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Protection of atrial natriuretic factor against degradation: Diuretic and natriuretic responses after *in vivo* inhibition of enkephalinase (EC 3.4.24.11) by acetorphan

(atrial natriuretic peptide/kidney/healthy volunteers/cardiovascular diseases)

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ABSTRACT **Atrial natriuretic factor (ANF) might be beneficial in several cardiovascular disorders, but its poor oral absorption and rapid inactivation *in vivo* have so far prevented its use in therapeutics. We have assessed the role of enkephalinase (membrane metallo-endopeptidase, EC 3.4.24.11) in the *in vivo* inactivation of ANF in mice and healthy human volunteers by evaluating the effects of acetorphan, a potent inhibitor. In mice, the degradation of ^{125}I-labeled ANF was markedly delayed, as shown by the levels of the intact peptide in the plasma and the kidney, a major target organ. The effect of acetorphan was due to the inhibition of enkephalinase activity, since it occurred at an ED_{50} very close to this drug's ID_{50} for the inhibition of the specific binding of radioactive material to the kidney or lung peptidase that was measured after administration of [^{3}H]acetorphan. The effects of acetorphan were also studied in eight healthy human volunteers by using a randomized double-blind, placebo-controlled design. Oral administration of acetorphan elicited a lasting elevation of plasma ANF-like immunoreactivity, with a time course parallel to that of the inhibition of plasma enkephalinase activity. These effects were accompanied by significant increases in urinary volume and sodium excretion, two well-established renal responses to ANF peptides. These results indicate that enkephalinase plays a critical role in ANF degradation *in vivo* and that its inhibition enhances the levels of circulating endogenous ANF, which, in turn, results in diuresis and natriuresis. Enkephalinase inhibition may constitute another therapeutic approach to the treatment of cardiovascular diseases, such as congestive heart failure or essential hypertension, on which ANF is postulated to have a beneficial effect.**

Atrial natriuretic factor (ANF, atrial natriuretic peptide) is a peptide hormone which is secreted by the heart and exerts potent actions on the kidney and cardiovascular system (1, 2). Upon atrial distension, a 28-amino acid peptide [ANF-(99–126)] is secreted into the circulation and acts on specific receptors in the kidney, vasculature, adrenal gland, and some structures in the central nervous system (3–5). It decreases blood pressure, raises the glomerular filtration rate and urinary excretion of water and sodium, and lowers plasma renin and aldosterone levels (6, 7). In patients with essential hypertension, ANF lowers blood pressure (8, 9). In humans with congestive heart failure, it decreases right atrial pressure, pulmonary wedge pressure, and peripheral vascular resistance (10, 11).

Hence ANF might constitute a useful therapeutic agent in several cardiovascular disorders affecting a large fraction of the population. However, its poor absorption *per os* and extremely rapid inactivation when injected (12–14), the latter explaining its essentially transient biological effects, have hitherto prevented its clinical use.

One approach to this problem consists of delaying the inactivation of endogenous ANF; this requires precise identification of the mechanisms by which it is inactivated. *In vitro*, tissue fractions rapidly hydrolyze ANF (15–21), but contradictory data were obtained about the enzymes responsible. For instance, ANF degradation by kidney extracts was variously attributed to aminopeptidases and angiotensin-converting enzyme (ACE) (13), to enkephalinase (membrane metallo-endopeptidase, EC 3.4.24.11) (15–18, 22), and, subsequently, to an endopeptidase resembling enkephalinase (19); in heart ANF degradation was attributed to an ACE isozyme (20) or to a metallopeptidase distinct from ACE, enkephalinase, and aminopeptidases (21); and in brain degradation was attributed to enkephalinase in conjunction with unidentified metallopeptidases and thiol proteases (23).

Numerous peptidases capable of hydrolyzing neurohormonal peptides are present in tissue homogenates or fractions. However, metabolic pathways in these preparations may not reliably reflect those responsible for inactivation of the endogenous peptides *in vivo*: with these preparations the peptides are exposed to enzymes with which they may never come into contact while circulating through the intact tissue (24, 25).

