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Renal effects of TAPP, a highly selective μ-opioid agonist

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1 The effect of i.v. administration of TAPP, a highly selective and exclusively peripherally-acting μ-opioid receptor agonist, on urine output, urinary sodium, potassium and cyclic GMP, and on plasma immunoreactive atrial natriuretic factor (IR-ANF) levels was studied in conscious normally hydrated female rats (200-250 g).

2 TAPP treatment produced a significant dose-dependent increase of urine output and urinary sodium, potassium and cyclic GMP excretion during the first hour. The highest TAPP dose used (2.5 mg kg^{-1} body weight) elicited a 10 fold elevation of urine output from 0.23 ± 0.06 ml h^{-1} to 2.5 ± 0.3 ml h^{-1} ($n=18$) accompanied by augmented sodium [from 17.0 ± 4.7 μEq h^{-1} to 79 ± 12.7 μEq h^{-1}, $n=18$ ($P<0.001$)], potassium [from 9.5 ± 2.5 μEq h^{-1} to 39.4 ± 6.6 μEq h^{-1}, $n=18$ ($P<0.005$)], and cyclic GMP excretion [from 191 ± 21 pmol h^{-1} to 1340 ± 322 pmol h^{-1}, $n=18$ ($P<0.001$)]. Plasma IR-ANF rose from 22 ± 4 pg ml^{-1} to 508 ± 22 pg ml^{-1} ($n=18$) ($P<0.001$) 5 min after administration of TAPP (2500 μg kg^{-1}).

3 TAPP lowered systemic blood pressure, also in a dose-related manner, 1-5 min after injection. This decrease in blood pressure was transient and did not last more than 10 min.

4 Pretreatment with the opioid antagonist naloxone (0.8 mg per rat) abolished the diuretic, natriuretic and kaliuretic effect of TAPP (250 μg kg^{-1}): urine output dropped from 1.16 ± 0.15 ml h^{-1}, $n=12$, to the control value of 0.15 ± 0.06 ml h^{-1}, $n=12$ ($P<0.001$), sodium excretion fell from 57.5 ± 11 μEq h^{-1}, to 21.3 ± 8.5 μEq h^{-1}, $n=12$ ($P<0.001$), and potassium excretion decreased from 45.4 ± 9.7 μEq h^{-1}, $n=12$, to 16.1 ± 7.0 μEq h^{-1}, ($P<0.001$).

5 Pretreatment with anti-ANF serum (0.4 ml) abolished the diuretic effect of TAPP: urine output diminished significantly from 1.93 ± 0.28 to 0.88 ± 0.29 ml h^{-1} ($P<0.01$) ($n=6$). The TAPP-induced diuretic action, increased sodium/potassium excretion and elevated urinary cyclic GMP levels were also reversed by anti-ANF antibodies.

6 Since TAPP is totally unable to cross the blood-brain barrier, the ensemble of these observations led to the conclusion that the diuretic, natriuretic, kaliuretic and hypotensive effects produced by this μ-opioid agonist through interaction with peripheral μ-opioid receptors occur via ANF release.

Keywords: Opioids; opioid receptors; μ-opioid agonists; ANF; diuresis, natriuresis, kaliuresis; hypotension; cyclic GMP

Introduction

During the past two decades, considerable evidence has accumulated showing that endogenous opioids are implicated in numerous physiological processes. This also holds true for blood pressure regulation and hydromineral homeostasis. Systemic administration or central application of opioids to brain areas involved in blood pressure regulation induces renal and cardiovascular responses (Holaday, 1983). The mechanisms of these effects are not well understood. It is known that renal control of sodium handling is important in the maintenance of blood pressure (Guyton *et al.*, 1972; Borst & Borst-De Geus, 1983) and that deficiencies in renal sodium excretion may lead to cardiovascular pathologies like hypertension (De Wardener & MacGregor, 1980).

