ml or less) were blotted onto filter paper disks (diameter, 5 mm) and were allowed to dry for 15 to 20 min, and the disks were applied to the culture medium. The petri dishes were incubated at 37°C overnight, and the antibiotic activity was quantified as the radius of growth inhibition around the disks. Each fraction pool was tested in triplicate, and the results are expressed as the mean radius ± standard error of the mean. Under these conditions, unfrac- tionated bacitracin USP exhibited an activity of 12.7 ± 0.3 mm for a 1-mg/ml solution and 14.7 ± 1.3 mm for a 10-mg/ml solution (n = 3). 5-HT (1 mg/ml) was inactive in this assay.

The presence of 5-HT and of the related molecules 5-hydroxytryptophan and 5-hydroxyindole acetic acid in vasoactive fractions of bacitracin has been tested by using an HPLC analysis coupled with electrochemical detection (9). The sensitivity of the assay is better than 2 ng/ml, and it is highly linear in the range of 0.02 to 5 ng of standard applied to the column for 5-HT, 5-hydroxytryptophan, and 5-hydroxyindole acetic acid. High-resolution mass spectrometry was performed on selected purified fractions of bacitracin by Michael Evans (Université de Montréal).

Drugs. Bacitracin USP, in the form of the sterile powder, was from Upjohn (Don Mills, Ontario, Canada). Ketanserin tartrate was a gift from Janssen Pharmaceutica (Beerse, Belgium). fMet-Leu-Phe, its antagonist Boc-Phe-d-LeuPhe-d-Leu-Phe (BPLLP) (1), 5-HT (carnitine sulfate complex), norepinephrine hydrochloride, and indomethacin were purchased from Sigma (St. Louis, Mo.).

Indomethacin and fMet-Leu-Phe were dissolved in 0.1 M Na2CO3, and BPLLP was dissolved in dimethyl sulfoxide. The maximum concentration of these solvents at the tissue level was 0.02%. Other drugs were dissolved in 0.9% saline.

**RESULTS**

**Effects of bacitracin USP and 5-HT on kidney perfusion pressure.** In rat kidneys, bacitracin is a concentration-dependent vasoconstrictor, as assessed by the increased perfusion pressure (Fig. 1 and 2). The threshold concentration was about 9 μg/ml, but the amplitude of the effects at higher concentrations was important.

For comparison, the effects of 5-HT and fMet-Leu-Phe were determined in other sets of kidneys (Fig. 2). 5-HT was a potent vasoconstrictor. The chemotactic peptide fMet-Leu-Phe was a significant vasoconstrictor only when it was used at a high concentration (1 μM) in rat kidneys (Fig. 2). Nevertheless, the amplitude of the effect of this peptide was important at this concentration.

**Effect of inhibitory drugs on bacitracin-induced renovascular responses.** Inhibitory drugs were added to the Krebs solution for the whole perfusion period in groups of rat kidneys in order to analyze the mechanism of the vasomotor effects (Fig. 2). Ketanserin and cyproheptadine, which were used at a concentration (100 nM) sufficient to abolish the
FIG. 1. Tracing of perfusion pressure of a rat kidney as a function of time. Bacitracin USP (BAC) or norepinephrine (NE) was infused during the periods indicated by the horizontal bars. The concentrations of the agents are indicated in micrometers per milliliter.

Effect of 5-HT, reduced significantly the pressor effect of bacitracin. However, the antagonist effect of cyproheptadine or ketanserin was not complete when the compounds were tested against a bacitracin concentration of 175 µg/ml. The fMet-Leu-Phe antagonist BPLLP had no effect against bacitracin at a concentration that reduced extensively the pressor effect of fMet-Leu-Phe. There was a consistent trend, suggesting that indomethacin inhibited partially all intense pressor responses in rat kidneys, with statistical significance being reached for the highest concentrations of 5-HT and fMet-Leu-Phe. Additionally, indomethacin significantly potentiated the effect of the lowest 5-HT concentration tested.

