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Concurrent Treatment with Verapamil Suppresses Immune and Behavioural Response to Endogenous Cannabinoid Anandamide

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The cellular effects of anandamide, endogenous cannabinoid receptor agonist, include changes in cellular immunity and calcium currents. The present study investigated the effects of anandamide, the calcium channel antagonist verapamil and the combined treatment with verapamil + anandamide on leukocyte phagocytosis and the social behaviour in aggressive singly-housed mice on dyadic interactions with non-aggressive group-housed partners. Verapamil was used at the dose (1 mg/kg) which did not markedly affect either phagocytosis or the behaviour of the mice. Anandamide given alone elicited a biphasic effect on phagocytosis: stimulation after the low dose (0·01 mg/kg) and inhibition after the high dose (10 mg/kg). Both doses of anandamide caused dose-related inhibition of aggressiveness in singly-housed mice. Anandamide combined with verapamil prevented both the stimulatory and inhibitory effect of anandamide on phagocytosis and on inhibition of aggression elicited by the low dose of anandamide. The dose of verapamil used did not influence behavioural changes caused by the high dose of anandamide.

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KEY WORDS — endogenous cannabimimetic; anandamide; calcium channel; verapamil; leukocyte phagocytosis; social behaviour in mice

INTRODUCTION

Anandamide, an endogenous agonist of cannabinoid receptors (Devane *et al*., 1992; Mechoulam *et al*., 1994), has been purified from bovine brain while screening for endogenous modulators of calcium channels. It has been reported to inhibit a voltage-gated calcium current (Brown, 1991) coupled directly to cannabinoid receptors (Caulfield and Brown, 1992; Mackie and Hille, 1992) and the binding of antagonists for N-type and L-type of calcium channels in brain, skeletal and smooth muscle membranes and cardiac membranes where it behaves as a partial agonist (Johnson *et al*., 1993; Mackie *et al*., 1993; Gebremedhin *et al*., 1995; Shimasue *et al*., 1996). Cannabinoid receptors have also been identified in immunocompetent cells (Bouaboula *et al*., 1993; Devane, 1994). The present study investigated the interaction of anandamide and the calcium channel antagonist verapamil, at the dose which does not elicit any behavioural changes when given alone (Šulcová, 1996). The phagocytic activity of mouse whole blood was investigated using the luminol aided, zymosan-induced chemiluminescence method (Knyszynski and Fischer, 1981; Weeks *et al*., 1986). The behavioural interaction was studied using a model of social ('agonistic') behaviour (for review see Miczek and Krsiak, 1979).

MATERIALS AND METHODS

Animals

Female mice of the inbred strain C57BL/10 (8 weeks old) were used for testing kinetics of whole blood phagocytic activity following the *in vivo* administration of the drugs. Male mice of albino outbred ICR strain (from Velaz, Prague)

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8 weeks of age were used for the model of agonistic behaviour. Animals received food and water *ad lib* and were maintained at a constant temperature (20–22°C) on a 12-h light/dark cycle. Tests were performed during the light phase in the same room where the mice were housed.

Drugs

Anandamide was dissolved as previously described (Devane *et al*., 1992) in equal volumes of ethanol and emulphor 620 and mixed thoroughly with 9 volumes of sterile phosphate buffered saline (final formulation 1:1:18) and administered intraperitoneally in doses of 0·01 and 100 mg/kg in a volume of 0·1 ml/10 g mouse, 15 min prior to the behavioural observation. Control animals received the vehicle alone. Verapamil was dissolved in physiological saline and administered intraperitoneally at dose of 1·0 mg/kg, 15 min prior to the behavioural observation.

Procedures

Chemiluminescence assay of leukocyte phagocytosis. Mice were injected daily for 7 days prior to the assay with the vehicle (controls), anandamide, verapamil or anandamide + verapamil. The assay was undertaken 2 h after the last dose which was administered between 07.00–08.00 hours.

Chemicals. HSS (Hanks balanced salt solution) was used for the dilution of the blood samples. Luminol (Sigma Chemical Co., Germany) was solubilized in borate buffer (pH 9·0) at a concentration of 1·7 mg/ml and stored at −20°C in the dark.

Zymosan (Sigma-Aldrich s.r.o., Prague, The Czech Republic) was suspended in HSS at a concentration of 20 mg/ml and boiled in a water bath for 20 min. The zymosan suspension was then centrifuged (10 min 2000 r.p.m.) and washed twice in HSS. The sediment was resuspended in mouse serum to a concentration of 20 mg/ml and incubated at 37°C for 45 min with moderate shaking. It was recentrifuged and washed twice in HSS. Finally, the opsonized zymosan was dissolved in HSS at a concentration of 20 mg/ml and stored at −20°C.