Relatively few studies have been devoted to the degradation of ANF *in vivo*. Radioiodinated ANF (^{125}I-ANF), which retains the biological activity of the parent hormone, is cleared from rat plasma with a $t_{1/2}$ of less than 1 min (14, 26), but no attempt has been made to identify the responsible peptidase(s).

Here we have assessed the role of enkephalinase in the inactivation of exogenous and endogenous ANF *in vivo* by using acetorphan, a potent inhibitor (27), and thus evaluated the protection of ^{125}I-ANF in mice and the changes in plasma ANF-like immunoreactivity (ANF-ir), diuresis, and natriuresis in healthy human volunteers.

MATERIALS AND METHODS

Preparation and Purification of ^{125}I-ANF. Synthetic α human ANF-(99–126) (Neosystem Laboratory, Strasbourg, France) was iodinated (3) and the reaction mixture was loaded onto a C_{18} μBondapak HPLC column (Waters) equilibrated in 0.01 M NH_4OAc containing 20% (vol/vol) CH_3CN. Elution was performed by using a linear gradient of CH_3CN

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Abbreviations: ANF, atrial natriuretic factor; ANF', ANF hydrolyzed between residues Cys-105 and Phe-106; ANF-ir, ANF-like immunoreactivity; TCA ppt, trichloroacetic acid-precipitated radioactive material.

(20–30%) at a flow rate of 1 ml/min. Retention times were 10.5 min for ANF and 13.5 and 15.0 min for its mono- and di-iodinated derivatives. The mono[^{125}I]iodinated derivative was collected and stored at 4°C after addition of bovine serum albumin at 1 mg/ml. The mono[^{125}I]iodinated derivatives of tyrosine or Phe-Arg-Tyr (Bachem)—i.e., the C-terminal tripeptide of ANF-(99–126)—were similarly obtained.

Analysis of Radioactivity in Plasma and Kidney of Mice Injected with ^{125}I-ANF. Male Swiss mice weighing 20 g (Iffa Credo, St. Germain sur l'Arbresle, France) were decapitated at various times after i.v. injection of 0.5 μCi (1 Ci = 37 GBq) of ^{125}I-ANF (corresponding to 0.25 pmol). The blood was collected in an equal volume of 0.2 M HCl; the kidneys were dissected and immediately homogenized in 5 vol of cold 0.1 M HCl. Blood and homogenates (previously frozen and then thawed to facilitate protein precipitation) were centrifuged and the supernatants were used for radioactivity quantification and analysis by either HPLC or determination of trichloroacetic acid-precipitated radioactive material (TCA ppt) (28). For HPLC analysis, aliquots of plasma or kidney extracts were fractionated on a C_{18} μBondapak column equilibrated in 0.01 M NH_4OAc buffer, pH 4.2, containing 10% CH_3CN. Elution was completed by using a linear gradient of CH_3CN (10–50%) at a flow rate of 1 ml/min for 40 min. The column was calibrated with ^{125}I-labeled standards added to control extracts—i.e., the mono[^{125}I]iodinated derivatives of Tyr, Phe-Arg-Tyr, and ANF, which were eluted with retention times of 10, 23, and 28 min, respectively (see Fig. 1). Radioactivity was continuously monitored, and its level in the peak corresponding to the intact ^{125}I-ANF was determined by integration, taking into account the total radioactivities injected and eluted. For determination of TCA ppt (28), 0.9 ml of a 10% (wt/wt) TCA solution was added to 0.1-ml aliquots of the kidney extracts and the tubes were left for 3 hr at 4°C. After centrifugation for 10 min at 3000 × g, the radioactivity in the pellets was measured. When standard ^{125}I-ANF was added to a control kidney extract in 0.1 M HCl, TCA ppt represented 90% of the total. In contrast, when the extract was left for 10 min in contact with a kidney homogenate (prepared in 0.1 M Tris·HCl) before acidification with HCl, TCA ppt represented only 3%. These two values were taken into account when calculating TCA ppt in kidney extracts from mice given ^{125}I-ANF. With ^{125}I-Tyr and ^{125}I-Phe-Arg-Tyr standards, the TCA ppt was 3% and 15%, respectively.