Fractionation of bacitracin USP. Pools of the fractions obtained by preparative chromatography of bacitracin USP were tested in three bioassays in order to resolve some of the multiple bioactivities present in the antibiotic mixture (Fig. 3). An analytical high-pressure liquid chromatogram of unfraccionated bacitracin is shown in Fig. 4A; it was obtained by using a resin and a mobile phase similar to those involved in the preparative chromatogram. The inhibitory activity against M. luteus was distributed in two wide areas of the preparative chromatograms: in fractions 26 to 45 and in fractions 56 to 65. The contractile activity for the isolated aorta was present essentially in fractions 11 to 25, which contained very little peptide and accounted for a very small fraction of the total weight. We obtained evidence that the vasoactive substance in this more polar peak was a 5-HT mimetic agent. Pretreatment of the rabbit aorta preparations with cyproheptadine (100 nM) prevented completely the contractile effect of this polar peak. However, 5-HT itself or the related molecules 5-hydroxytryptophan and 5-hydroxyindole acetic acid could not be detected in fractions 1 to 30 by using a very sensitive chromatographic method coupled with electrochemical detection (9).

Two peaks of activity were identified by using the rat perfused kidney. A vasoconstrictor substance exhibited a chromatographic profile very similar to the one recorded for the contractile activity in the aorta preparation. At least one other vasoactive substance appeared to be present in fractions 46 to 65.

Analytical high-pressure liquid chromatograms were obtained for the most active pools of this less polar area (fractions 51 to 55, Fig. 4B; fractions 56 to 60, Fig. 4C). At least three minor peaks were present in this area. The peak in fractions 51 to 55 was characterized further. In the perfused kidney test, this fraction exerted a pressor effect (+4 ± 1 mm Hg [1 mm Hg = 133.32 Pa] at 3.5 µg/ml; +14 ± 10 mm Hg at 35 µg/ml; means of duplicate determinations on different organs) that was reduced by cyproheptadine (100 nM) coinfused into two other organs (pressor responses of 0 and 2 ± 2 mm Hg, respectively; n = 2). The peak in fractions 51 to 55 exhibited a molecular weight of 1,419 by mass spectrometry. A more abundant fraction that migrated slightly later (the major peak in Fig. 4C) was also purified to homogeneity and also exhibited a molecular weight of 1,419 by mass spectrometry.

The major peak of the unfraccionated chromatogram (Fig. 4A), which was assumed to be bacitracin A, could be recovered in a pure form in fractions 32 and 33. These fractions had no vasoactive properties but inhibited the growth of M. luteus (radius of 10 to 12 mm when 1-mg/ml solutions were tested). Their identities were confirmed by mass spectrometry (molecular weight, 1,423) (6).

There was an apparent discrepancy between the vasoactive behaviors of bacitracin fractions in the aorta and renal assays (Fig. 3B and C). The aorta bioassay was apparently refractory to components that were vasoconstrictors in the rat kidneys and that were recovered in fractions 46 to 65. We tested the hypothesis that the peptide(s) of the bacitracin family could be metabolically transformed into a 5-HT...
FIG. 3. Bioassays of fraction pools generated by the preparative chromatography of bacitracin USP. The inhibition of M. luteus growth is expressed as a radius (in millimeters), the contractile effect on the rabbit isolated aorta is expressed in grams of tension, and the pressor effect on the perfused rat kidney is expressed in millimeters of mercury. Values are the means of two (B and C) or three (A) determinations. Vertical bars in panels A and C are standard errors of the means.
DISCUSSION

Rat kidneys were found to respond by an increased perfusion pressure, corresponding to an increased renovascular resistance, to bacitracin USP. The threshold concentration for this effect was similar to the concentration determined for bacitracin-induced contraction of the rabbit aorta strip (7). However, the mechanism of the vasomotor effect in the rat kidney appears to be more complex than that reported for the aorta preparation. Bacitracin USP behaved as a pure 5-HT mimic in the rabbit aorta (7), whereas the 5-HT competitive antagonists cyproheptadine and ketanserin (21) prevented completely the response to bacitracin at low concentrations (8.8 or 35 μg/ml) in rat kidneys and prevented the response only partially at the higher concentration (175 μg/ml). The latter concentration appears to be above the in vivo levels achievable following the intramuscular injection of 200 to 400 mg of bacitracin in humans, a formerly accepted practice (11). However, the smaller but consistent vasoconstrictor effects of the drug at 8.8 and 35 μg/ml were observed in a possible range of concentrations in tissue, assuming a distribution of the peptide antibiotic in extracellular water.

