

Cortisol and ACTH release in dairy cows in response to machine milking after pretreatment with morphine and naloxone

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SUMMARY. The aim of the study was to examine the effect of morphine and naloxone pretreatment on cortisol and ACTH concentrations in response to machine milking in dairy cows. In the first part of the experiment, the effects of i.v. morphine doses (0, 21, 70 and 210 mg, one dose each day) 10 min before morning milking were studied in six Brown Swiss dairy cows. In the second part, four cows were treated 1 d after the control milking with 210 mg morphine at 10 min before milking and the next day with 210 mg naloxone at 15 min before milking followed by 210 mg morphine at 10 min before milking. In addition, four other cows were treated 1 d after the control milking with 210 mg naloxone at 10 min before milking. Pretreatment with morphine significantly suppressed the machine milking-induced increase of cortisol in blood plasma as compared with controls. Naloxone pretreatment overcame the inhibitory effect of morphine and elevated milking-induced cortisol concentrations. Naloxone administration alone significantly increased cortisol concentration resulting from milking as compared with controls. However, ACTH concentrations did not change in either control or treated animals, suggesting an ACTH-independent release mechanism for cortisol during milking. We conclude that the release of cortisol in response to machine milking seems to be modulated by endogenous opioids at the adrenal level and does not appear to be under the control of ACTH.

Cortisol levels increase in blood during normal milking (Gorewit *et al.* 1992; Bruckmaier *et al.* 1993; Tančín *et al.* 1995), but the role of cortisol release during milking is still unclear. Cortisol cannot inhibit milk ejection, even in supraphysiological doses (Mayer & Lefcourt, 1987), but ejection can be inhibited by stress (Bruckmaier *et al.* 1993) or administration of adrenocorticotrophic hormone (ACTH; Van der Kolk, 1990) or corticotrophin-releasing hormone (CRH; Almeida *et al.* 1994). This suggests the involvement of additional factors such as catecholamines. Previous work has clearly shown that milking does not stimulate peripheral catecholamine release (Gorewit & Aromando, 1985; Blum *et al.* 1989). Increased levels of catecholamines were connected with central inhibition of oxytocin release (Lefcourt

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& Akers, 1991; Lefcourt *et al.* 1997). Glucocorticoids play an important role in the formation and maintenance of mammary tight junctions *in vitro* (Zettl *et al.* 1992). Tight junctions may be involved in mammary gland secretion and regulation of milk yield. As shown recently (Stelwagen *et al.* 1998), cortisol seems to be important in the reduction of leakiness in mammary epithelial cell tight junctions in the udders of dairy cows.

ACTH is the principal regulator of cortisol synthesis and secretion, but there is no well documented information on how ACTH concentrations respond to milking. It has been demonstrated in several species that the secretion of ACTH and cortisol can be modulated by opioids. In sheep and cows, the hypothalamo–pituitary–adrenal axis appears to be under suppressive opioidergic control. However, the opioidergic system involved in hypothalamo–pituitary–adrenal functions of an animal under chronic stress behaves in the opposite manner (Nanda *et al.* 1989, 1992; Matthews & Parrott, 1991).

Opioids can inhibit oxytocin release and milk ejection. The cortisol released during milking may have important functions for the mammary gland itself. The role of opioids in the secretion of ACTH and cortisol during milking is not known. The aim of the present study was to investigate whether morphine and naloxone can modulate the release of cortisol and ACTH in response to machine milking in dairy cows.

MATERIALS AND METHODS

Animals

A total of 14 Brown Swiss dairy cows in their first to fifth lactation (body weight 700 ± 35 kg; mean \pm SEM) were used for the experiments. The cows were non-pregnant, in the second to tenth month of lactation and had daily milk productions of 17–41 kg immediately before the experiments. They had free access to a mixed ration providing energy and nutrients for the production of 22 kg milk and received additional concentrates depending on their milk production. Before starting the experiment, the cows were separated from the herd and brought to another part of the building, where they could be housed in individual tie stalls. They were milked using a bucket milking installation. The experiment started when the milk flow profile characteristics and the milk yields were at the same levels as during previous milking in the dairy parlour. At 1 d before the experiment, the cows were fitted with a cannula inserted into the jugular vein (Tančin *et al.* 2000).

