Effects of diltiazem, a Ca\(^{2+}\) channel blocker, on naloxone-precipitated changes in dopamine and its metabolites in the brains of opioid-dependent rats

Abstract
The effects of diltiazem, an L-type Ca\(^{2+}\) channel blocker, on naloxone (an opioid receptor antagonist)-precipitated withdrawal signs and changes in extracellular levels of dopamine (DA) and its metabolites in various brain regions of morphine (a μ-opioid receptor agonist) or butorphanol (a μδκ mixed opioid receptor agonist) dependent rats were investigated using high performance liquid chromatography fitted with an electrochemical detector (HPLC-ED). Rats were rendered opioid-dependent by continuous intracerebroventricular (ICV) infusion with morphine (26 nmol/μl per h) or butorphanol (26 nmol/μl per h) for 3 days. The expression of physical dependence produced by these opioids, as evaluated by naloxone (5 mg/kg, IP)-precipitated withdrawal signs, was reduced by concomitant infusion of diltiazem (10 and 100 nmol/μl per h). Under the same condition, naloxone decreased the levels of DA in the cortex, striatum, and midbrain; 3,4-dihydroxyphenylacetic acid (DOPAC) in the cortex, striatum, limbic areas, and midbrain; and homovanillic acid (HVA) in the striatum, limbic areas, and midbrain regions. In animals rendered dependent on butorphanol, the results obtained were similar to those of morphine-dependent rats except for the changes in DOPAC levels. Furthermore, concomitant infusion of diltiazem and opioids blocked the decreases in levels of DA, DOPAC, and HVA in a dose-dependent manner. These results suggest that the augmentation of intracellular Ca\(^{2+}\) mediated through L-type Ca\(^{2+}\) channels during continuous opioid infusion results in a decrease in extracellular levels of DA and its metabolites in some specific regions, which are intimately involved in the expression of withdrawal syndrome precipitated by naloxone.

Key words
Opioid dependence · Ca\(^{2+}\) channel · Diltiazem · Dopamine · HPLC-ED · Morphine · Butorphanol

Introduction
Several studies have demonstrated that the administration of opioids modifies levels and movement of Ca\(^{2+}\) in the brain of several animal species (Sanghvi and Gershon 1976; Harris et al. 1977; Bhargava 1978). This biochemical action of opioids has been correlated both with their antinociceptive effects and with their dependence liabilities. Presently, Ca\(^{2+}\) channels are classified into six electrophysiologically and pharmacologically different types: T-, L-, P-, Q-, and R-type (Varadi et al. 1995). It has been noted that L-type Ca\(^{2+}\) channel plays an important role in the expression of withdrawal syndrome from various centrally acting drugs, such as ethanol (Little et al. 1986; Littleton et al. 1990; Watson and Little 1994), benzodiazepines (Dolin et al. 1990), clonidine (Barrios et al. 1993), and opioids (Bongianni et al. 1986; Baeyens et al. 1987; Barrios and Baeyens 1991). In addition, we have also demonstrated that diltiazem, an L-type Ca\(^{2+}\) channel blocker, inhibited an expression of withdrawal syndrome precipitated by naloxone (an opioid receptor antagonist) in rats rendered dependent on morphine (a μ agonist) or butorphanol (an agonist/antagonist) (Tokuyama et al. 1995a).

It is well known that monoaminergic and opioid neuronal pathways are closely linked in several brain regions of rats (Miller 1981). Both acute and chronic administrations of morphine and other opioids alter the turnover of three monoamine neurotransmitters: norepinephrine, dopamine (DA), and 5-hydroxytryptamine, suggesting that these amines are intimately involved in the development of tolerance to and dependence on opioids (Garcia-Sevilla et al. 1978;
Wakabayashi et al. 1995). To the best of our knowledge, no information is available on effects of Ca\(^{2+}\) channel blockers on naloxone-precipitated changes in dopamine and its metabolites in various brain regions of opioid-dependent rats, although verapamil and nimodipine, Ca\(^{2+}\) channel blockers, have been reported to prevent the modifications of norepinephrine metabolism which are associated with the withdrawal syndrome (Bongianni et al. 1986).

The present study was designed to elucidate the involvement of Ca\(^{2+}\) channel in opioid physical dependence; specifically, effects of diltiazem on naloxone-precipitated changes in levels of DA and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), were examined in various brain regions of animals that had been rendered dependent by continuous ICV infusion of morphine or butorphanol.

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**Materials and methods**

**Animals**

Male Sprague-Dawley rats weighing 230–250 g (Charles River, Wilmington, Mass.) were purchased and housed in a group of three or four animals in a cage. They were kept in a room maintained at 21 ± 2°C and a 12-h light-dark cycle with free access to food and tap water. After reaching 280–300 g, they were used for experiments.

