resulted in the altered ontogeny of sex-differentiated drug and xenobiotic metabolizing enzymes. Neonatal chlordecone altered the imprinting of UDP-glucuronyltransferase (defeminization) and caused induction of cytochrome P-450 content, benzo[*a*]pyrene hydroxylase and glutathione *S*-transferase.

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Structure–activity evidence against opiate receptor involvement in Leu^5-enkephalin-induced pulmonary vasoconstriction

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The recognition that endogenous opioids may play an important role in the cardiovascular system [1] has led to attempts to identify the receptors mediating such actions. Opioid-mediated cardiovascular responses usually are blocked by naloxone [1–4], thereby implying opiate receptor involvement. More recent evidence suggests that these receptors may be of the δ subtype [5–7]. However, there are some instances in which naloxone is ineffective, thus raising the possibility that non-opiate receptors may be involved. Dean *et al.* [8] have shown that inhibition of nicotine-induced catecholamine release from adrenal chromaffin cells by Leu^5-enkephalin is not antagonized by naloxone. Similarly, neither morphine-induced tachycardia in conscious squirrel monkeys [9] nor systemic hypertension induced in conscious dogs by des-Tyr^1-D-Ala^2-Leu^5-enkephalinamide is blocked by naloxone [10]. We have demonstrated recently that pulmonary vasoconstriction provoked by Leu^5-enkephalin in isolated rat lungs is not blocked by either naloxone or naltrexone, nor is it mimicked by morphine [11]. To more firmly establish the non-opiate nature of the receptor mediating Leu^5-enkephalin-induced pulmonary vasoconstriction, this report examines in isolated rat lungs the pulmonary vasoactivity of several cogeners of Leu^5-enkephalin as well as a number of smaller fragments of the pentapeptide. In addition, we have evaluated the inhibitory effects of diprenorphine on responses to Leu^5-enkephalin in an effort to rule out a role for δ opiate receptors.

Methods

Leu^5-enkephalin, Met^5-enkephalin, D-Ala^2-D-Leu^5-enkephalin, bestatin and captopril were purchased from the Sigma Chemical Co. Diprenorphine was obtained from Reckett & Colman, Ltd. The peptide fragments Gly-Gly-L-Phe-L-Leu, L-Phe-L-Leu, L-Tyr-Gly and L-Tyr-Gly-Gly were synthesized as described previously [12–15], and their structures were confirmed by elemental and amino acid analysis, proton magnetic resonance spectrometry and GLC-mass spectrometry [16].

Pulmonary vasoactivity of Leu^5-enkephalin and cogeners

as well as fragments of the pentapeptide were evaluated in isolated perfused lung preparations from male Sprague–Dawley rats weighing 225–325 g. Animals were anesthetized with an intraperitoneal injection of pentobarbital-sodium (100 mg/kg). The trachea was cannulated, and positive pressure ventilation (peak inspiratory pressure, 9 cm H_2; expiratory pressure, 2 cm H_2O) with warmed (37°), humidified room air was initiated at 65 breaths/min. The heart and lungs were then removed and suspended in a humidified chamber. Ventilation was then changed from room air to a gas mixture of 21% O_2, 5% CO_2, and 74% N_2. Recirculating perfusion with 80 ml of pre-warmed (37°) physiologic salt solution was initiated at a constant flow rate of 0.05 ml/min/g body weight with a Cole–Parmer tubing pump system. The composition of the physiologic salt solution in millimoles/liter was: NaCl, 119; KCl, 4.7; $MgSO_4$, 1.17; $NaHCO_3$, 17; dextrose, 5.5; $CaCl_2$, 1.6; and bovine serum albumin, 3% (weight-to-volume). All chemicals were of reagent grade and were dissolved in distilled, deionized water.

Pulmonary artery perfusion pressure was measured from a side arm of the infusion cannula and was recorded continuously on a Grass polygraph. Because ventilation was at constant pressure and perfusion was at constant flow, changes in perfusion pressure reflected changes in pulmonary vascular resistance.

Results and discussion

Our initial aim was to determine whether the pulmonary vasoactivity of Leu^5-enkephalin was also exhibited by the cogeners, Met^5-enkephalin and D-Ala^2-D-Leu^5-enkephalin. As illustrated by the experimental records shown in Fig. 1, only Leu^5-enkephalin evoked dose-dependent (10–100 μg i.a. bolus injections) pulmonary vasoconstriction. Both Met^5-enkephalin and D-Ala^2-D-Leu^5-enkephalin were inactive, even at doses which were twice that of the highest Leu^5-enkephalin dose examined. In a previous study [11], we found that morphine also does not evoke pulmonary vasoconstriction.