Experiment 1

On four consecutive days, six cows were given i.v. injections of saline (control) or morphine (Merck, D-85737 Ismaning, Germany) at 21, 70 or 210 mg (equivalent to 0.03, 0.1 and 0.3 mg/kg body weight) dissolved in 10 ml sterile saline 10 min before (–10.00) the start of manual stimulation (0 min) of the udder to prepare the cows for morning milking (at 07.00). During intensive blood collections around the morning milking, 10 ml samples were collected into tubes containing 200 μ l of a solution containing ethylene dinitrilotetra-acetic acid (disodium salt, 300 μ mol/l; Merck, D-64293 Darmstadt, Germany) plus acetylsalicylate (10 g/l; Serva, D-69042 Heidelberg 1, Germany), centrifuged at 3000 g, and stored at –20 °C until assayed. Blood samples were taken at –20, –15, –10 (except for control milking), –5, 0, +2, +4 and +6 min relative to the start of milking and at 0, 5, 10, 20 and 30 min after the end of milking. Milking times for the control and the three doses (21, 70 and 210 mg) were 7.3 ± 0.4 , 7.2 ± 0.5 , 7.8 ± 0.6 and 10.1 ± 0.5 min respectively.

Experiment 2

In the first part, three treatments were carried out on three consecutive days before morning milking with four cows: saline (–10 min), 210 mg morphine (–10 min) and 210 mg naloxone hydrochloride (Biotrend, D-50933 Köln, Germany; –15 min) followed by 210 mg morphine (–10 min). In the second part, another four cows were treated with saline or 210 mg naloxone alone (–10 min).

Hormone determinations

Plasma cortisol concentrations were measured by a competitive enzyme immunoassay previously characterized and validated (Sauerwein *et al.* 1991). The mean recovery after extraction was $77.5 \pm 8.6\%$ ($n = 18$). The intra-assay and interassay CV were 6–8 and 12.5% respectively.

ACTH was determined by commercial equilibrium radioimmunoassay (Dia Sovin., Stillwater, MI 55082-0285, USA) validated for human plasma by using human ACTH as standard. To test specificity for bovine plasma, validation experiments were performed by injecting human CRH (Bachem, D-69126 Heidelberg, Germany) into cows and by diluting plasma with elevated ACTH levels. ACTH was measured in 0.1 ml bovine plasma. The sensitivity of the assay was 15 pg/ml plasma. The intra-assay and interassay CV were 4–8% and 7.0–12.5% respectively.

Statistical evaluation

Concentrations were measured as area under the curve/min for five phases and expressed as ng/ml or pg/ml. Phase I included values at 20, 15 and 10 min before milking; phase II, 5 and 0 min before milking; phase III, 2, 4 and 6 min after the start of milking; phase IV, 0, 5 and 10 min after milking ended; phase V, 20 and 30 min after milking. For statistical evaluation, a repeated measures analysis of variance was calculated using the MIXED procedure of the SAS program package (SAS, 1995). The model included terms for treatment, phase, and treatment \times phase. The animal was the repeated subject. Least square means differences were localized using Bonferoni's *t* test. Values are presented as means \pm SEM.

RESULTS

Validation of ACTH assay

Three non-lactating, non-pregnant dairy cows (body weight \sim 700 kg) were used for the CRH test. Each cow received one of the three doses of human CRH only (150, 300 or 450 mg). Since there was no clear dose effect, values for the ACTH and cortisol released were combined and are shown in Fig. 1. There was a clear parallel increase of ACTH and cortisol followed by a decrease. Diluted plasma samples with a high ACTH content gave values parallel to the standard curve.

Experiment 1

The effect of different morphine doses on the release of cortisol and ACTH induced by machine milking is shown in Fig. 2. Morphine did not influence the basal levels of cortisol before milking (Fig. 2*a*, phase II). Cortisol concentrations increased significantly after the end of milking (phase IV) in the control group. However, all morphine doses tested inhibited the release of cortisol after milking significantly as compared with controls ($P < 0.05$). In contrast, ACTH concentrations (which were