Over the past decade, a prodigious amount of work led to the isolation and characterization of a cardiac hormone, the presence of which was demonstrated by De Bold *et al.* (1981) in mammalian atria, and the existence of which had been postulated long ago. Atrial natriuretic factor (ANF) is a potent diuretic, natriuretic and vasorelaxant hormone believed originally to be produced only by the heart. Although ANF and ANF receptors, which mediate its effects, are distributed throughout the body (Gutkowska & Nemer, 1989), there is a consensus that the heart is the main source of ANF. The hormone is released from this organ in response to atrial stretch induced by volume expansion or increased blood pressure (Ruskoaho, 1992). In addition to this mechanism, accumulated evidence shows that opioid agonists stimulate ANF release (Horky *et al.*, 1985; Xie *et al.*, 1988; Louisy *et al.*, 1989; Yamada *et al.*, 1991; Ationu *et al.*, 1994).

In our early work, we observed that morphine is a very potent stimulus of ANF release in the rat (Horky *et al.*, 1985; Gutkowska *et al.*, 1986), a finding which was subsequently confirmed in both human subjects and animals (Vollmar *et al.*, 1987; Crum & Brown, 1988; Chen *et al.*, 1989; Ögutman *et al.*, 1990). Furthermore, we demonstrated that morphine, administered intracerebroventricularly to conscious, normally hydrated rats, had a potent diuretic effect which was accompanied by a significant increase of plasma ANF and was abolished by pretreatment with anti-ANF antibodies (Gutkowska *et al.*, 1993). Morphine exerts biphasic renal actions in human subjects and rats. At low doses, it has diuretic properties, while in volume-expanded subjects and at higher doses, it acts as an antidiuretic. Since morphine crosses the blood-brain barrier to some extent (Miller & Elliott, 1995; Johannesson & Wood, 1964), its effects could be mediated by central opioid receptors. However, the actions of morphine are not specific because it is able to interact not only with μ but also with δ- and κ-opioid receptors. Determination of the opioid receptor type responsible for the renal and cardiovascular effects produced by enhanced plasma ANF release re-

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quires highly receptor-selective opioid agonists. In the present studies, we used the opioid tetrapeptide TAPP (H-Tyr-D-Ala-Phe-Phe-NH_2), a potent and highly selective μ-opioid agonist (Schiller *et al.*, 1989). Since TAPP is totally unable to cross the blood-brain barrier, it is ideally suited for determining the cardiovascular and renal effects mediated by peripheral μ-opioid receptors.

Methods

TAPP was synthesized by the solid-phase method and purified by gel filtration on a Sephadex-G25 column and by reversed-phase chromatography on an octadecasilyl silica column, as described elsewhere (Schiller *et al.*, 1989). The peptide was at least 98% pure, as judged from the h.p.l.c. elution profile.

Experimental protocol

Female Sprague-Dawley rats weighing 200–250 g were used in these studies. They were housed 4 per cage at 22°C, and a 12 h light-dark cycle (room illumination 06 h 00 min – 18 h 00 min) was maintained. Purina laboratory chow (Ralston Purina, St. Louis, MO, U.S.A.) and water were available *ad libitum* before the start of the experiments. Each experiment was performed in an isolated room and started at 09 h 00 min. The drug solutions were freshly prepared before the experiments. The animals were placed in restraint cages and after placing the tail in lukewarm water, various TAPP doses ranging from 25 μg kg^{-1} to 2500 μg kg^{-1} body weight in 300 μl saline were administered into the tail vein. The whole procedure lasted 60–90 s. The animals were previously acclimatized to the restraint cages; to avoid additional stress, the same technician worked on them. Immediately after the injection, they were put in metabolic cages without food or water and their urine was funneled into graduated cylinders. Urine was collected each hour following the injection for a total of 5 h to measure urinary volume, sodium, potassium and cyclic GMP. The control group received 300 μl of saline i.v. Three experiments were performed for each dose of TAPP with 6 rats in each experimental and control group.