The above results, combined with our earlier observation that Leu^5-enkephalin-induced pulmonary vasoconstriction is insensitive to naloxone [11], argued strongly against a role for opiate receptors. However, there is growing evidence that some of the cardiovascular effects of opioids, particularly those of Leu^5-enkephalin, may be mediated in part through activation of δ-type opiate receptors [5–7]. Furthermore, naloxone appears to be relatively ineffective against some δ-mediated effects [17]. Thus, to determine, whether δ opiate receptors mediated Leu^5-enkephalin-induced pulmonary vasoconstriction, we evaluated the inhibitory effects of diprenorphine, an agent with documented inhibitory activity against δ-mediated effects in

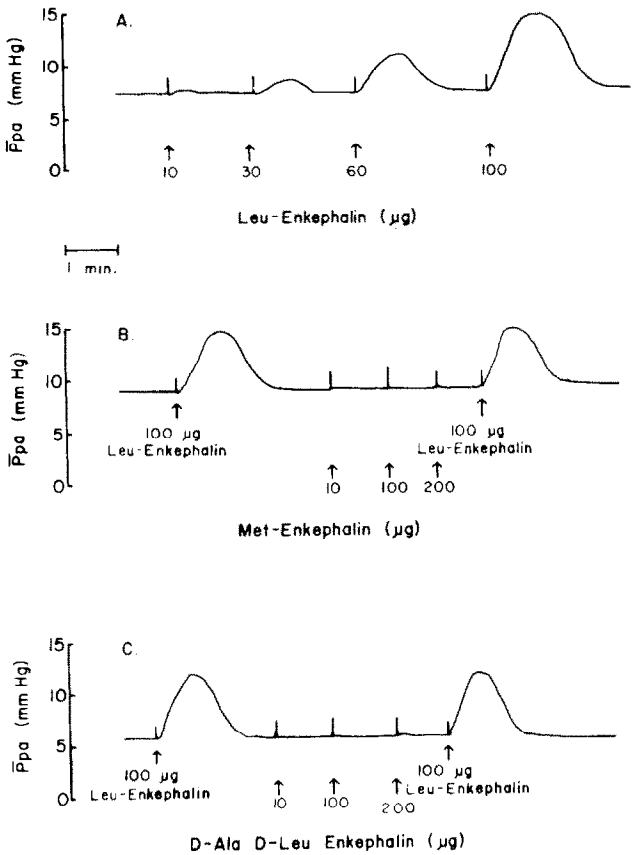

Fig. 1. Representative experimental records illustrating dose-dependent pulmonary vasoconstriction induced by Leu^5-enkephalin (A) and lack of vasopressor activity of the cogeners Met^5-enkephalin (B) and D-Ala^2-D-Leu^5-enkephalin (C). Identical results were obtained in at least three additional preparations for each of the opioids.

other preparations [17]. In these experiments, dose–response curves for Leu^5-enkephalin were generated before and after addition of 10^{-5} M diprenorphine to the perfusate reservoir. Preliminary studies demonstrated that two successive curves could be generated with no significant differences between the individual curves, thereby validating the use of a "paired" experimental protocol. As shown in Fig. 2, diprenorphine did not antagonize Leu^5-enkephalin-induced pulmonary vasoconstriction. We note, however, that because there are no known opiate receptor-mediated effects in the perfused rat lung, it was impossible to confirm the efficacy of the 10^{-5} M dose of diprenorphine used in this study or the 10^{-4} M doses of naloxone and naltrexone employed in our previous report [11]. Nonetheless, these doses of opiate receptor blockers are rather large, and, combined with the failure of Leu^5-enkephalin cogeners to exhibit pulmonary vasoactivity, support the view that conventional opiate receptors are not related to the Leu^5-enkephalin-induced pulmonary pressor response. However, until the receptor mediating this effect is identified, the possibility must remain that a heretofore unrecognized opiate receptor subtype could be involved.

