EFFECT OF CALCIUM CHANNEL BLOCKERS ON STRESS-INDUCED VISCERAL, ENDOCRINOLOGICAL AND IMMUNE RESPONSES

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ABSTRACT

In the present study effects of five commonly used calcium channel blockers (CCBs) belonging to different chemical classes have been investigated on stress-induced modulation of some visceral, endocrinological and immunological parameters in rats and mice. Restraint stress (RS) produced gastric mucosal lesions, increased plasma corticosterone levels and reduced antiSRBC antibody titre, a measure of humoral immune response and % leucocyte migration inhibition (% LMI) and foot pad thickness, measures of cell-mediated immunity. Nimodipine (10, 20, 40 mg/kg, i.p.) and flunarizine (10, 20, 40 mg/kg, i.p.) attenuated the effect of RS on all the studied parameters. However, verapamil (5, 10, 20 mg/kg, i.p.), diltiazem (10, 20, 40 mg/kg, i.p.) and nifedipine (10, 20, 40 mg/kg, i.p.) antagonised the effect of RS on gastric ulcerogenesis and plasma corticosterone levels but failed to modulate the stress-induced suppression of both humoral and cell-mediated immune responses. Thus, CCBs appear to modulate stress-induced visceral, endocrinological and immune responses, albeit in a differential manner.

KEY WORDS: Restraint stress; Gastric ulcerogenesis; Corticosterone levels; Immune responses

INTRODUCTION

Acute and chronic stress experiences are known to alter the visceral, endocrinological and immunological status of the organism (1-5). Both central and peripheral neurotransmitter mechanisms appear to play important modulatory roles in the mediation of stress responses. These mechanisms include adrenergic (6), dopaminergic (7), cholinergic (8), GABAergic (9) and opioidergic (10) systems.

Calcium is an ubiquitous second messenger which links membrane excitation with subsequent intracellular enzymatic responses. Changes in the cytosolic Ca^{2+} concentration depend on ion influx via voltage-sensitive and receptor-operated Ca^{2+} channels as well as release of Ca^{2+} from intracellular

stores (11). Sensory neurons have been shown to possess 5 distinct types of voltage dependent Ca^{2+} channels designated as L, N, T, P and Q. The L, N- and P/Q-type Ca^{2+} channels have been implicated to play an important role in mediating Ca^{2+} influx that triggers depolarization evoked neurotransmitter release from nerve terminals (12,13). Calcium channel blockers (CCBs) are the agents which block the entry of Ca^{2+} into the cell by interacting with voltage-dependent Ca^{2+} channels. Strong evidence suggests that CCBs by this mechanism influence higher nervous system functions and are reported to possess antidepressant, neuroleptic, anticonvulsant, sedative, analgesic and anxiolytic-like activities (14). By the same Ca^{2+} -influx inhibiting effect they have also been shown to modulate NMDA receptor antagonist, diazocipine-induced behaviours in rats (15). In our previous studies, antianxiety agents, like benzodiazepine group of drugs have been noted to modulate stress induced changes in somatic and immune responses (5, 16, 17). Taking cue from the

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anxiolytic-like effect of CCBs (14) the present study was undertaken to investigate the effects of five clinically used CCBs belonging to different chemical groups on stress induced modulation of some visceral, endocrinological and immunological parameters.

MATERIALS AND METHODS

Animals

The study was carried out in male Wistar rats (180-220g) and Swiss albino mice (25-30g). The animals were procured from the local animal dealer and housed in light (12h light - 12h dark cycle) and temperature ($22\pm 2^\circ\text{C}$) controlled conditions. Pellet diet (Brooke Bond Lipton India Ltd.) and water were available ad libitum except when indicated. Animal care was as per the 'Guidelines for the Care and Use of Laboratory Animals' prepared by the Indian National Science Academy, New Delhi (18).

Drugs

Diltiazem (Torrent, India), verapamil (Isoptin, German Remedies), nimodipine (Cipla Ltd.), nifedipine (Cipla Ltd.), flunarizine (FDC) were used and dissolved in normal saline, except the nimodipine which was solubilized in 100% ethanol. Doses of different drugs were selected on the basis of pilot studies and previous reports.