To determine whether opioid receptors were involved in the renal effects of TAPP, the opioid antagonist, naloxone, was used. In this experiment, 0.8 mg of naloxone hydrochloride (Dupont, No. 124) dissolved in 300 μl saline was injected i.v. into the tail vein of 12 rats. Three groups of animals were used: the first group ($n=12$) was placed in restraint cages, injected with 300 μl saline (i.v.) and immediately returned to their usual cages for 10 min. They were then put once again into restraint cages and injected a second time with saline (300 μl i.v.). After the second injection, the animals were kept in metabolic cages for urine collection. The second group received saline (300 μl) and 10 min later, following the above-described procedure, TAPP (250 μg kg^{-1}) was administered in 300 μl saline. The

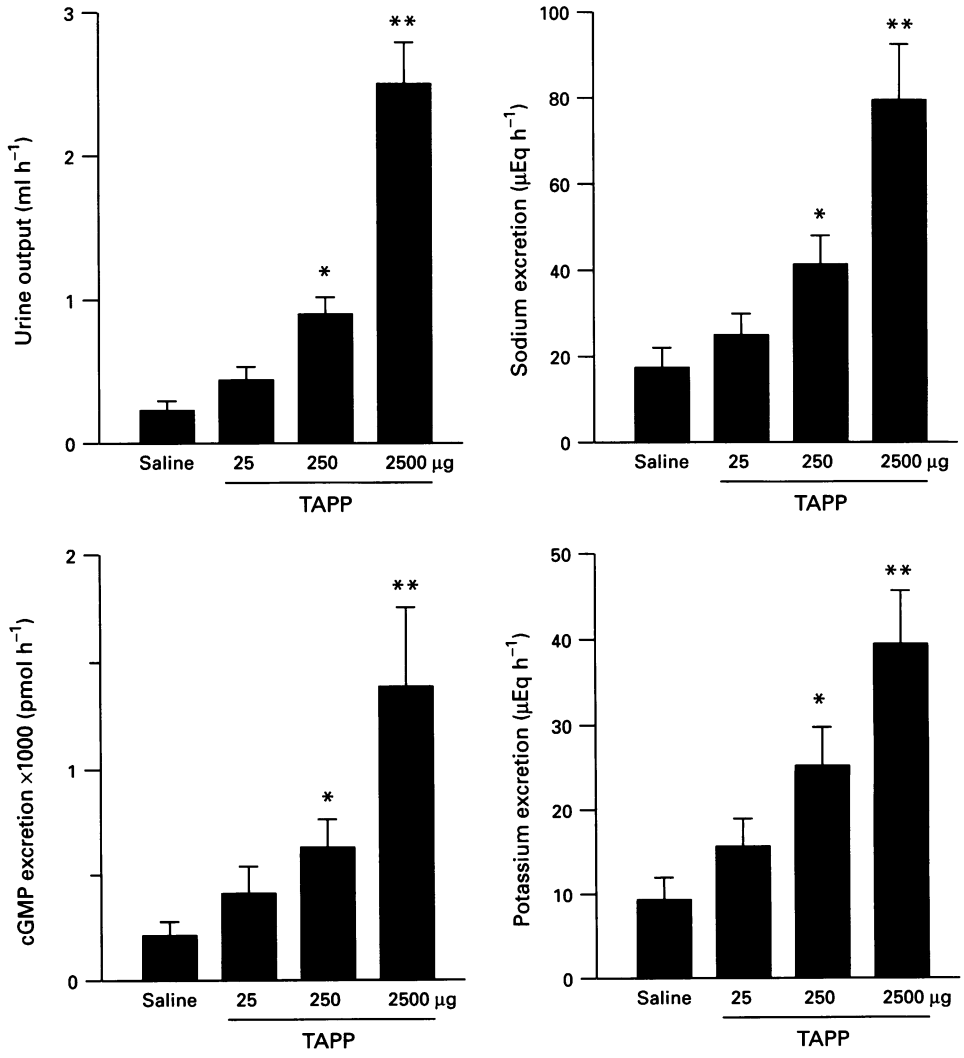

Figure 1 Effect of various doses of TAPP during the first hour after drug administration on urine output, sodium, potassium and urinary cyclic GMP (cGMP) excretion in conscious, normally hydrated rats ($n=18$). Values are expressed as means ± s.e.mean. $*P<0.01$, $**P<0.001$ experimental versus control group receiving saline.

third group received naloxone (0.8 mg) in 300 μl saline and TAPP (250 μg kg^{-1}) 10 min later. At the end of the experiment, the rats were returned to their cages.