Blood samples. Blood (20 µl) was withdrawn from the retro-orbital plexus under ether anaesthesia into 500 µl of HSS. Another 2 µl of blood was diluted in 10 ml of a solution of Unisol I (Lachema a.s., Czech Republic), + three drops of Ekoglobin, (Hemax s.r.o., Czech Republic) for leukocyte counts (Coulter Counter 2F, Coulter Electronics Ltd.), and blood smears were prepared and stained for microscopic differential counting.

Chemiluminescence (CL) measurements. CL measurements were performed using Biolumat LB 9 500 C (Berthold Co., Germany). Blood (20 µl) was suspended in 500 µl of HSS and incubated at 37°C. A sample of 200 µl was mixed with 40 µl of luminol. Five minutes after the background chemiluminescence was recorded, CL was initiated by adding 40 µl of opsonized zymosan and the CL was then measured every fifth minute for 1 h.

Agonistic behaviour. The test animals were individually housed for 4 weeks in metal self-cleaning cages (8 × 6 × 13 cm). They were not handled except on the experimental days. This housing procedure is known to stimulate intensive agonistic behaviour in male mice when tested on interactions with group-housed opponents of the same age and origin (Miczek and Krsiak, 1979). The opponents were housed in groups of 10 in standard plastic cages (38 × 22 × 14 cm) with the floors covered with sawdust. Group housing nearly abolishes aggression of mice towards isolated males, hence the competitive conditions during the agonistic encounter are unequal. The group-housed males behaved as standard non-aggressive opponents. Agonistic behaviour was tested in transparent observation boxes (20 × 30 × 20 cm) with clean sawdust provided before each encounter. After a 30-min adaptation period in the box, the opponent was introduced and interactions were videotaped for 4 min. The system for collection and analysis of behavioural observational data (The Observer 3.1, Noldus Information Technology v.v., The Netherlands) was used to score 11 behavioural elements. The following behavioural categories, similar to those described by Grant and Mackintosh (1963), were recorded: sociability — social sniffing, following the partner, climbing over the partner; timidity — defensive posture (upright), escape, alert posture; aggression — attack, aggressive unrest (threat), tail rattling; locomotion — walk, rear. The singly-housed males showed spontaneous agonistic behaviour towards non-aggressive opponents. Only those isolated mice which attacked the opponents (aggressive mice) were evaluated in this study.

Statistics. The kinetics of chemiluminescence were analysed by multifactor analyses of variance — Tukey's significant differences test ($p < 0{\cdot}0$). Agonistic behaviour was evaluated using two-tailed non-parametric Wilcoxon matched-pairs signed-ranks test (Siegel, 1956).

RESULTS

Anandamide elicited a biphasic dose-related effect on phagocytosis of mouse leukocytes, namely a significant stimulation at the dose of 0·01 mg/kg and inhibition at the dose of 10 mg/kg ($p < 0{\cdot}05$). Verapamil administered alone did not change phagocytosis. However, when given concurrently with anandamide it prevented both the stimulatory and inhibitory effects of anandamide (Figures 1 and 2). Neither anandamide nor verapamil induced significant changes in the number of leukocytes or in the numbers of lymphocytes, monocytes or polymorphonuclear neutrophil cell types. In the model of agonistic behaviour anandamide at a dose of 0·01 mg/kg selectively suppressed ($p < 0{\cdot}05$) aggression in singly-housed mice (Figure 3). Greater inhibition of aggression ($p < 0{\cdot}01$) was observed after a dose of 10 mg/kg of anandamide (Figure 4) which also inhibited locomotion ($p < 0{\cdot}05$) and stimulated timid behaviour ($p < 0{\cdot}05$). Verapamil did not markedly change agonistic behaviour in mice. However, in the combined treatment verapamil antagonized the inhibition of aggressive behaviour caused by the administration of anandamide at a dose of 0·01 mg/kg, but it did not alter the behavioural changes elicited by the high dose (10 mg/kg) of anandamide.

DISCUSSION

The results of the present study demonstrated the biphasic effects of anandamide and the suppression of such effects by verapamil on both the behaviour and on immunocompetent cells. These findings are consistent with our previous studies of the behavioural effects of verapamil (Šulcová, 1991) and anandamide on agonistic behaviour in aggressive singly-housed mice (Šulcová and Mechoulam, 1995) and on the effects of anandamide on leukocyte phagocytosis (Šulcová, 1996). Previous reports of the biphasic effects of anandamide were published by Fride and Mechoulam (1993), Mechoulam and Fride (1995), Smith *et al*. (1994) and Šulcová *et al*., (1996) when they tested locomotor and exploratory activity of mice in an open field and some other models.

