Gibbson and Edsall[14], and CA-B may contribute less to the total CO_2 hydrase activity; thus, the physiological meanings of the changes in CA-B levels remain to be elucidated. In normal subjects, inactive CA-B enzymes also seem to participate in the degradation process of the enzyme. There was a significant decrease in the total esterase activity in our previous study[7] and a decreasing tendency in the specific activity of CA-B in the present study, and one probable explanation for these results is that the active CA-B enzymes were converted in part to inactive enzymes with the exercise, possibly by binding to inhibitors or decreased zinc binding. Carbonic anhydrase has 1 atom of zinc per molecule of enzyme in its active center[4]. In an earlier study we found an inactive form of CA-B in the erythrocytes of children with primary renal tubular acidosis[15]. The addition of zinc chloride to hemolysates from these patients resulted in a marked increase in the activity of this enzyme. Quite recently we found that the zinc levels in erythrocytes vary during physical exercise (unpublished data). It was suggested that physical stress might have some effect on the affinity between CA-B enzyme protein and zinc. The simultaneous estimation of active and inactive enzyme in vivo provides useful data for physiological and experimental problems.

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Endotoxin inhibits the fluoride-stimulated adenylate cyclase activity of rat liver plasma membranes enriched with bile canaliculi

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Summary. Escherichia coli endotoxin inhibited the fluoride-stimulated adenylate cyclase activity of liver plasma membranes enriched with bile canaliculi. Inhibition was of a mixed competitive and uncompetitive type. This effect, which may result from changes of membrane organization, may have relevance in the understanding of endotoxin-cell membrane interactions.

Bacterial endotoxin (lipopolysaccharide, LPS) affects many aspects of hepatic functions[2]. Since LPS has high affinity for biological membranes[3] it may act by altering membrane configuration and membrane-bound enzyme activities, thereby triggering intracellular events[4]. In studying the mechanisms of LPS effect on the liver, we have previously found that the cholestatic effect induced by LPS[5,6] was paralleled by changes of membrane-bound Na^+,K^+-AT-Pase, which is involved in bile formation, while other membrane enzymes (i.e. Mg^{++}-ATPase and 5′-nucleotidase) were unaltered[7,8]. Since adenylate cyclase also plays a role in water and electrolyte transport[9], and may modulate LPS effects[4], we studied the effect of LPS on the activity of adenylate cyclase in preparations of rat liver plasma membranes (LPM) enriched with bile canaliculi.

Material and methods. Purified *Escherichia coli* 0127:B8 LPS, Boivin type, was purchased from Difco. ATP, phosphoenolpyruvate and pyruvate kinase were purchased from Boehringer; (α-^{32}P)ATP and (^{3}H)cyclic AMP from Amersham.

LPM enriched with bile canaliculi were prepared from male Sprague Dawley rats (250–300 g; Morini, Italy) as described by Boyer and Reno[10] with minor modifications[7]. LPM were suspended in the same buffer used for adenylate cyclase assay at concentration of 1–2 mg/ml and used the same day. Protein was determined by the method of Lowry et al.[11].

Adenylate cyclase activity was determined by measuring the conversion of ATP to cyclic AMP. Each sample (100 µl) contained 5 mM $MgCl_2$, 1 mM EDTA, 2.5 mM theophylline, 5 mM ATP containing 1.5 µCi of (α-^{32}P)ATP, 50 mM Tris-HCl buffer, pH 7.6. Phosphoenolpyruvate (5 mM) and pyruvate kinase (60 µg/ml) were used as ATP-regenerating system. LPS, when used, was dissolved in Tris buffer and added at concentrations from 20 to 200 µg/ml. The reaction was started by adding 40–100 µg of membrane proteins. The samples, in triplicate, were incubated for 10 min at 30 °C and the reaction was stopped by the addition of 0.1 ml of 0.5 M EDTA, pH 7.6, immediately followed by 3 min boiling. Then, 0.8 ml of cold 50 mM Tris-HCl buffer were added to the samples and they were centrifuged at 4 °C for 10 min. (^{3}H)Cyclic AMP (24 Ci/mM) equivalent to 30,000 cpm was added for the determination of recovery. The (^{32}P)cyclic AMP formed was isolated according to Salomon et al.[12]. Phosphodiesterase activity was measured using an incubation mixture identical to that for adenylate cyclase except that the ATP-regenerating system was omitted and the ATP was replaced with a trace amount of

(^{3}H)cyclic AMP (30,000 cpm). Cyclic AMP was isolated by chromatography on alumina columns[13]. TLC of reaction products was done on silica gel plates (Merck F254) using isopropyl alcohol, water, ammonium hydroxide (7/2/1) as solvent mixture and unlabeled ATP, ADP, AMP, and cyclic AMP as standards.