Hydrolysis of ^{125}I-ANF by Enkephalinase. ^{125}I-ANF (5 nCi) was incubated at 37°C in 0.1 ml of 50 mM Tris·HCl buffer, pH 7.5, containing 1 ng of recombinant human enkephalinase (29), a generous gift of B. Malfroy (Genentech). After addition of 0.05 ml of 0.1 M HCl, the mixture was analyzed by HPLC as described in the previous section.

***In Vivo* Binding of [^{3}H]Acetorphan Products in Mice.** *In vivo* labeling of enkephalinase in kidney or lung membranes was evaluated essentially as described (30). Briefly, mice received [^{3}H]acetorphan (i.v.) and the radioactive material, mainly consisting of the active compound [^{3}H]thiorphan, specifically bound to enkephalinase was evaluated by a filtration assay.

Assay of Enkephalinase Activity in Human Plasma. Blood samples were collected in heparinized tubes and centrifuged for 15 min at 3000 × g and 4°C. The enkephalinase activity in 50-μl plasma samples was immediately determined by a two-step fluorometric assay with succinyl-Ala-Ala-Phe-amidomethylcoumarin as the substrate (31, 32).

Assay of ANF-ir in Human Plasma. Blood samples were collected in tubes containing peptidase inhibitors (EDTA, phenylmethanesulfonyl fluoride, pepstatin) and immediately centrifuged for 30 min at 3000 × g and 4°C. Plasma was collected and stored at −70°C for only a few days before assays. ANF-ir was extracted on Vycor glass powder and assayed by using a commercial radioimmunoassay (RIA) kit (Amersham).

Experimental Protocol of the Study in Human Volunteers. We studied eight healthy male volunteers aged 20–35 yr, with their informed consent, after a 15-day drug-free period. They were kept on their usual sodium and water intake. The double-blind, randomized placebo-controlled design consisted of three sequences of treatment with single drug (or placebo) separated by an interval of at least 4 days. After an overnight fast, the subjects were placed in a supine position at 7:30 on the morning of the trial, and they stood up only for urination and during lunch. Their antecubital veins were catheterized. At 8:00 a.m. (t = 0) their bladders were emptied and they were given either acetorphan (100 or 300 mg) or placebo orally, with 200 ml of water.

Blood pressure and heart rate were recorded every 2 hr. Blood samples were taken at t = 0, 0.5, 1, 2, 4, and 8 hr. Urine was collected at t = 2, 4, and 8 hr, and its volume was recorded and samples were analyzed for electrolytes (Na^+, K^+, and Cl^-). Changes in plasma ANF-ir levels were evaluated by determining the area under the curve, using the trapezoidal rule. The within-subject differences were calculated by a repeated measure analysis of variance model (ANOVA) followed by the Newman–Keuls test.

RESULTS

***In Vitro* Hydrolysis of ^{125}I-ANF by Human Enkephalinase.** After a 5-min incubation of ^{125}I-ANF with recombinant human enkephalinase, two main hydrolysis products were detected by HPLC analysis: one with a retention time corresponding to ^{125}I-Phe-Arg-Tyr, the other with a retention time of 25.5 min, presumably corresponding to ^{125}I-ANF cleaved between residues Cys-105 and Phe-106 (^{125}I-ANF′) (Fig. 1*A*) (15, 16).