(i) Stress procedure

The rats were sensitized with sheep red blood cells (SRBC; 0.5×10^9 cells/ml/100g, i.p.) on day 0 (sensitizing dose). On day 7, the animals received the same dose of the antigen again (booster dose). Immediately after the booster dose the rats were deprived of food but not water for 18h and then subjected to restraint stress (RS) individually in plexiglas restrainers (Inco, Ambala) for 24 h at room temperature ($22\pm 2^\circ\text{C}$). A control group sensitized as above but not put to RS was run parallel. The animals in this group were kept in home cages during the period of stress but deprivation of food and water was similar to the stressed animals. Just before subjecting the animals to RS, they were injected with respective vehicle or drug. After the completion of

stress procedure all animals (stressed as well as non-stressed) were lightly anaesthetized with ether and blood was collected from the retroorbital plexus. Following estimations were performed:

(a) **Immunological assay:** The serum was assayed for haemagglutination titre. Two fold dilutions (0.025 ml) of sera were made in the microtitre plates with saline. To each well 0.025 ml of 1% (v/v) SRBC was added. The plates were incubated at 37°C for 1h and then observed for haemagglutination. The highest dilution giving haemagglutination was taken as the antibody titre. The antibody titres were expressed in a graded manner, the minimum dilution (1/2) being ranked as 1. The mean ranks of different groups were compared for statistical significance.

(b) **Biochemical assay:** The plasma was assayed for corticosterone using the method of Glick et al with some modification (5). To 100 μl of plasma 300 μl of isooctane was added. After mixing and centrifugation the isooctane layer was discarded and 600 μl chloroform was now added to each tube and after extraction 400 μl of chloroform was transferred to another stoppered tube. To this 800 μl of acid-alcohol (50%) solution (2:1 v/v) was added. After 1h acid layer fluorescence was measured at 462 nm (excitation) and 518nm (emission) using a spectrofluoro-photometer (Shimadzu - RF 540, Japan).

(c) **Estimation of gastric ulcerogenesis:** After collecting the blood the rats were sacrificed with an over dose of ether. The stomachs were dissected out, cut open along the greater curvature, washed with cold water and examined microscopically (X10) using a dissecting microscope with a micrometer (Zeiss, GDR). The number of mucosal erosions and their severity (cumulative length in mm, to the nearest 0.1 mm) were determined.

(ii) Cell-mediated immune response

(a) **Leucocyte migration inhibition test:** The rats were sensitized with 0.5 ml egg albumin (25 mg/ml) and 0.5 ml Freund's complete adjuvant given subcutaneously (s.c.) on day 0. The animals were then divided into different groups. Rats in one group

were subjected to RS for 24h at room temperature ($22\pm 2^\circ\text{C}$) on day +1 and +13 (first and 13th day after the day of sensitization, i.e. day 0). Vehicle/drugs were administered just prior to subjecting the animal to RS on day +1 and +13. Rats in another group were sensitized as above but were not subjected to RS and acted as no-stress control. However, they were also food and water deprived, like stressed animals. On day 14 all the animals were bled under ether anaesthesia and 5-6 ml of blood was drawn in a syringe containing 250 units of heparin by cardiac puncture after opening the chest and the LMI test was performed (17).

(b) **Footpad thickness test:** Mice were immunized by injecting 1×10^8 SRBC s.c. in the back. After immunization animals were divided into different groups. The non-stressed (NS) animals were administered vehicle from day 0-5. The animals of the stressed group were injected with vehicle/drug and then subjected individually to RS for 1 h every day in round pipe mouse restrainer from day 0-5 at room temperature ($22\pm 2^\circ\text{C}$). On the 5th day animals in all the groups were challenged with 1×10^8 SRBC s.c. in the right hind foot pad, whereas normal saline was injected in the left hind paw. Increase in footpad thickness was measured 24h after the challenge by fluid displacement method.