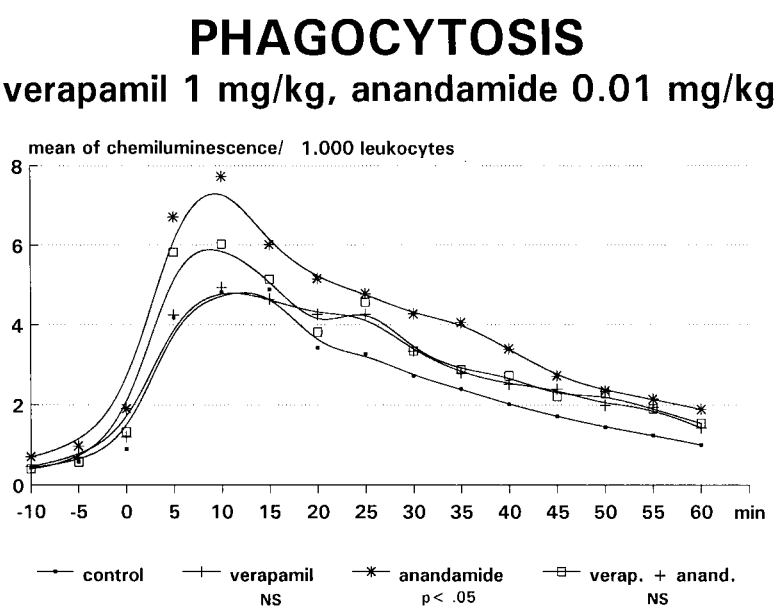

Figure 1. The kinetics of zymosan-induced luminol-aided chemiluminescence of whole blood cells. Vehicle or verapamil (1 mg/kg) or anandamide (0·01 mg/kg) or verapamil + anandamide were injected (i.p.) into 8-week-old female mice (C57BL/10 inbred strain) once daily for 7 days. NS, not significantly different from control. *Significantly different from vehicle: $p < 0{\cdot}05$; (Tukey test)

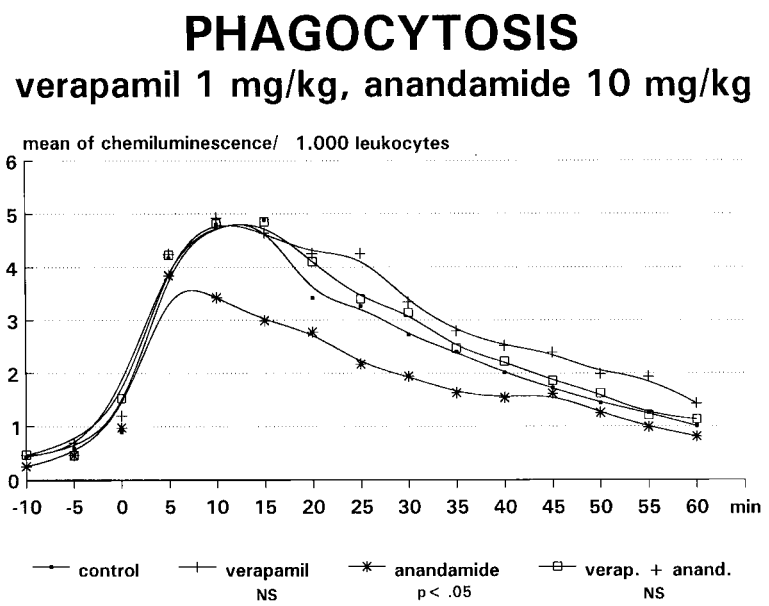

Figure 2. The kinetics of zymosan-induced luminol-aided chemiluminescence of whole blood cells. Vehicle or verapamil (1 mg/kg) or anandamide (10 mg/kg) or verapamil + anandamide were injected (i.p.) into 8-week-old female mice (CB7BL/10 inbred strain) once daily for 7 days. NS, not significantly different from control. *Significantly different from vehicle: $p < 0{\cdot}05$; (Tukey test)