Effects of Acetorphan on Kidney Radioactivity in Mice Injected with ^{125}I-ANF. Two minutes after the i.v. injection of ^{125}I-ANF, the total radioactivity recovered in kidneys was 32,000 ± 3000 dpm (mean ± SEM of 12 experiments). HPLC analysis revealed a major peak with a retention time corresponding to ^{125}I-Tyr and/or free ^{125}I accompanied by two minor peaks, one corresponding presumably to ^{125}I-ANF′ and the other to intact ^{125}I-ANF. Radioactivity at the retention time of intact ^{125}I-ANF was 8000 ± 1500 dpm—i.e., 25% of the total (Fig. 1*B*). This pattern changed markedly in mice pretreated with acetorphan at 15 mg/kg, as total radioactivity was 80,000 ± 5000 dpm (a 150% increase over control) and the major radioactivity peak now corresponded to intact ^{125}I-ANF and represented 43% of the total. It was accompanied by two smaller peaks, presumably corresponding to ^{125}I-ANF′ and ^{125}I-Phe-Arg-Tyr (Fig. 1*C*). When the extracts were analyzed in two other HPLC systems, the main peak was also found to correspond to intact ^{125}I-ANF (not shown). Levels of intact ^{125}I-ANF in blood (dpm/μl) were 16 ± 1 in controls and 40 ± 3 in acetorphan-treated mice, corresponding to 14% and 34% of total radioactivity, respectively.

In kidneys from controls, intact ^{125}I-ANF was identified clearly by HPLC only for up to 2 min after the injection of the peptide; its level was maximal at 1 min and then declined with a $t_{1/2}$ of about 1 min (Fig. 2). In acetorphan-treated mice the intact ^{125}I-ANF (and total radioactivity) was maximal at 2 min, representing an ≈4-fold increase over the control level ($P < 0.01$), and then declined with an apparent $t_{1/2}$ of 3.5 min. At 10 min, ^{125}I-ANF levels were about the same as those found at 2 min in controls.

In controls TCA ppt corresponded fairly closely to intact ^{125}I-ANF evaluated by HPLC analysis for up to 2 min. In acetorphan-treated mice, the level of TCA ppt was about 50% higher than that of intact ^{125}I-ANF, a difference presumably due to the presence of ^{125}I-ANF′, which was found to

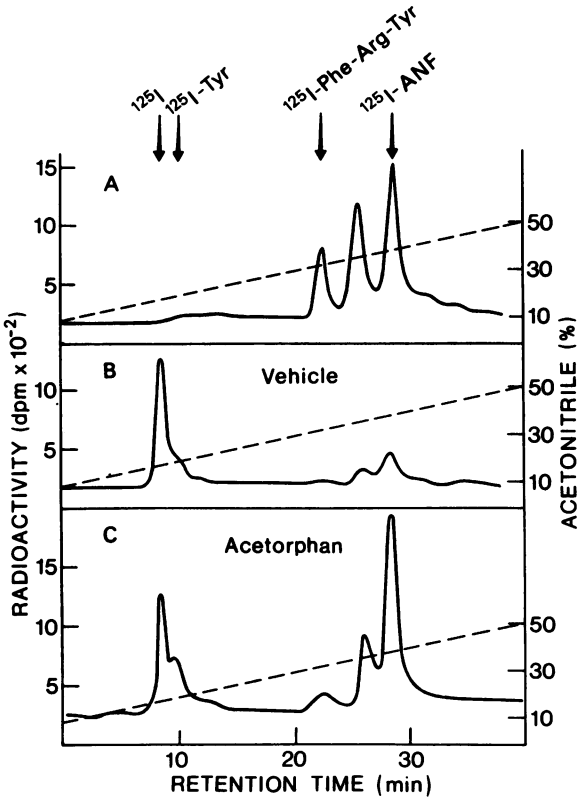

FIG. 1. HPLC analysis of ^{125}I-ANF hydrolysis products formed by pure enkephalinase (*A*) or recovered in the kidney of mice after injection with ^{125}I-ANF. Mice were given either vehicle (methylcellulose) (*B*) or acetorphan at 15 mg/kg (*C*) 30 min before the injection of ^{125}I-ANF (0.5 μCi, i.v.) and were killed 2 min later. Arrows indicate the retention times of standards. Data are the results of a single typical experiment, which was replicated 5–10 times.