To demonstrate that ANF mediates the renal effects of TAPP, anti-ANF serum was used. In this experiment, rats ($n=6$) were administered 0.4 ml of anti-ANF i.v. The control group ($n=6$) received 0.4 ml of normal rabbit serum. Ten min later, both groups were injected i.v. with TAPP (250 μg kg^{-1}). The anti-ANF serum was produced in New Zealand white rabbits by immunization with an antigen obtained by coupling the synthetic 26-amino acid peptide ANF ($Arg_{101}-Tyr_{126}$) to thyroglobulin via its C-terminal carboxyl group (Gutkowska, 1987). This antibody is specific to the C-terminal ANF peptide, but also recognizes the 126-amino acid prohormone ANF (Asn_1-Tyr_{126}).

Urinary sodium and potassium were measured with a flame photometer (Perkin-Elmer, Narville, CT, U.S.A.). To record blood pressure, the animals were cannulated with polyethylene catheters (PE-50) in the left carotid artery under pentobarbitone anesthesia (Somnotol; 65 mg kg^{-1}, i.p.). The catheter was connected to a pressure transducer through a continuous flush valve (Model 7, Grass Instruments, Quincy, MA, U.S.A.). For the determination of plasma ANF, blood samples were obtained from decapitated rats 5 min after TAPP administration. Two ml of blood were collected in a chilled tube containing protease inhibitors: 1 mg EDTA, 10 μl 1 mM phenylmethylsulphonyl fluoride (PMSF) (No. P7626; Sigma Chemical Co., St. Louis, MO, U.S.A.) and 10 μl 0.5 mM pepstatin-A (No. P-4265, Sigma Chemical Co.) per ml of blood. After centrifugation for 20 min at 4000 r.p.m. at 4°C, the separated plasma was immediately extracted or stored at −70°C until assayed. Plasma ANF was measured by radioimmunoassay with prior extraction by heat-activated Vycor glass powder (Corning Glass Works, Corning, NY, U.S.A.) according to the method outlined earlier (Gutkowska, 1987). Urinary cyclic GMP excretion was measured by a previously-described technique (Hamet *et al.*, 1989).

All values are expressed as means±s.e.mean. Statistical differences (to at least $P<0.05$) between group means were assessed by 2-way analysis of variance and the unpaired t test.

Results

TAPP administration to conscious, normally hydrated rats evoked a dose-related increase of urine output. The effect was most prominent during the first hour. As seen in Figure 1, a 10 fold rise in urine volume was observed with the highest TAPP dose (2500 μg kg^{-1}) during the first hour after administration of the drug, as compared to the group receiving saline. This was accompanied by significantly enhanced urinary sodium excretion from 17±4.7 μEq h^{-1} ($n=18$) to 79±12.7 μEq h^{-1} (Figure 1), and urinary potassium excretion from 9.5±2.5 μEq h^{-1} in control rats to 39.4±6.6 μEq h^{-1} in animals receiving 2500 μg kg^{-1} TAPP ($n=18$) (Figure 1).

Since urinary cyclic GMP levels are an index of ANF system activation, we measured this parameter in TAPP-treated and control animals during the first hour after drug administration. Urinary cyclic GMP was augmented in a dose-related manner with a 7 fold increase from 191±21 pmol h^{-1} in control rats to 1340±322 pmol h^{-1} ($n=18$, $P<0.001$) in animals given the highest TAPP dose (2.5 mg kg^{-1}) (Figure 1). Parallel to the renal effects, a significant (23 fold) elevation of plasma ANF was observed 5 min after TAPP administration, rising from 22±4 pg ml^{-1} in animals which received saline to 508±22 in those treated with 2500 μg kg^{-1} TAPP ($n=18$, $P<0.001$) (Figure 2).