Knowing that the lung possesses a number of enzymes capable of metabolizing Leu^5-enkephalin [18], we considered the possibility that the pentapeptide might be degraded to a smaller fragment which ultimately mediated the pressor response. This idea was tested in two ways. First, we examined the influence of peptidase inhibition with bestatin and captopril on the Leu^5-enkephalin-induced response. We reasoned that, if metabolism were involved, then inhibition of the enzymes responsible would blunt the pressor responses. A "paired" experimental protocol analogous to the one described above for diprenorphine was used in these experiments. A combination of 10^{-4} M captopril and 10^{-6} M bestatin was added to the perfusate reservoir between the first and second Leu^5-enkephalin dose–response curves. As shown in Fig. 3, peptidase inhibition augmented rather than inhibited Leu^5-enkephalin-induced pulmonary vasoconstriction. We attribute the potentiation to the fact that lung peptidases probably limit the amount of exogenous Leu^5-enkephalin available to interact with receptor sites. Because we used a combination

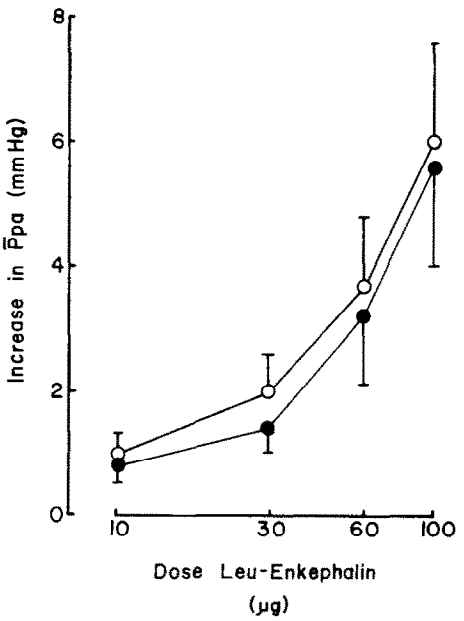

Fig. 2. Lack of antagonism by diprenorphine (10^{-5} M) of Leu^5-enkephalin-induced pulmonary vasoconstriction (N = 5). Key: (●—●) control and (○—○) diprenorphine.

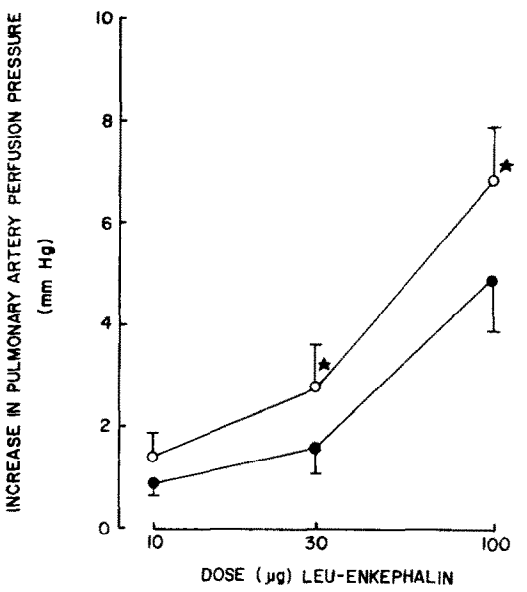

Fig. 3. Potentiation by a combination of bestatin (10^{-6} M) and captopril (10^{-4} M) of Leu^5-enkephalin-induced pulmonary vasoconstriction (N = 5). Key: (●—●) control, (○—○) bestatin-captopril, and (*) significantly different at $P < 0.05$.

of relatively high doses of the peptidase inhibitors, no information regarding the identify of the metabolizing enzyme(s) can be derived from these experiments. It is noteworthy, however, that captopril has been shown recently to potentiate the vasodepressor action of Met^5-enkephalin in anesthetized rats [19].

Our second approach was to evaluate the pulmonary vasoactivity of a number of fragments of the Leu^5-enkephalin pentapeptide. We considered these studies to be important because, in addition to addressing the hypothesis that metabolism may be required for Leu^5-enkephalin-induced vasoconstriction, they also provided insight into the structural requirements of the receptor mediating this response. The pulmonary vasoactivities of four fragments, Gly-Gly-L-Phe-L-Leu, L-Tyr-Gly-Gly, L-Tyr-Gly, and L-Phe-L-Leu, and the amino acid, L-leucine, were each evaluated in at least three preparations over a dose range of 10–200 μg (i.a. bolus injections). Responsiveness to the parent compound, Leu^5-enkephalin, was documented in each preparation before and after exposure to the fragments. None of these agents exhibited any pulmonary vasoactivity, with the exception of Gly-Gly-L-Phe-L-Leu, which produced an approximate 1 mm Hg pressor response at a dose of 200 μg in the three preparations in which it was studied. This response is only about 20% of that associated with the 100 μg dose of the parent compound.