precipitate like ^{125}I-ANF (Fig. 1). Nevertheless the effect of acetorphan treatment on this index was still clearly shown by the marked increase over corresponding controls (e.g., 5-fold at 2 min). The effect of acetorphan on TCA ppt, measured 30 min after its oral administration and 2 min after ^{125}I-ANF

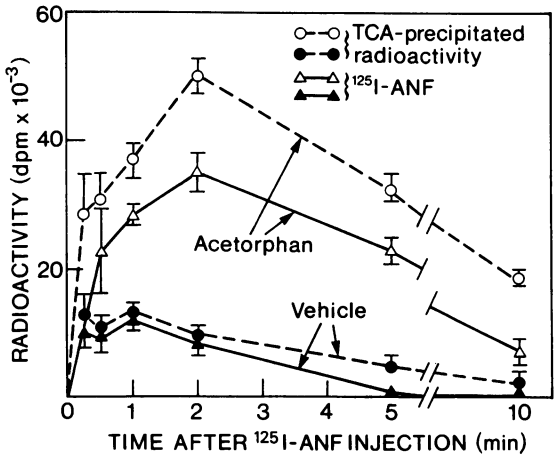

FIG. 2. Effects of acetorphan on the kidney radioactivity of mice injected with ^{125}I-ANF. Groups of four to eight mice were given, *per os*, either acetorphan at 15 mg/kg or vehicle 30 min before the injection of ^{125}I-ANF (0.5 μCi, i.v.) and were killed at various times after injection. Data are mean ± SEM of the values for intact ^{125}I-ANF (isolated by HPLC as shown in Fig. 1) or TCA ppt in kidney extracts. All values in acetorphan-treated mice differed from the corresponding values in controls ($P < 0.01$).

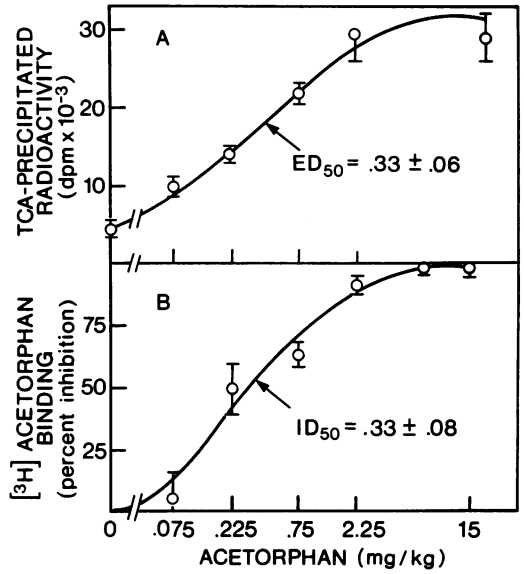

FIG. 3. Comparison of the dose–response curves for ^{125}I-ANF protection and occupation of enkephalinase in the kidney of mice treated with acetorphan. In both tests, acetorphan was administered orally 30 min before mice were killed. (*A*) ^{125}I-ANF protection was studied by evaluating the TCA ppt in kidney 2 min after injection of 0.5 μCi of ^{125}I-ANF. (*B*) Occupation of enkephalinase was studied by evaluating the inhibition of specific binding of radioactivity to kidney membranes after administration of [^{3}H]acetorphan (6 μCi, i.v.) 30 min before sacrifice. Mean ± SEM of four to eight values.

injection, was almost maximal at 2 mg/kg, the ED_{50} being 0.33 ± 0.06 mg/kg (Fig. 3*A*).

Inhibition of *in Vivo* Binding of [^{3}H]Acetorphan Products in Mice. The inhibition of binding of radioactive material in kidney and lung after administration of [^{3}H]acetorphan was measured under conditions similar to those used to assess ^{125}I-ANF protection. In controls membrane-bound radioactivity was 375,000 ± 28,000 dpm in kidney and 66,000 ± 1,000 dpm in lung. Oral administration of acetorphan in increasing doses, 30 min before killing, gradually inhibited total binding to a maximum of 97% in kidney and 88% in lung. The ID_{50} (mg/kg) for inhibition of specific binding was 0.33 ± 0.08 in kidney (Fig. 3*B*) and 0.77 ± 0.15 in lung (not shown).