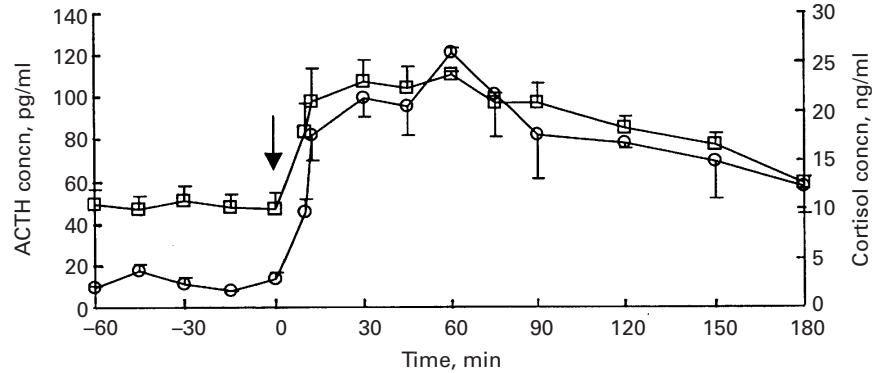


Fig. 1. Effect on the levels of \square , ACTH and \circ , cortisol in the blood of three cows given i.v. human corticotrophin-releasing hormone (150, 300 and 450 mg; one dose for each cow). No dose effect was found, and values are given as means with SEM indicated by vertical bars. \downarrow , Time of injection.

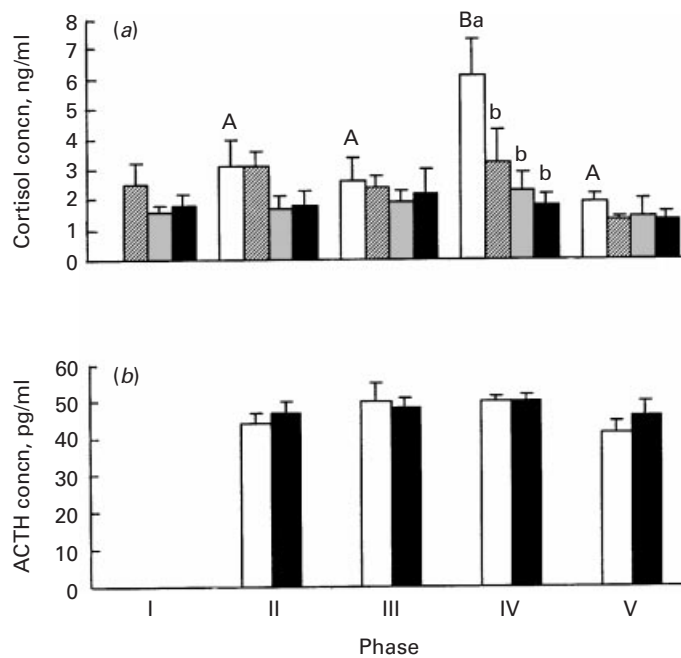


Fig. 2. Concentrations during milking of (a) cortisol and (b) ACTH in the blood of cows after injections of \square , saline and \square , 21; \square , 70 and \blacksquare , 210 mg morphine (equivalent to 0.03, 0.1 and 0.3 mg/kg body weight) at 10 min before milking. Concentrations were measured during five different phases: I, 20, 15 and 10 min before milking; II, 5 and 0 min before milking; III, during milking, 2, 4 and 6 min after the start; IV, 0, 5 and 10 min after the end of milking and V, 20 and 30 min after the end of milking. Values are means for six cows with SEM indicated by vertical bars. In (a), means without common letters were significantly different at $P < 0.05$: a, b, within phase between treatments; A, B, within treatment between phases.

measured only in controls and after 210 mg morphine; Fig. 2b) were unaffected (saline, 47.6 ± 2.8 ; morphine, 4.9 ± 3.3 pg/ml).

Experiment 2

Neither morphine nor naloxone plus morphine influenced the basal levels of cortisol or ACTH (Fig. 3). Cortisol increased significantly after milking (phase IV) in controls (cf. Expt 1). This rise was blocked by morphine. However, naloxone