In summary, our results suggest that Leu^5-enkephalin-induced pulmonary vasoconstriction in the isolated buffer perfused rat lung does not involve activation of conventional opiate receptors nor does it relate to obligatory metabolism of the pentapeptide by endogenous lung peptidases. We note, however, that the results also could be explained by the existence of a heretofore unrecognized opiate receptor subtype. Such a receptor would be distinct from known subtypes in its absolute insensitivity to opioid ligands other than Leu^5-enkephalin and the inability of a variety of antagonists to block its activation. Structure–activity data suggest that the receptor mediating the pressor response is quite specific in terms of its ability to discriminate between Leu^5-enkephalin, its cogeners, and

selected fragments. Leu⁵-enkephalin induced pulmonary vasoconstriction thus joins a growing list of opioid-mediated responses which are insensitive to naloxone and do not appear to relate to conventional opiate receptor activation.

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Effects of erythromycin derivatives on cultured rat hepatocytes

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Erythromycin and its derivatives are useful antibiotics in the treatment of a variety of human infections. However, these macrolides have some limitations: the base (EB) is inactivated by gastric juice and the estolate (EE) and other derivatives, which are gastroresistant, may produce hepatic injury and jaundice in patients [1, 2]. The mechanisms involved in erythromycin hepatotoxicity are not clear [2]: hypersensitivity of patients and intrinsic toxicity of the drug [3] have been considered.

In rats [4, 5] and humans [6], erythromycins induce biosynthesis of a particular type of cytochrome P-450 which binds their own metabolites, forming an inactive complex with the iron (II) of cytochrome P-450 [7]. Erythromycin derivatives differ in induction of their own transformation and formation of stable complexes [4, 5, 7] but it is not known whether hepatotoxicity and metabolism rate of these compounds are directly correlated.

Experimental *in vitro* models have been used to study the intrinsic toxicity of erythromycin compounds but they have some major shortcomings. Chang liver cells [8, 9] do not express the properties of differentiated parenchymal cells. Freshly isolated hepatocytes [3] surviving only a few hours may respond atypically to chemicals, on account of the morphological and biochemical trauma of isolation. Conventional hepatocyte cultures normally also undergo rapid phenotypic alterations including a large decrease in several drug-metabolizing enzymes [10]. In this regard, hepatocytes co-cultured with rat liver epithelial cells, which maintain various specific liver functions for several days or weeks [11, 12] seem a more reliable experimental model. Pure and mixed hepatocyte cultures have never been used for studying the metabolism and toxicity of erythromycins.

Recently, a new fluorinated derivative of erythromycin ((8S)-8 fluoroerythromycin A) (P-0501A) (EF) [13] was found to be more gastroresistant and more available in animals than the erythromycin base [14, 15]. We used various liver cell cultures to compare the cytotoxicity and cytochrome P-450 complex formation of this new compound with EB and EE.

Methods

Cell cultures: liver cells. Adult hepatocytes were isolated from 2-month-old male Sprague–Dawley rats by perfusing the liver with an Hepes-buffered collagenase solution. Parenchymal cells were seeded in Ham's F 12 medium containing 0.2% bovine serum albumin and 10% foetal calf serum. For the preparation of co-cultures [11], rat liver epithelial cells (see below) were added 3 hr after hepatocyte seeding. Cell confluency was reached within the first 24 hr of co-culture. The medium, supplemented with 7×10^{-5} M or 3.5×10^{-6} M hydrocortisone hemisuccinate for pure and mixed cultures respectively, was renewed every day [11].

Liver epithelial cell lines, derived from 10-day-old Fisher rats by trypsinization of the liver, were used before transformation. These cells did not express specific liver functions [16].

Human liver fibroblasts were obtained by growing liver fragments (from autoptic material of a 3-day-old baby) in minimum essential Eagle's medium containing 10% foetal calf serum [17]. The cells were used after about 10 passages.

Skin fibroblasts. For comparison with human liver fibroblasts, skin fibroblasts (from autoptic material of a 3-year-old infant) were also used after about 10 passages in the same conditions.

Treatments. All erythromycins (from Pierrel S.p.A. Milan, Italy) were dissolved in dimethylsulfoxide (DMSO) and added once to the cultures in concentrations ranging from 1×10^{-4} to 8×10^{-4} M, at various times after cell