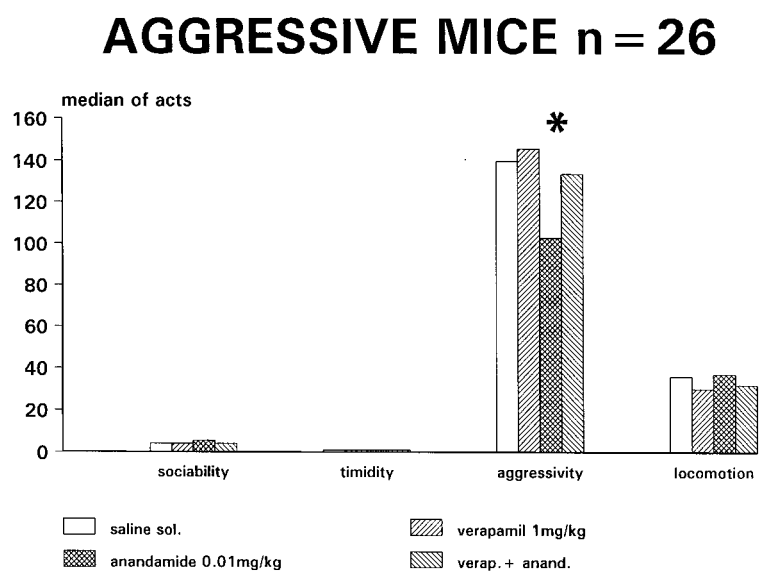

Figure 3. Vehicle or verapamil (1 mg/kg) or anandamide (0·01 mg/kg) or verapamil + anandamide were injected (i.p.) into adult singly-housed male mice (ICR strain) 15 min prior to 4- min dyadic interactions with non-aggressive group-housed partners. Significantly different from vehicle: *$p < 0{\cdot}05$; **$p < 0{\cdot}01$ (two-tailed non-parametric Wilcoxon matched-pairs signed-ranks test)

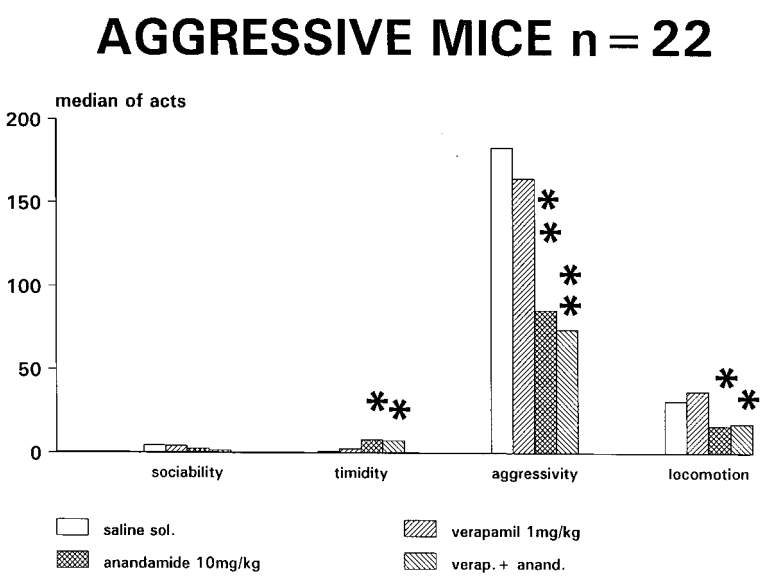

Figure 4. Vehicle or verapamil (1 mg/kg) or anandamide (10 mg/kg) or verapamil + anandamide were injected (i.p.) into adult singly-housed male mice (ICR strain) 15 min prior to 4-min dyadic interactions with non-aggressive group-housed partners. Significantly different from vehicle: $^{*}p < 0{\cdot}05$; $^{**}p < 0{\cdot}01$ (two-tailed non-parametric Wilcoxon matched-pairs signed-ranks test)

The reasons for differential effects of low and high doses of anandamide are presently under investigation. Anandamide can activate both, CB1 and CB2 type of cannabinoid receptors (Watson and Girdlestone, 1994). CB1 receptors exist both centrally and peripherally and may mediate the behavioural effects of anandamide while CB2 receptors occur only in the periphery (Pertwee, 1995). One of the suggested roles of CB2 receptors is that they may mediate cannabinoid-induced changes in immune function (Pertwee, 1995). The assessment of whole blood phagocytic activity which is associated with the activation of leukocyte metabolism can serve as a marker of cell-mediated immune response (Knysyznski and Fisher, 1981; Masihi *et al.*, 1984). Such assessment is widely used in clinical immunological diagnostics using the chemiluminescence method (Ernst *et al.*, 1983; Tono-Oka *et al.*, 1983). In the present study, the chemiluminescence method has been found to be useful as a test for the cannabinoid-induced cell-mediated immune response.

Inhibition of a calcium current coupled directly to cannabinoid receptors was demonstrated in various studies while others showed an increase in intracellular calcium when cannabinoid agonists were administered (for review see Howlett, 1995). While there does not appear to be any studies of the relationship between calcium channel function and leukocyte phagocytosis, there is growing evidence for altered neuronal calcium channel function and the influence of drugs of abuse (Hitchcott *et al.*, 1992). The antagonism of both the stimulatory and the inhibitory effects of anandamide on phagocytosis and behaviour may suggest that the competition between anandamide and verapamil exists at functionally-competent binding sites; a similar conclusion has been reported from a study of the effects of anandamide on rabbit skeletal muscle membranes (Shimasue *et al.*, 1996).

The molecular basis for the interaction between verapamil and anandamide on immune function and behaviour cannot be deduced from the present study. However, the present results produce further evidence for the involvement of calcium channel function in cannabimimetic effects of anandamide which require further investigation.

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