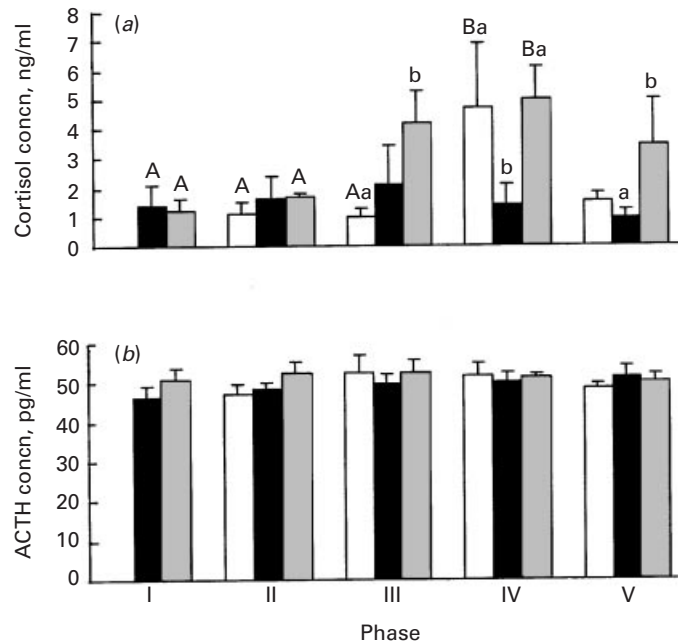


Fig. 3. Concentrations during milking of (a) cortisol and (b) ACTH in the blood of cows after injections of \square , saline (control), \blacksquare , 210 mg morphine at 10 min before milking and \boxplus , 210 mg naloxone at 15 min before milking followed by 210 mg morphine at 10 min before milking. Concentrations were measured during five different phases: I, 20, 15 and 10 min before milking; II, 5 and 0 min before milking; III, during milking, 2, 4 and 6 min after the start; IV, 0, 5 and 10 min after the end of milking and V, 20 and 30 min after the end of milking. Values are means for four cows with SEM indicated by vertical bars. In (a), means without common letters were significantly different at $P < 0.05$: a, b, within phase between treatments; A, B, within treatment between phases.

pretreatment prevented the inhibiting action of morphine. Cortisol had already increased during milking, in contrast to controls (Fig. 3a). The response of ACTH (Fig. 3b) to machine milking was unchanged by the different treatments (control, 50.1 ± 3.9 ; morphine, 49.9 ± 3.2 ; naloxone–morphine, 51.8 ± 2.8 pg/ml).

In controls, the increase in cortisol during phase IV was not significant (Fig. 4), perhaps because of the shorter milking time (5.5 ± 0.8 min). In contrast, treatment with naloxone alone resulted in a significant increase at this stage compared with controls. As before, ACTH levels were not influenced during milking in either group (control, 38.8 ± 2.3 ; naloxone, 38.9 ± 2.5 pg/ml).

DISCUSSION

The basal levels of ACTH found in these experiments were lower (Abebe *et al.* 1993; Wellnitz *et al.* 1997) or higher (Munksgaard & Simonsen, 1996) than in previous studies. Human CRH injections increased ACTH in peripheral blood, as reported for cattle by Abebe *et al.* (1993). The absence of a dose response suggests that a dose of $150 \mu\text{g}$ CRH is sufficient to induce a maximum ACTH response. We concluded that the sensitivity of our test was sufficient to determine ACTH changes in response to milking in dairy cows.

Different premilking treatments with morphine or naloxone did not influence the basal levels of cortisol and ACTH, probably because of the short time between drug administration and the start of milking. A significant decrease of basal cortisol in heifers or cows has been reported 30–40 min after morphine administration