**Surgical procedures**

Rats were anesthetized with Equithesin (4.25 g chloral hydrate, 2.23 g MgSO\(_4\) 7H\(_2\)O, 0.972 g sodium pentobarbital, 44.4 ml propylene glycol, 10 ml 95% ethanol, in a final volume of 100 ml), 0.3 ml/100 g body weight, IP, and then placed in a stereotaxic apparatus. An indwelling stainless steel guide cannula (26 gauge, 10 mm long) was implanted into the right lateral cerebral ventricle (AP: –0.5 mm, LAT: +1.3 mm, and DV: –4.5 mm) with the bregma chosen as the stereotaxic reference point (Paxinos and Watson 1986). Dental acrylic cement (Lang Dental MFG Co., Wheeling, Ill.) was applied to the surface of the skull, and a protective cap was placed around the cannula. After the acrylic had hardened, the animal was removed from the stereotaxic frame. A stylet (32 gauge stainless steel tubing) was placed into the guide cannula to maintain patency. The presence of cerebrospinal fluid in the guide cannula was examined to assure proper placement.

After surgery, rats were given 300,000 units of procaine penicillin G (Pfizer-AS, Pfizer Corp., New York, N.Y.), SC, to prevent infection and were allowed at least 1 week to recover before infusion of morphine-HCl (Sigma, St Louis, Mo.), butorphanol-tartrate (17-cyclobutylmethyl-3,14-dihydroxy morphinan; a generous gift from Bristol-Myers-Squibb Corp., Evansville, Ind.) and/or diltiazem-HCl{(2S-cis)-3-(acetyloxy)-5-[2-(dimethylamino)-ethyl]-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4(5H)-one; Sigma, St Louis, Mo.} commenced.

**Administration schedule and induction of morphine and butorphanol dependence**

Animals were infused ICV continuously with saline (1 µl/h), morphine (26 nmol/µl per h), butorphanol (26 nmol/µl per h), and/or diltiazem (10 or 100 nmol/µl per h) for 3 days via osmotic minipumps (Alzet 2001, Alza Corp., Palo Alto, Calif.). This infusion period and dose paradigm were determined to be optimal from our previous experiments (Jaw et al. 1993a,b). In the case of concomitant infusion of opioid and diltiazem, the drugs were mixed in the same pump. Under ether anesthesia, animals were implanted SC with minipumps between the scapulae. A 4-cm piece of Tygon tubing (0.38 mm inner diameter, Cole-Palmer, Chicago, Ill.) was applied to connect the minipump to a piece of L-shaped stainless steel injector tubing (32 gauge, 30 mm long) with one end having the same length as the guide cannula. All drug solutions were passed through a 0.2 mm sterile Acrodisk filter (Gelman Sciences, Ann Arbor, Mich.) before being introduced into the minipumps, and the delivery apparatus was assembled under sterile conditions. Minipumps were primed overnight at room temperature in normal saline so that an optimal flow rate (1 µl/h) was obtained. Rats were injected with naloxone (Sigma, St Louis, Mo.), 5 mg/kg, IP, 2 h after the termination of drug infusion, performed by cutting the tubing.

**Measurement of behavioral signs during morphine and butorphanol withdrawal**

Ten distinct behaviors (escape behavior, wet dog shakes, teeth chattering, rearing, locomotion, stretching, scratching, salivation, penile licking, and ptosis) were scored during a 15-min period following the naloxone injection as behavioural signs of withdrawal. The reactions of each animal were evaluated by an independent observer who did not have prior knowledge of the nature of the treatment received.

**Extraction of biogenic amines and their metabolites**

Immediately after the 15-mm observation period for withdrawal behavior, rats were killed by decapitation, and their brains were rapidly removed and rinsed with ice-cold saline. Six brain regions (cortex, striatum, limbic areas, midbrain, pons/medulla, and cerebellum) were separated according to the method of Glowinski and Iversen (1966). The brain regions were immediately frozen in liquid nitrogen and stored at –80°C until extraction. Frozen tissues were weighed and transferred to a 1.5-mL polypropylene tube, and each tissue was homogenized in 0.5 mL of 0.1 M perchloric acid containing a pre-determined quantity of isoproterenol as an internal standard. The homogenates were centrifuged at 14000 rpm for 15 min at 4°C and supernatants were filtered through a Nalgene syringe filter (0.45 µm). A 5 to 20-µl aliquot of the clear filtrate was injected into the HPLC-ED system (see below).

**Measurement of DA and its metabolites**

Measurements were conducted using an HPLC-ED system which consists of a model PM-80 pump (Bioanalytical Systems; BAS, West Lafayette, Ind.) and a Rheodyne 7125 injection valve (Cotati, Calif., USA) with a 100-µm sample loop. A separation column of Eicom MA-50 ODS (150 × 4.6 mm id, Eicom, Kyoto, Japan) and a guard column (10 × 4.6 mm id, ODS, Eicom, Kyoto, Japan) were operated at ambient temperature. The electrochemical detector was a Model LC-4C (BAS, West Lafayette, Ind.) with a glassy carbon working electrode (teflon cell gasket, 0.002) and an Ag/AgCl reference electrode. The applied oxidation potential was set at +0.75 V. A mixture of 0.1 M citrate buffer (pH 3.5)–methanol (85:15, v/v) containing 1.0 mM sodium octyl sulfate and 0.02 mM EDTA was used as the mobile phase at a flow rate of 1.0 mL/min. The mobile phase was passed through a 0.45 µm membrane filter and degassed under a vacuum. Data were recorded and evaluated using a Hewlett Packard Model 3390 A integrator (Avondale, Pa.).