Effects of Acetorphan on Enkephalinase Activity and on ANF-ir Levels in Plasma, Urinary Volume, and Electrolyte Excretion in Human Volunteers. No side effects were recorded during the trials. Blood pressure and pulse rate were not significantly modified after acetorphan treatments. After administration of placebo, plasma enkephalinase activity did not significantly differ from the pre-administration level (380 ± 55 pmol/min per ml). After 100 mg of acetorphan, the activity was significantly inhibited between 0.5 and 4 hr, inhibition reaching 70% at 1 hr (Fig. 4 *Left*). After 300 mg of acetorphan the inhibition was significant between 0.5 and 8 hr, reaching 80% at 2 hr (Fig. 4 *Right*).

ANF-ir levels tended to rise transiently after administration of the placebo, the increase being 40% at 1 hr, as compared with the baseline level, while the subjects remained in a supine position. After 100 or 300 mg of acetorphan the areas under the curves of ANF-ir were increased ≈6-fold compared with values in the placebo session ($P < 0.05$) (Fig. 4). The rise in ANF-ir was still significant 4 hr after 100 mg of acetorphan and 8 hr after 300 mg of acetorphan. In both cases, its peak was observed at 2 hr, corresponding to 127% and 81% increases (as compared with pre-administration levels) after 100 and 300 mg of acetorphan, respectively.

The urinary volumes recorded during the trials (0–8 hr) were significantly increased ($P = 0.002$) after either 100 or 300