Results. In the LPM preparations used in this study, which displayed an enrichment of bile canaliculi and basolateral membranes[7], basal adenylate cyclase activity was proportional to membrane protein concentration from 40 to 100 µg. Sodium fluoride (10 mM) stimulated adenylate cyclase activity 6–7-fold in these preparations. Although LPS did not affect basal adenylate cyclase activity, it caused a dose-dependent inhibition of fluoride-stimulated adenylate cyclase at concentrations of 20–80 µg/ml. Maximal inhibition (50%) was observed at LPS concentrations from 80 to 200 µg/ml (fig. 1). In the presence of heat-treated enzyme, LPS did not affect cyclic AMP recovery using chromatographic procedures, nor the stability of ATP or of cyclic AMP, as determined by TLC. Over a 5-min incubation period, LPS (at 80 µg/ml) inhibited the fluoride-stimulated adenylate cyclase to the same extent whether in the presence or in the absence of an ATP-regenerating system. In addition, no effect was found on the phosphodiesterase activity in LPM preparations. Kinetic studies showed that LPS (at 80 µg/ml) caused a mixed type of inhibition. The double-reciprocal plot indicated that LPS decreased both V_{max} and the apparent K_m (fig. 2). This could reflect an interaction of LPS with either the enzyme or the enzyme-substrate complex.

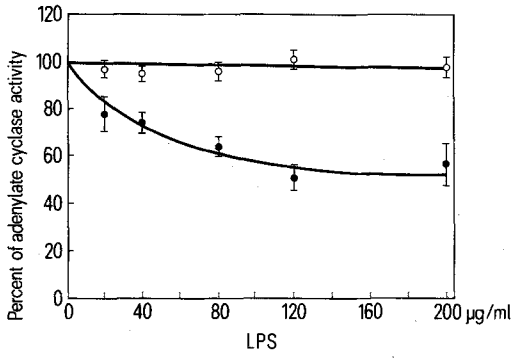

Figure 1. The effect of *Escherichia coli* LPS on the basal (○----○) and the fluoride-stimulated (●----●) adenylate-cyclase activity in rat liver plasma membranes enriched with bile canaliculi. Data are expressed as percent of controls which corresponded to 66± 7 and 401± 19 pmoles cyclic AMP/mg protein/min for the basal and the fluoride-stimulated activity, respectively. Incubation mixture contained in a final volume of 0.1 ml the following: 5 mM $MgCl_2$, 1 mM EDTA, 2.5 mM thophylline, 5 mM ATP containing 1.5 µCi of (α-^{32}P)ATP, 50 mM Tris-HCl buffer pH 7.6, 5 mM phosphoenolpyruvate and 60 µg/ml pyruvate kinase. The reaction was started by addition of membrane proteins (40–100 µg), carried out for 10 min at 30 °C and stopped by addition of 0.1 ml of 0.5 M EDTA containing 30,000 cpm (^{3}H)cyclic AMP (24 Ci/mM) for determination of recovery, and by boiling for 3 min. Results are mean ± SE of 6 experiments, each in triplicate.

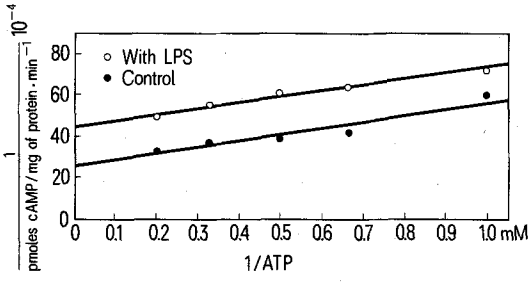

Figure 2. Double reciprocal plot of the fluoride-stimulated adenylate-cyclase activity vs increasing ATP concentrations in absence or presence of LPS (80 µg/ml). In controls, V_{max} and K_m-values were 444 pmoles cyclic AMP/mg protein/min and 1.2×10^{-3} M, respectively; while, in presence of LPS, V_{max} was 235 and K_m 0.64×10^{-3} M.

Discussion. The present study has demonstrated that LPS inhibits the fluoride-stimulated adenylate cyclase in rat LPM and that it exerts a mixed type of inhibition (figs 1 and 2). Endotoxin with its lipidic groups interacts with the hydrophobic phase of membranes and decreases their fluidity[14,15]. Since adenylate cyclase, as well as Na^+,K^+-ATPase[16], is highly sensitive to changes of membrane lipidic composition[17], it seems reasonable to suggest that the inhibition of the fluoride-stimulated adenylate cyclase could be the consequence of a LPS-induced alteration of membrane organization. Consistent with this view are the kinetic data which show a mixed type of inhibition (fig. 2) and the fact that only the fluoride-stimulated activity was affected by LPS (fig. 1). Indeed, decreases in membrane fluidity which alter the physical interaction between the regulatory and the catalytic units of adenylate cyclase[18] may inhibit the fluoride-stimulated more easily than the basal activity[19]. At similar concentrations LPS reduces bile flow[5,6] which is also affected by changes of LPM lipidic organization[20]. A similar effect has been observed for chlorpromazine and ethynil estradiol which intercalate into the lipidic phase of the membrane and cause both cholestasis[21,22] and inhibition of the fluoride- or glucagon-stimulated adenylate cyclase[23,24]. In other biological systems[25–27] LPS has been reported to increase basal adenylate cyclase activity, while contrasting reports exist on LPS effects on hormone- and fluoride-stimulated adenylate cyclase in mouse liver[28,29]. These discrepancies may in part be the result of different methodological procedures. However, like other membrane-active agents[19], endotoxin may exert a different effect on adenylate cyclase depending upon the LPS concentration and the phospholipidic composition of the membrane. The change in membrane organization induced by LPS may be very important in the elucidation of the mechanism of action of LPS on various biological targets.