Statistical analysis

The data were analysed using Mann-Whitney 'U' test, Student's 't' test and Chi-square test whenever appropriate. A 'p' value of 0.05 was used as the level of significance in all statistical tests.

RESULTS

Effect of restraint stress (RS)

RS for 24h at room temperature produced a significant decrease in the anti-SRBC antibody titre ($p < 0.001$) as compared to the control animals which were not subjected to stress but were given similar sensitization treatment with SRBC (Table 1). RS also caused a significant suppression of cell-mediated immunity, the % LMI and food pad thickness responses were significantly reduced as compared to the NS group. A significant increase in the plasma corticosterone level was observed in RS group of animals as compared to NS group. Analysis of the gastric pathology showed that animals submitted to RS exhibited development of gastric ulcers, whereas non-stressed animals kept in home cages but subjected to similar food and water deprivation, like stressed animals, did not show such effect (Table 1).

Table 1. Effect of restraint stress (RS) on visceral, endocrinological and immune responses

Treatment group number	Stress parameters					
	Gastric ulcer [#]		Plasma corticosterone titre [#]	Antibody	Immune response	
	Ulcer length (mm)	Cumulative level ($\mu\text{g/dl}$) [#]			% LMI	Change in paw volume (mm) [#]
Control(NS)	1.3 ± 0.4	0.1 ± 0.03	19.3 ± 0.4	8.0 ± 0.3	58.0	0.062 ± 0.003
RS	$18.8\pm 2.5^{**}$	$6.7\pm 0.8^{**}$	$40.8\pm 0.9^{**}$	$4.8\pm 0.3^{**}$	11.8 ^{**}	$0.044\pm 0.006^*$

[#] Values are Mean $\pm$ SE

* $p < 0.05$ ** $p < 0.001$

Effect of CCBs on stress responses

Nimodipine (10, 20, 40 mg/kg, i.p.) and flunarizine (10, 20, 40 mg/kg, i.p.) pretreatment significantly attenuated the effect of RS on all the studied parameters i.e., there was a significant reduction in gastric ulcerogenesis, decrease in the rise of plasma corticosterone levels and a reversal of RS-induced suppression of anti-SRBC antibody titre (Table 2) and % LMI and footpad thickness responses (Table 3). Unlike nimodipine and flunarizine, verapamil

(5, 10, 20 mg/kg, i.p.), diltiazem (10, 20, 40 mg/kg, i.p.) and nifedipine (10, 20, 40 mg/kg, i.p.) failed to modulate stress-induced changes on immune responsiveness, i.e. the immunosuppressive effects of RS on anti-SRBC antibody titre, % LMI and footpad thickness were not antagonized by pretreating the animals with these CCBs (Tables 2, 3). However, like nimodipine and flunarizine all the three CCBs, i.e. verapamil, diltiazem and nifedipine attenuated the effect of RS on gastric ulcerogenesis and plasma corticosterone levels (Table 2).

Table 2. Effect of various calcium channel blockers on restraint stress (RS)- induced changes in gastric ulcers, plasma corticosterone and humoral immune responses