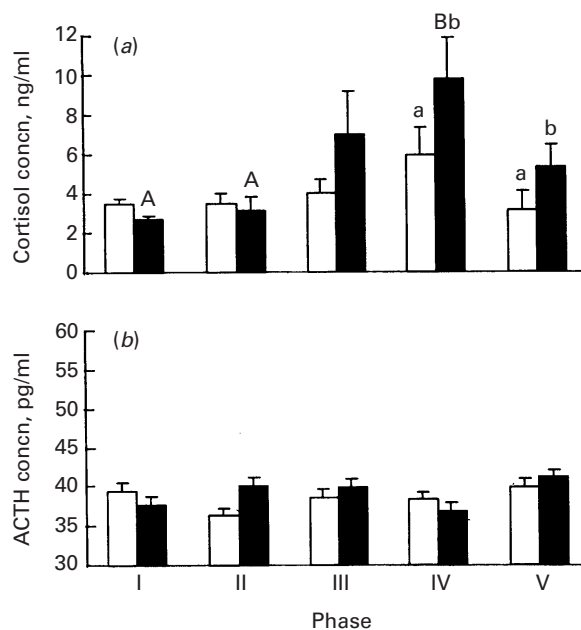


Fig. 4. Concentrations during milking of (a) cortisol and (b) ACTH in the blood of cows after injections of \square , saline (control) and \blacksquare , 210 mg naloxone at 10 min before milking. Concentrations were measured during five different phases: I, 20, 15 and 10 min before milking; II, 5 and 0 min before milking; III, during milking, 2, 4 and 6 min after the start; IV, 0, 5 and 10 min after the end of milking and V, 20 and 30 min after the end of milking. Values are means for four cows with SEM indicated by vertical bars. In (a), means without common letters were significantly different at $P < 0.05$: a, b, within phase between treatments; A, B, within treatment between phases.

(Armstrong & Johnson, 1989; Nanda *et al.* 1992), and a stimulatory effect of naloxone on cortisol has been demonstrated: slight after 15 min and clear after 30 min (Nanda *et al.* 1992). Cortisol concentrations were elevated in response to machine milking. However, in some cows this cortisol increase was not clear, which is not surprising (Faltys *et al.* 1987). Morphine injected 10 min before milking in our study suppressed cortisol release in response to hand and machine stimulation. There are no directly comparable studies available, only those under stress conditions, where a μ -receptor agonist suppressed psychological stress-induced cortisol release (Parrott & Thornton, 1989; Matthews & Parrott, 1991; Domanski *et al.* 1993), or after CRH administration (Redekopp *et al.* 1985). In the present experiment naloxone abolished the inhibitory effect of morphine on cortisol release and even accelerated cortisol release in response to machine milking. As compared with control milking, cortisol concentrations were clearly elevated after naloxone administration in all our experimental cows. These results agree with observations in dairy cows when under basal conditions morphine failed to alter the stimulatory effect of naloxone on cortisol release (Nanda *et al.* 1992), and when naloxone alone potentiated cortisol release in response to the stress evoked by transport of cows (Nanda *et al.* 1989). From the cortisol results it could be concluded that the hypothalamo–pituitary–adrenal axis of dairy cows during milking appears to be under suppressive opioidergic control. Surprisingly, control milking and milking with naloxone pretreatment had no effect on ACTH, though cortisol levels were significantly increased. Thus, the stimulatory effect of naloxone on cortisol release seems not to be ACTH-dependent.

To our knowledge, this is the first evidence that the cortisol increase during milking under normal conditions seems to be regulated by mechanisms other than those involved in stress. It seems that the hypothalamo–pituitary–adrenal axis is not activated in response to machine milking. Thus, the stimulatory effect of naloxone on cortisol concentrations in dairy cows seems to be at the level of the adrenal glands. However, it has been claimed that naloxone probably has no direct effect on the adrenal cortex in the rat (Douglas *et al.* 1998), because naloxone could not stimulate cortisol release after hypophysial stalk transection in gilts (Estienne *et al.* 1988). However, there is evidence that opioids may act on steroid secretion at the adrenal cortex level through a specific μ -opioid receptor (Kapas *et al.* 1995). Moreover, in non-stressed sheep intracerebroventricular injection of naloxone did not affect cortisol secretion as i.v. injection did (Parrott & Goode, 1993). We assume from our results that the increase of cortisol in response to machine milking with or without naloxone pretreatment could be regulated directly from the adrenal cortex. There is evidence that the autonomic system can influence adrenocortical steroidogenesis by splanchnic nerve activation (Ehrhart-Bornstein *et al.* 1995; Edwards, 1997). However, under such conditions naloxone has suppressive rather than stimulatory effects on cortisol release in calves (Bloom *et al.* 1988; Edwards & Jones, 1989) and the response of the adrenal cortex to ACTH is not affected by naloxone (Edwards & Jones, 1989).

In conclusion, i.v. morphine suppressed cortisol release in response to machine milking and the effect was reversible by naloxone. The accelerating effect of naloxone on cortisol release seems to be evoked by further inhibition of endogenous opioids at the adrenal cortex level. The cortisol increase in response to machine milking seems not to be regulated by ACTH. Hence the mechanisms involved in cortisol release in response to machine milking need further investigation.

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