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Use of the specific benzodiazepine antagonist, Ro 15-1788, in studies of physiological dependence on benzodiazepines

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Summary. The specific benzodiazepine antagonist, Ro 15-1788, elicited withdrawal symptoms in squirrel monkeys, cats, rats and mice made tolerant and physically dependent by subchronic administration of high doses of diazepam, lorazepam or triazolam.

Physiological (physical) drug dependence is the altered physiological state which results from the long-lasting presence of high drug concentrations in the body and which necessitates the maintained administration of the drug in order to prevent the appearance of unpleasant somatic and/or psychic symptoms[1]. For most drugs, the only practicable way of detecting and quantifying physiological dependence in animals is to search for, and to measure, withdrawal (abstinence) symptoms that appear upon abrupt cessation of repeated drug administration. The occurrence, intensity and profile of the abstinence syndrome are, however, not a simple function of the severity of the state of physiological dependence, but also depend critically on the rate at which the pharmacological effects of the last drug dose disappear; this rate is in turn determined by the kinetics of dissociation of the drug from its receptors, the reversibility of the cellular events that are induced by the drug-receptor interaction, and the rate of elimination and/or metabolic inactivation of the drug. It is evident that the intensity of withdrawal symptoms should be most pronounced when the drug, which induces physiological dependence, is displaced from its receptors within the shortest possible time. Until recently opiates were the only psychotropic drugs for which specific antagonists were available. The dramatic withdrawal symptoms that are precipitated in opiate-dependent animals by opiate antagonists, such as naloxone, greatly contributed to the view of opiates being the drugs with a dependence liability par excellence.

Many other centrally (and even peripherally) acting agents are also capable of inducing physiological dependence under certain conditions. Among them, barbiturates lead to a type of physiological dependence which is clearly different from that induced by opiates. A physiological dependence similar in quality to, but milder in intensity than, the barbiturate type is induced by e.g. meprobamate and benzodiazepines[2]. Recently we have found potent and highly specific blockers of benzodiazepine receptors[3-6]; we anticipated that such compounds should be able to precipitate withdrawal symptoms in animals made physiologically dependent on benzodiazepines. We here report our findings with the specific benzodiazepine antagonist, Ro 15-1788.

Methods. Mice (SPF albino Füllinsdorf, 18–20 g), rats (SPF albino Füllinsdorf, 70–90 g) and mongrel cats (2.5–4.8 kg) of either sex as well as male squirrel monkeys (0.8–1.3 kg) were used. Mice and rats were administered a daily dose of 10 or 100 mg/kg diazepam p.o. for 12 days. Cats and monkeys were given a fixed dose of diazepam, lorazepam, triazolam or midazolam i.p. or p.o. for 15, 16, 18, or 21 days. The benzodiazepine antagonist, Ro 15-1788, dissolved in 0.3% v/v Tween 80 in distilled water, was injected i.v. or i.p. at various times after the last dose of the benzodiazepines. The animals were observed until withdrawal symptoms disappeared; the monkeys were controlled for 72 h by video-recording. In 3 cats, the bipolar EEG of the parietal and occipital cortex and dorsal hippocampus was also recorded.

Results. All doses of the benzodiazepines used were considerably higher than effective anxiolytic and anticonvulsant doses; they produced acute effects such as ataxia or decrease of vigilance. Complete or nearly complete tolerance to the latter effects developed in all species with repeated administration (diazepam and lorazepam within 5–8 days, triazolam within 3–5 days and midazolam within 3 days.

Withdrawal symptoms induced by 10 mg/kg Ro 15-1788 i.v. in mice and rats treated for 12 days with diazepam (10 or 100 mg/kg p.o. daily) are listed in the table. In mice, seizures lasting 2–3 sec occurred about 15 min after injection of the antagonist. All other symptoms appeared a few minutes after injection of the antagonist and lasted approximately 30 min. In rats, exophthalmos and a decrease of respiratory rate were seen for 45 min and 1.5–2 h, respectively.

In cats (N = 2) injected with 10 mg/kg lorazepam i.p. twice daily or 1 mg/kg triazolam i.p. once daily for 16 days, 100 mg/kg Ro 15-1788 i.p. either immediately or 1.5 h, 6 h, 12 h, 48 h or 60 h after the last dose evoked rigidity, vocalization and tachypnoe for 30 min as well as hypersalivation for 2 h (clonic seizures were observed only in the triazolam group, when Ro 15-1788 was injected 1.5 h after the last dose). Ro 15-1788 elicited similar withdrawal symptoms in cats treated with 30 mg/kg diazepam i.p., but only when injected within 12 h after the last dose; seizures were not observed. However, in 3 cats spike-wave activities