To examine whether or not the increase in plasma ANF was a consequence of haemodynamic changes, blood pressure was monitored for 10 min after drug administration. A transient, brief, dose-related decrease of blood pressure was seen 1–5 min after TAPP injection (Figure 3). No changes in blood pressure were found in control rats.

The involvement of opioid receptors in the actions of TAPP was tested by prior administration of the opioid antagonist, naloxone. Pretreatment with naloxone blocked the renal effects of TAPP. Intravenous injection of 0.8 mg naloxone 10 min prior to TAPP administration totally abolished the diuretic action of TAPP. As seen in Figure 4, urinary output of 1.16±0.15 ml h^{-1}, $n=12$, induced by 250 μg TAPP was reduced to 0.15±0.06 ml h^{-1} in naloxone-treated rats, a value which was not different from that determined in rats receiving saline. Sodium excretion was significantly decreased from 57.5±11 μEq h^{-1} to 21.3±8.5 μEq h^{-1}, $n=12$ ($P<0.001$) (Figure 4). Similarly, potassium was decreased from 45.4±9.7 μEq h^{-1}, $n=12$, to 16.1±7 μEq h^{-1} by naloxone pretreatment (Figure 4). The increase in cyclic GMP was also prevented by naloxone (Figure 4).

Pretreatment with anti-ANF serum (0.4 ml) 10 min before TAPP administration significantly blocked urine output, urinary sodium excretion and urinary cyclic GMP, whereas no effect on potassium excretion was observed (Table 1).

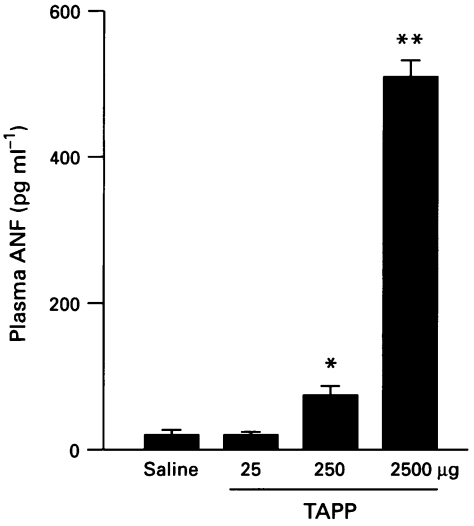

Figure 2 Plasma ANF levels as a function of increasing doses of TAPP 5 min after drug administration. Values are expressed as means±s.e.mean. *$P<0.001$ vs control group.

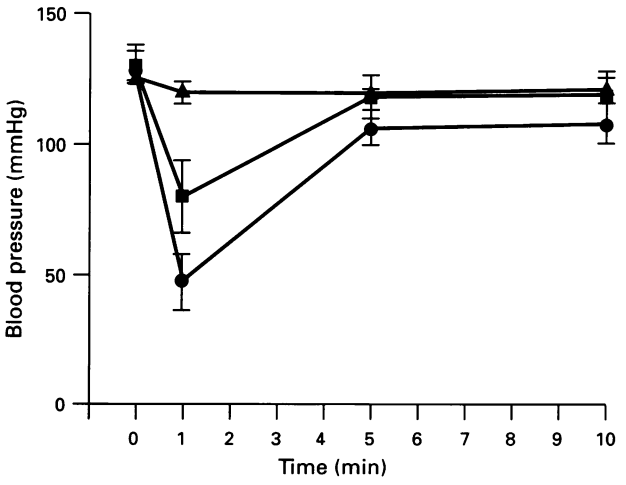

Figure 3 Time course of the effect of 2 doses of TAPP: 25 μg (■) and 250 μg (●) on blood pressure. The control group received 300 ml saline (▲).