A 5-HT-mimetic agent that is active on the rabbit aorta preparation coeluted with the more polar renal vasoconstrictor constituent in the chromatographic fractionation of bacitracin. In this region of the chromatogram, there was very little peptide content on the basis of the absorbance readings and the appearance of the lyophilized fractions. Therefore, this 5-HT-mimetic agent appears to be a very small fraction of the total weight of bacitracin USP and, apparently, is not 5-HT itself. The picture is further complicated by the fact that one or more peptides less polar than bacitracin A, but also from the bacitracin family (apparent molecular weight, 1,419), also behaved as a 5-HT-mimetic agent in the rat kidney but not in the rabbit aorta preparation. This peptide(s) is different from bacitracins A, B, and F (molecular weights, 1,423, 1,409, and 1,407, respectively) (6). It is possible that this peptide(s) could be cleaved under the effect of an enzyme(s) present in the kidney preparation, but not in the aorta preparation, to liberate a low-molecular-weight 5-HT-mimetic agent. This hypothesis is supported by the results of a preliminary experiment reported above in which the effluent of the renal vein was transferred into a tissue bath. It has been noted previously that the substituted thiazole ring of bacitracins exhibits structural resemblance to 5-HT and to some synthetic 5-HT agonists (7). A low-molecular-weight fragment that would include such a structure could be a vasoactive constituent that is both present in crude bacitracin and that is releasable from certain forms of the peptide antibiotic.

The effect of 5-HT on the renal circulation in the rat is well documented (4, 10, 13, 22). This amine is a potent and selective renal vasoconstrictor when it is injected by the parenteral route. The afferent arterioles of the glomeruli are contracted at doses that do not affect the general circulation, leading to a fall in the glomerular filtration rate (10). Rat mesangial cells are also stimulated by 5-HT (13). The 5-HT2-type of receptor is involved in the vasoconstrictor effect of 5-HT in rat perfused kidneys (4). Administration of large or repeated doses of 5-HT to rats results in ischemic renal cortical necrosis, and it is remarkable that other organs are not affected (10). This pattern of toxicity shows some similarity to the one reported for bacitracin in humans (16), supporting a possible 5-HT receptor-mediated mechanism. In addition, other bacitracin side effects, such as pain at the
site of injection and an adverse effect on gastrointestinal motility, are also reminiscent of 5-HT pharmacology.

The relationship between prostaglandins and bacitracin effects in the perfused kidneys did not appear to be specific for each stimulus. In the rat kidney, all intense pressor stimuli were depressed by indomethacin, suggesting the release of contractile prostanoids in response to high concentrations of any of the vasoconstrictors. In addition, indomethacin potentiated a low-intensity stimulation with 5-HT in rat kidneys, suggesting additional types of interaction between cyclooxygenase products and this amine. 5-HT is known to release vasodilator prostaglandins from isolated rat mesangial cells (13), a finding that is possibly related to the observations described here. Indomethacin did not modify the contractile effect of bacitracin on the rabbit aorta preparation (7).

The antagonist of fMet-Leu-Phe, BPLLP, failed to modify the renovascular action of bacitracin. In other respects, it is interesting that an immunological stimulus like fMet-Leu-Phe is active on the kidney circulation. This peptide also contracts coronary and umbilical arteries isolated from humans (3, 15). However, a large concentration of fMet-Leu-Phe is required to influence the perfused kidney, and in addition, bacitracin retains only a small fraction of the relative potency of fMet-Leu-Phe as a receptor agonist (1). This may explain why fMet-Leu-Phe receptor stimulation does not account for a significant fraction of the effect of bacitracin in the concentration range tested. The 5-HT-like actions of bacitracin on the renal vasculature might be important for explaining the nephrotoxic action of this drug, but additional mechanisms likely play a role; a component of the pressor effect of bacitracin at the very high concentration of 175 µg/ml in the rat kidney was resistant to all inhibitory drugs tested. The chromatographic fractionation did not indicate vasoactive peaks that were not 5-HT mimetics, possibly owing to dilution (the maximal fraction concentration tested was 17.5 µg/ml).

Results of the present study suggest that bacitracin A, the major antibiotic in bacitracin USP, has no vasomotor action. A pharmacologically refined preparation of the antibiotic without the two major vasoactive fractions might be worth testing in vivo and might lead to a “rehabilitation” of the system to use of bacitracin for specific indications. However, it has not been ruled out that the vasoconstrictor fractions can derive metabolically from bacitracin A. In this case, pretreating the subject with a 5-HT antagonist may be an alternate strategy to alleviate the nephrotoxicity of bacitracin.

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