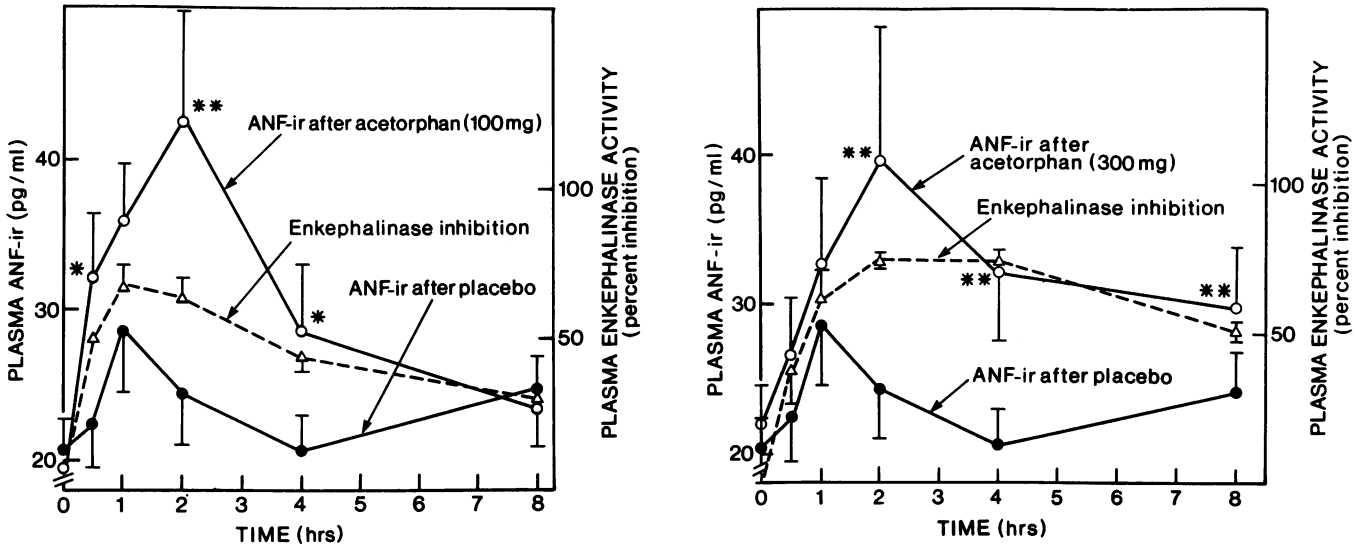

FIG. 4. Effects of acetorphan on ANF-ir levels and on enkephalinase inhibition in the plasma of eight healthy human volunteers. In a double-blind randomized trial the same subjects were given placebo or either 100 mg of acetorphan (*Left*) or 300 mg of acetorphan (*Right*). Venous blood was sampled just before administration and at the indicated times thereafter. Data are mean ± SEM. Plasma enkephalinase inhibition is expressed as percent of the corresponding control values in each subject before drug administration. The areas under the curves of ANF-ir levels were significantly increased after acetorphan relative to placebo ($P < 0.05$). *, $P < 0.05$; **, $P < 0.01$ as compared with corresponding placebo values.

mg of acetorphan: 559 ± 67 ml and 659 ± 82 ml as compared with 406 ± 26 ml after placebo. These increases took place mainly between 2 and 8 hr, corresponding to 34% and 76% after 100 and 300 mg of acetorphan (Fig. 5); the volumes were not significantly modified between 0 and 2 hr (not shown).

Urinary excretion values of Na^+ between 0 and 8 hr were 73.4 ± 10.0 mmol and 83.0 ± 12.5 mmol after 100 and 300 mg of acetorphan, respectively, values to be compared with 54.5 ± 7.3 mmol after placebo. However, these changes (not shown), corresponding to +35% and +52%, did not reach significance ($P = 0.08$). By contrast, urinary Na^+ excretion was significantly increased ($P < 0.05$) between 2 and 8 hr after administration of 100 mg of acetorphan (+35%) or 300 mg of acetorphan (+73%) (Fig. 5). Urinary excretion of Cl^- or K^+ did not significantly change.

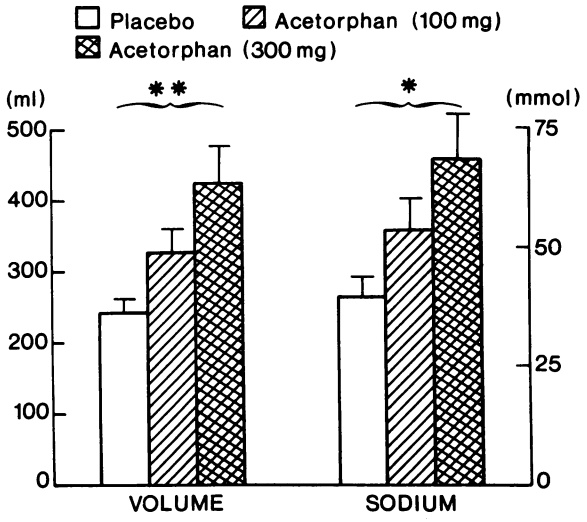

FIG. 5. Effects of acetorphan on urinary volume and Na^+ excretion in eight healthy volunteers. Values are mean ± SEM of values obtained during the 2- to 8-hr period of the trials. Other results of the trials are reported in Fig. 4. Asterisks denote significant effects of acetorphan versus placebo (*, $P < 0.05$; **, $P < 0.01$).

DISCUSSION

The two sets of data obtained in mice and humans with the inhibitor acetorphan strongly support the idea that enkephalinase has a crucial role in the *in vivo* inactivation of ANF.

In mice, simple tests were developed to assess the recovery of the intact peptide in the kidney, a major target organ for ANF, following its bolus i.v. injection in highly radiolabeled form. Although analysis of radioactivity by TCA ppt cannot be considered as reliable as analysis by HPLC, comparison of experimental data (Fig. 2) shows that it nevertheless constitutes an acceptable simplified means of evaluating the effects of inhibitors of ANF degradation in large series of experiments.