Treatment (mg/kg)	Stress parameters (mean $\pm$ SE)			
	Gastric ulcers		Plasma Corticosterone levels (μ g/dl)	Anti-SRBC antibody titre
	Ulcer number	Cumulative length (mm)		
NS (Control)	1.3 $\pm$ 0.4	0.1 $\pm$ 0.03	19.3 $\pm$ 0.4	8.0 $\pm$ 0.3
RS	18.8 $\pm$ 2.5 ^a	6.7 $\pm$ 0.8 ^a	40.8 $\pm$ 0.9 ^a	4.8 $\pm$ 0.2 ^a
Nimodipine (10)	16.7 $\pm$ 2.4	5.4 $\pm$ 0.7	38.2 $\pm$ 0.7	5.0 $\pm$ 0.3
+ RS (20)	6.7 $\pm$ 0.8 ^{ab}	1.3 $\pm$ 0.3 ^{ab}	25.9 $\pm$ 0.3 ^{ab}	7.8 $\pm$ 0.3 ^{ab}
(40)	6.7 $\pm$ 0.5 ^{ab}	1.4 $\pm$ 0.2 ^{ab}	24.7 $\pm$ 0.5 ^{ab}	7.5 $\pm$ 0.4 ^{ab}
Flunarizine (10)	17.0 $\pm$ 2.0	4.9 $\pm$ 0.6	38.8 $\pm$ 2.9	4.8 $\pm$ 0.5
+ RS (20)	6.7 $\pm$ 0.6 ^{ab}	1.1 $\pm$ 0.1 ^{ab}	25.5 $\pm$ 0.3 ^{ab}	7.3 $\pm$ 0.4 ^{ab}
(40)	6.5 $\pm$ 0.4 ^{ab}	0.9 $\pm$ 0.1 ^{ab}	25.7 $\pm$ 0.5 ^{ab}	7.8 $\pm$ 0.2 ^{ab}
Verapamil (10)	16.0 $\pm$ 1.8	5.3 $\pm$ 0.6	37.4 $\pm$ 1.1	5.2 $\pm$ 0.4
+ RS (20)	5.7 $\pm$ 0.3 ^{ab}	1.2 $\pm$ 0.1 ^{ab}	25.6 $\pm$ 0.2 ^{ab}	5.0 $\pm$ 0.4
(40)	6.0 $\pm$ 0.3 ^{ab}	1.1 $\pm$ 0.1 ^{ab}	24.6 $\pm$ 0.4 ^{ab}	4.8 $\pm$ 0.4
Diltiazem (10)	15.3 $\pm$ 1.2	4.9 $\pm$ 0.6	37.6 $\pm$ 0.7	5.0 $\pm$ 0.2
+ RS (20)	5.5 $\pm$ 0.6 ^{ab}	0.9 $\pm$ 0.2 ^{ab}	25.5 $\pm$ 0.3 ^{ab}	5.2 $\pm$ 0.5
(40)	5.5 $\pm$ 0.3 ^{ab}	0.9 $\pm$ 0.2 ^{ab}	24.7 $\pm$ 0.3 ^{ab}	4.8 $\pm$ 0.3
Nifedipine (10)	18.7 $\pm$ 2.6	5.2 $\pm$ 0.6	38.2 $\pm$ 0.9	5.2 $\pm$ 0.4
+ RS (20)	6.5 $\pm$ 0.5 ^{ab}	1.2 $\pm$ 0.2 ^{ab}	26.8 $\pm$ 0.2 ^{ab}	5.0 $\pm$ 0.6
(40)	6.3 $\pm$ 0.5 ^{ab}	1.1 $\pm$ 0.1 ^{ab}	25.8 $\pm$ 0.5 ^{ab}	5.2 $\pm$ 0.3

n = 6; * p < 0.001

a. Compared to NS (control) group; b. Compared to RS group

Table 3. Effect of various calcium channel blockers on restraint stress (RS)-induced changes in cell-mediated immune responses

Treatment (mg/kg)	% LMI ^a	Change in paw volume (mm) [*] (Mean $\pm$ SE)
NS (Control)	58.0	0.062 $\pm$ 0.003 [*]
RS	11.8 ^{**a}	0.044 $\pm$ 0.006 ^{*a}
Nimodipine (20) + RS	38.4 ^{**b}	0.071 $\pm$ 0.003 ^{ab}
Flunarizine (20) + RS	42.8 ^{**b}	0.064 $\pm$ 0.006 ^{ab}
Verapamil (20) + RS	13.2	0.048 $\pm$ 0.002
Diltiazem (20) + RS	10.4	0.045 $\pm$ 0.007
Nifedipine (20) + RS	9.8	0.045 $\pm$ 0.007

^a n = 6; ^{*}n = 10

^{*}p < 0.005, ^{**}p < 0.001a. Compared to NS (control) group; b. Compared to RS group

LMI = Leucocyte migration inhibition.