Discussion

The present studies provide strong evidence for a peripheral action of μ-opioids on the renal system and demonstrate that this action is mediated by the cardiac hormone ANF. Systemically-administered TAPP, a very selective μ-opioid agonist which is unable to cross the blood-brain barrier, induces potent dose-related diuresis, natriuresis and kaliuresis. These actions are accompanied by an increase in plasma ANF and urinary cyclic GMP excretion, both indices of ANF system activation. Furthermore, confirmation of the involvement of ANF was obtained by using anti-ANF serum which, at least in part, inhibited the TAPP-induced increment of urine output and natriuresis without an effect on kaliuresis. In fact, kaliuresis was slightly decreased under the conditions of this experiment but did not reach statistical significance. The opioid receptor antagonist, naloxone, completely blocked the renal effects of TAPP, indicating that the observed results were due to the activation of peripheral μ-opioid receptors.

Ever since the endogenous opioid peptides Met- and Leu-enkephalin were identified in the brain by Hughes *et al.* (1975), the physiological role of opioids has been studied extensively. There is strong evidence supporting the concept that endogenous and exogenous opioids, acting at various sites in the brain, play an important part in regulating cardiovascular, renal, respiratory and autonomic activities as well as other functions (Holaday, 1983). This contention is supported by: (i) the fact that opioid receptors are found in brain nuclei that are known to control haemodynamic, cardiovascular, respiratory and renal functions (Simantov *et al.*, 1977; Atweh & Kuhar, 1983; Faden & Feuerstein, 1983); (ii) the demonstration that the central opioid system mediates the hypotensive effect of centrally-acting antihypertensive drugs like clonidine (Kunos *et al.*, 1981; Pan & Gutkowska, 1988); and finally (iii) the observation that central administration of opioids has potent cardiovascular and renal consequences (Feuerstein & Faden, 1982; Hassen *et al.*, 1982a,b).

Table 1 Effect of anti-ANF on renal parameters induced by TAPP (250 μg kg^{-1} body weight) administration during the first hour

Treatment	n	UV (ml)	Na^+ (μEq)	K^+ (μEq)	Cyclic GMP (pmol)
NRS	6	1.93±0.28	67.6±10.1	41.6±5.3	750±45.9
Anti-ANF	6	0.88±0.29*	37.9±6.2*	34.2±11.3	472±80.5**

Anti-ANF (0.4 ml) or normal rabbit serum (NRS) (0.4 ml) was administered i.v., 10 min before TAPP (250 μg kg^{-1} body weight). Data are means±s.e. *$P<0.01$ and **$P<0.001$ significantly different from the control group of rats receiving NRS; n = number of animals in each group.