In the controls, intact ^{125}I-ANF could be reliably identified by HPLC analysis of kidney extracts only 2 min after the injection, its level being sharply decreased already after 2 min. This rapid degradation is consistent with the rapid clearance of the hormone and appearance of various fragments in plasma (14, 26).

In our experiments, prior treatment of mice with acetorphan raised intact ^{125}I-ANF in plasma and even more in kidney. In kidney, its peak level at 2 min corresponded to a 4-fold increase compared to the control level measured at the same time. These changes seem to indicate not only that ^{125}I-ANF was protected from degradation inside the kidney but also that a larger amount of the intact hormone was reaching this organ as a result of treatment with acetorphan.

These effects are selectively attributable to inhibition of enkephalinase, since they occurred with an ED_{50} closely similar to the ID_{50} for inhibition of *in vivo* binding generated by [^{3}H]acetorphan, a test which faithfully reflects enkephalinase occupation by inhibitors in various tissues of living mice (30). In addition, we observed that several other enkephalinase inhibitors had similar effects on ^{125}I-ANF metabolism, but a variety of inhibitors of peptidases other than enkephalinase were not effective (C.G, A.S, and J.-C.S., unpublished data). However, these effects cannot be attributed to selective inhibition of enkephalinase in the kidney, since acetorphan inhibits this enzyme with a similar potency in several other organs, such as the lung (see *Results*). Whole body radioautography of rats injected with ^{125}I-ANF revealed

that the kidney is one of the main organs involved in ANF clearance (26), but nephrectomy reduced ANF clearance from the plasma only slightly (13) or not at all (14). The plasma, which contains slight enkephalinase activity (32, 33), is not likely to contribute significantly to this clearance since it degrades ANF only slowly *in vitro* (14, 26). In rodent kidney the peptidase (34) is predominantly located in the brush border membranes and not basolateral or glomerular membranes, where ANF receptors are located. Consequently ANF would have to be filtered before reaching its main degradation site (28).

The clear protection of ^{125}I-ANF afforded by acetorphan in mice as well as the good tolerance of acetorphan prompted us to assess its effects on plasma endogenous ANF-ir in human volunteers as well as on diuresis and natriuresis, both known to increase in response to exogenous ANF (1, 2, 6). In these subjects, the dose-related lasting inhibition of plasma enkephalinase activity elicited by acetorphan was accompanied by a concomitant rise in plasma ANF-ir, exceeding the slight rise which occurred during the placebo session as a result of the change in posture (35). Comparison of the evolution of the two parameters suggests that approximately half of the inhibition of plasma enkephalinase was sufficient to induce a significant rise in plasma ANF-ir. This rise, which was within the upper limits of reported physiological variations (35), sufficed to induce a clear diuretic and natriuretic effect over a long period. This effect was only slightly lower than that observed after injection of 100 μg of ANF to human volunteers, which resulted in a much higher, but also much more transient, rise in plasma ANF-ir than that observed here, whereas lower doses were ineffective (36). This indicates that a rather modest increase in plasma ANF-ir, if sufficiently sustained, may result in significant renal effects.

Although the data obtained in mice suggest that the effects of acetorphan result mainly from the protection of endogenous ANF against degradation, the protection of other endogenous peptides, also known to be cleaved by enkephalinase, may contribute in either the rise of ANF-ir or the renal responses. Endogenous enkephalins, the first peptides shown to be inactivated by enkephalinase (37), may contribute indirectly by promoting ANF release from the heart (38). Protection of endogenous kinins was also suggested to account for the diuretic and natriuretic effects on rats of phosphoramidon, an enkephalinase inhibitor (39).

Whatever the exact mechanisms governing the actions of acetorphan, its clear effects on humans indicate that enkephalinase inhibition may constitute another therapeutic approach to treating disorders such as essential hypertension, congestive heart failure, cirrhosis, or cerebral edema. In these disorders a beneficial effect of ANF has been postulated despite evidence of an increased ANF secretion in some cases, because such secretion may be considered to constitute an insufficiently compensatory mechanism (2, 7, 9, 11).

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