DISCUSSION

In the present study effect of some CCBs has been examined on RS-induced changes in gastric ulcerogenesis, plasma corticosterone level and humoral and cell-mediated immune responses. RS produced gastric ulcerations, increased plasma corticosterone levels and caused suppression of humoral and cell-mediated immune responses. These results are in consonance with earlier observations (2-5, 16, 17, 19). All the studied CCBs, viz. nimodipine, flunarizine, nifedipine, verapamil and diltiazem significantly antagonized the effect of RS on gastric ulcerogenesis, and plasma corticosterone levels. However only nimodipine and flunarizine were able to attenuate the effect of stress on immune responses. Complex central and peripheral neurotransmitter mechanisms are reportedly involved in the mediation of stress responses (1, 20). Hence the CCBs induced modulation of stress responses could be mediated by central and/or peripheral actions. Previous studies have reported that CCBs protected against gastric ulcerations induced by cold restraint stress (21-23), ethanol (23), aspirin and pyloric ligation (24, 25) and cysteamine-induced duodenal ulcers (26). This ulcer protective effect of CCBs could be because of their ability to decrease histamine release from gastric mast cells (21), gastric acid production (21-23, 27-29) and gastric smooth muscle contraction (21, 22) or to prevent

gastric mucosal damage due to strengthening the mucosal barrier (25, 30). Szabo et al (31) have suggested that acetylcholine, histamine, dopamine and GABA-sensitive passage of acidic gastric juice into the duodenum following delayed gastric emptying seems to be a key element in the pathogenesis of duodenal ulcer disease. This sequence of event appears to involve both peripheral and central (e.g. brain) element. An initial 'hit' lesion in the duodenum seems to be followed by increased gastric acid output probably originating from the cortex, midbrain, hypothalamus and medulla oblongata via mediators like, acetylcholine, histamine, dopamine and GABA. In fact it has been shown that benzodiazepines which act by facilitating central GABA_A receptor mediated chloride ion conductance protect against stress induced gastric ulcerogenesis (2, 5). Hence, ulcer protective effects of CCBs observed in the present study could be because of a peripheral action affecting gastric acid secretion, gastric motility or strengthening of mucosal barrier by acting locally or a central action by modulating neurotransmitter(s) release.

Besides playing a critical role in contractile process, calcium ions also play a crucial role in excitation-secretion coupling, including the release of various hormones. The effect of CCBs on decreased Ca²⁺ influx thus could account for the

suppression of increased secretion of corticosterone due to stress. Since in previous studies, benzodiazepine group of drugs have been shown to suppress the stress-induced increase in the corticosterone levels by acting through central GABA_A receptor mechanism (5) the possibility of CCBs to produce similar effect by acting through central neurotransmitter systems cannot be ruled out.

Unlike their effect on gastric ulcers and corticosterone levels, out of all the studied CCBs, only nimodipine and flunarizine antagonized the effect of stress on immune responses. It is now well known that central nervous system (CNS) is involved in the regulation of stress-induced immune responsiveness (32, 33). The observed effect of nimodipine and flunarizine on immune parameters could be due to their increased selectivity for the CNS (34, 35). The L-, N- and P/Q-type calcium channels reportedly play a pivotal role in mediating calcium influx that triggers

depolarization evoked neurotransmitter release in nerve terminals (12, 13). It has been shown that neuronal communication of lymphoid tissue is possible and this may be an important route which allows functional interaction between the CNS and the immune system. Receptors for several hormones and neurotransmitters are present on lymphocytes and pharmacological alterations in neural activity influences immune system (32, 33). Thus, CCBs, like flunarizine and nimodipine may be modulating the stress induced changes in immune responses by affecting release of neurotransmitters like GABA, opioids etc. which have been shown to reverse the immunosuppressive effect of RS (5, 16, 17, 19).

In conclusion, RS was observed to suppress humoral as well as cell-mediated immune responses, induce gastric ulcerations and increase plasma corticosterone levels. Various CCBs appear to modulate these stress-induced responses, albeit in a differential manner.

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