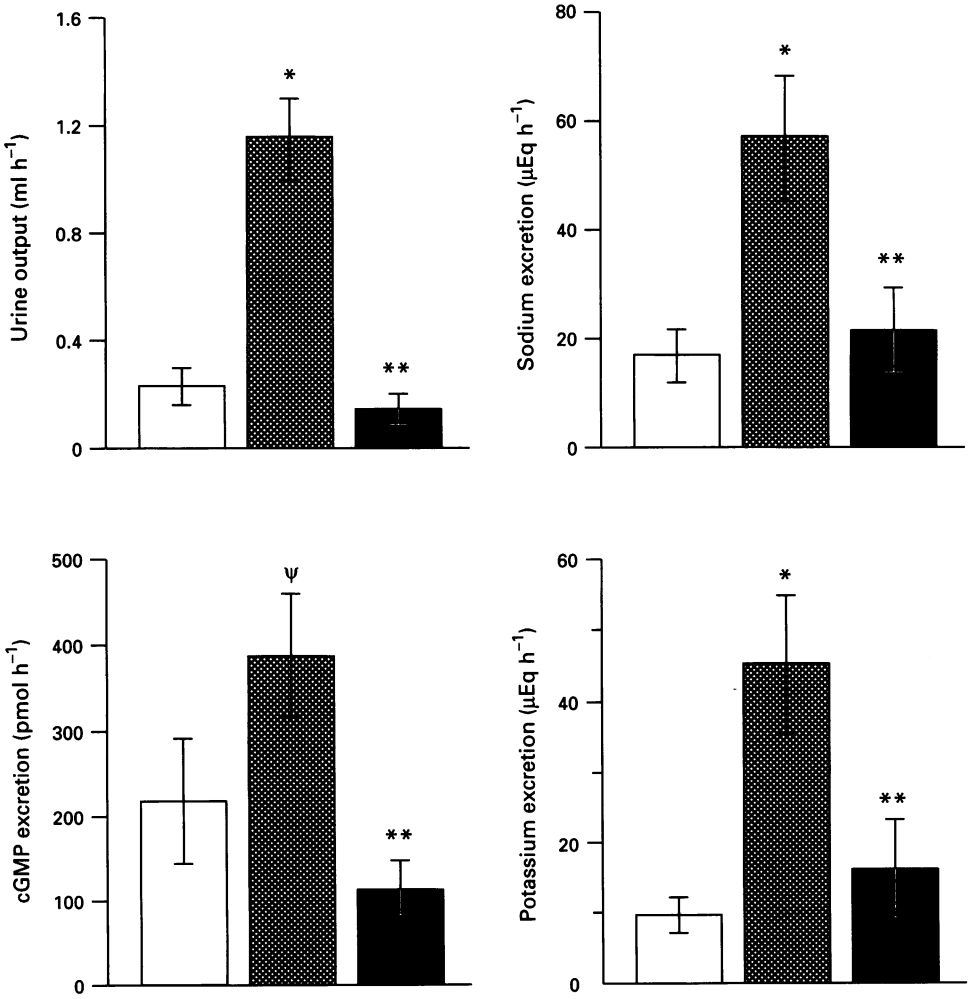

Figure 4 Effect of naloxone (0.8 mg per rat) administered i.v. prior to TAPP (250 μg kg^{-1}) injected i.v. in conscious and normally hydrated rats on urine volume, sodium excretion, potassium excretion and urinary cyclic GMP (cGMP). Values are expressed as means±s.e.mean: $^{\Psi}P<0.02$ and *$P<0.001$, TAPP-treated rats versus controls. **$P<0.001$ naloxone-injected vs TAPP-treated animals. Open columns: animals receiving saline; cross-hatched columns: animals treated with TAPP (250 μg kg^{-1}); solid columns: animals receiving 0.8 mg naloxone and TAPP (250 μg kg^{-1}) 10 min later.

Recent studies from our laboratory have shown that centrally-acting morphine has a strong impact on renal functions (Gutkowska *et al.*, 1993) and that these effects are mediated by ANF which was first discovered by de Bold in 1981 in mammalian atria (De Bold *et al.*, 1981). Over the past decade, a prodigious amount of work has led to the isolation, sequencing and synthesis of the active form of the peptide. Much has been learned about the physiology, pharmacology and mechanism of action of this hormone and about its role in cardiovascular and renal regulation (Ballermann & Brenner, 1985; Maack *et al.*, 1985). ANF is a powerful diuretic, natriuretic and vaso relaxant hormone which, as part of a complex cardiovascular control mechanism, regulates blood pressure and plasma volume by its direct effect on the kidney, adrenal gland and brain, or by interacting with other hormonal systems. It is believed that the main stimulus promoting ANF release directly from the heart is stretch-induced atrial distention. There is also evidence that the pressor agents phenylephrine, angiotensin II or vasopressin elicit a rise in plasma ANF levels *in vivo* (Manning *et al.*, 1985; Garcia *et al.*, 1986). Furthermore, a good correlation between ANF release and atrial pressure has been reported (Katsube *et al.*, 1985).

However, our data obtained in rats (Horky *et al.*, 1985; Gutkowska *et al.*, 1986; 1993) and subsequently confirmed by other investigators (Vollmar *et al.*, 1987), show that opioids like morphine and dynorphin are potent stimuli of ANF release in animals in the absence of any evident haemodynamic changes (Chen *et al.*, 1989; Pesonen *et al.*, 1990). The effect is reversible by naloxone, indicating that it is mediated by opioid receptors (Gutkowska, 1987). Other studies suggest that opioid agonists may stimulate ANF release by a central mechanism. Small doses of centrally-administered morphine increase plasma ANF levels, whereas the same doses given peripherally have no effect (Crum & Brown, 1988; Gutkowska *et al.*, 1993). Since morphine is able to cross the blood-brain barrier to a certain extent, it is conceivable that the ANF release observed after peripheral administration of larger doses may be due to interaction with central opioid receptors.

Another opioid, dynorphin, an endogenous κ agonist which has been found in the heart (Chavkin & Goldstein, 1981), produces a large increase of ANF release from isolated atria or rat cardiocytes when administered *in vivo* or *in vitro* (Tang *et al.*, 1987; Stasch *et al.*, 1989; Yamada *et al.*, 1991). Yamada *et al.* (1991) reported that dynorphin has a direct effect on ANF release, mediated by κ-opioid receptors via inhibition of adenylyl cyclase activity and decreased cyclic AMP production. While animal studies have clearly demonstrated opioid effects on ANF release, the published data from human subjects are controversial. Clinical investigations suggest that opioids have no influence on ANF release in human subjects under basal conditions (Kidd *et al.*, 1987; Borges *et al.*, 1988), although Ögutman *et al.* (1990) found significant ANF release upon morphine administration. There is evidence of a β-endorphin effect on ANF secretion. During exhaustive exercise in humans (Louisy *et al.*, 1989), a significant increase of plasma β-endorphin and ANF has been observed. Administration of opioid antagonists abolished this augmented ANF release. Supporting the role of opioids, Fontana *et al.* (1993) showed that high plasma opioid levels affected ANF release in acute congestive heart failure (CHF) patients, and this effect depended on the intensity of sympathico-adrenergic activity related to the severity of the disease. It has been suggested that in moderate cases of CHF, β-endorphin may directly trigger ANF release, while in severe cases of acute CHF the opioid system may inhibit ANF release not directly but by reducing noradrenaline secretion. Similarly, the endogenous opioid system has been shown to attenuate ANF secretion in the normotensive offspring of hypertensive parents during exercise, whereas no change occurs at rest (Fontana *et al.*, 1994). However, this effect is most likely mediated by reducing noradrenaline release. Nevertheless, a direct action of dynorphin on human cardiac atrial explants has recently been demonstrated in culture (Ationu *et al.*, 1994).

Morphine has been used in many of the studies. However, it is not a very specific μ-opioid agonist. Three major classes of opioid receptors (μ, δ, κ) have been identified in the central nervous system as well as in peripheral tissues. Consequently, the role of these various receptor types in the mediation of cardiovascular and renal effects is of interest. To understand the mechanism and site of opioid-induced renal actions better, we used TAPP, a potent and highly selective μ-opioid agonist which works exclusively in the periphery since it does not cross the blood-brain barrier (Schiller *et al.*, 1989). The results of our studies indicate that TAPP acts peripherally on the renal and cardiovascular system by inducing a dose-related increase of urine output and of urinary sodium, potassium and cyclic GMP excretion. These effects are mediated by opioid receptors since they are blocked by naloxone. The observed increase in plasma ANF levels and partial inhibition of TAPP's renal actions by anti-ANF antibodies led us to propose a new mechanism of μ-opioid-induced renal effects namely, that they are due to enhanced ANF release. Interestingly, the renal actions of TAPP are independent of systemic haemodynamic changes. No increase in blood pressure was observed and, in fact, a small but significant transient decrease was noted. Therefore, we may speculate that TAPP has a direct stimulatory influence on cardiocytes or other cells that may release ANF. Alternatively, the TAPP-induced increase of ANF may occur indirectly via the release of some other endogenous agent. The observation that dynorphin stimulates ANF release from isolated atria is relevant to our findings. However, it is important to note that Ferrari & Agnoletti (1989) were unable to induce ANF release directly from atria by the administration of morphine as well as Met- and Leu-enkephalin. More studies with highly-selective opioid agonists are required to determine which type of opioid receptor present in cardiocytes activates the heart ANF system.

In conclusion, these studies demonstrate marked peripheral effects of a highly selective μ-opioid agonist on ANF secretion in conscious, normally hydrated rats, suggesting that other factors besides haemodynamic changes contribute to the mechanism of ANF release.

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