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Research report

Anandamides: tolerance and cross-tolerance to Δ^9-tetrahydrocannabinol

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Abstract

In this study we examined whether tolerance develops to chronic exposure to anandamides [20:4, n-6 (ANA) and 20:3, n-6 (HLEA)] two of the recently discovered endogenous cannabinoid receptor ligands in brain. Tolerance to ANA and cross-tolerance to Δ^9-tetrahydrocannabinol (Δ^9-THC) was examined in female Sabra or C57BL/6 mice which had received daily injections (i.p.) of low (0.001–1 mg/kg) or high doses (20 mg/kg) of ANA or HLEA for 2 weeks. Twenty four h after the last injection, the mice were challenged with 20 mg/kg ANA or Δ^9-THC. Animals were subjected to a series of tests frequently used to assess cannabinoid-induced effects. The results indicated that the high dose, but not the low doses of anandamides produced tolerance to ANA and cross-tolerance to Δ^9-THC for motor activity in an open field, catalepsy on a ring, hypothermia and analgesia on a hot plate. One week after the last ANA treatment, tolerance was not present anymore. No tolerance to ANA was observed for reduced defecation in the open field, a measure of intestinal hypomotility. This phenomenon may possibly be attributed to a difference between activities produced through different types of cannabinoid receptors.

Keywords: Tolerance; Anandamide; Δ^9-THC; Cannabinoid; Cannabinoid receptor; G_i protein; G_s protein

1. Introduction

There is extensive evidence that cannabinoid drugs induce tolerance to most of their pharmacological effects in man [25,26] and in a variety of animal species. Thus tolerance was found to anti-convulsant activity, catalepsy, hypoactivity, hypothermia, hypotension and shock avoidance behavior in rats and mice [3,18,24,48] but not to isolation-induced aggression [45] (see also [14]). Profound tolerance to Δ^9-tetrahydrocannabinol (Δ^9-THC) develops rapidly (one to four administrations) and persists for a long time after cessation of drug treatment [4,31,41].

Since the identification of a cannabinoid receptor (CB_1) [12,30], it has been possible to link the development of behavioral tolerance to a decrease in the number of cannabinoid receptors within 7–14 days of cannabinoid treatment in rats. Thus decreased binding was found in a number of brain nuclei including the nucleus accumbens, caudate-putamen, septum and olfactory tubercle [37,41], but no changes were found when whole brain tissue was measured [2].

An endogenous ligand for the CB_1 receptor was recently identified as the ethanolamide of arachidonic acid and named anandamide [13]. It was found to display pharmacological effects similar to those of the cannabinoids, both in vivo [10,20,43] and in vitro [17,46]. Further observations have suggested that anandamide is a member of a family of compounds, all of which are ethanolamides of long chain fatty acids with similar activities. They were designated 'anandamides' and the nomenclature used for the shorthand description of unsaturated all-*cis* fatty acids was applied [21]. Following this nomenclature, archidonoyl ethanolamide (ANA) is anandamide (20:4, n-6) and homo-γ-linolenoyc ethanolamide (HLEA) is anandamide (20:3, n-6) (when just the term 'anandamide' is used it indicates the ethanolamide of arachidonic acid (20:4, n-6)). Thus the anandamides (22:4, n-6, 20:3, n-6, and 22:6, n-3) depressed motor activity in an open field, induced catalepsy on a ring, and induced hypothermia and hypoalgesia on a hot plate [7,20]. While the anandamides have pharmacological profiles closely related to that of Δ9-THC, differences have been noted. For example, the anandamides act as partial agonists in at least some assays studied [7,28,33].

Some partial agonists have been shown to cause only a low degree or lack of tolerance after chronic administra-

Abbreviations: ANA, arachidonyl ethanolamide; HLEA, homo-γ-linolenic ethanolamide; Δ^9-THC, tetrahydrocannabinol; CB_1, cannabinoid receptor 1; CB_2, cannabinoid receptor 2; i.p., intraperitoneal; ANOVA, analysis-of-variance

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tion. Well known examples are the benzodiazepine Ro16-6028 [22,34] and the β-adrenergic xamoterol [6]. Hence it was of interest to determine the response to chronic treatment with the anandamides.

Cross-tolerance to anandamide (20:4, n-6) after two pretreatments with Δ^9-THC (20 mg/kg) has been recorded for the twitch response in the murine vas deferens, but not for hypothermia in mice [40].

However, to our knowledge, the response to chronic treatment with anandamides has not been studied before.

2. Materials and methods

2.1. Animals

Female Sabra or C57BL/6 mice (6–7 week old, Harlan-Sprague Dawley) received food and water *ad libitum* and were maintained at constant temperature (20–22°C) on a 12 h light/dark cycle. Tests were performed during the light phase. At the start of the experiments, mice were randomly divided into treatment groups of 6 mice. Animals were weighed just before the first injection and weekly thereafter. No significant differences in body weights were noted between treatment groups in any of the experiments.

2.2. Drugs

Δ^9-THC, ANA and HLEA were synthesized in our laboratory as previously described [13,21]. The drugs were dissolved in ethanol and emulphor 620 (1:1) and mixed thoroughly with 9 volumes of phosphate buffered saline. Control animals received this formulation (vehicle) without the drug. Solutions were administered i.p. in a volume of 0.1 ml/10 g mouse. Fresh solutions were prepared every day from stock solutions (drug in ethanol) kept at −20°C.

2.3. Chronic treatments

Chronic treatment consisted of daily injections for 2 weeks of ANA or HLEA, at doses of 0.001, 1 or 20 mg/kg. Drugs were administered at about 10 AM. Control animals received chronic injections of vehicle. In one experiment, an untreated control group was also included. Since the results from this group were similar to those from the vehicle-treated group, data from these 2 groups were pooled. Twenty four h after the last treatment, a challenge dose (of 20 mg/kg ANA or Δ^9-THC) was given and the mice tested as described below.

2.4. Behavioral testing

As reported previously, maximal pharmacological effects of ANA are observed 10 min after i.p. injections [20]. I.p. injections of Δ^9-THC reach their maximal effectivity, in our laboratory, at 30 min. This observation is in agreement with previous data by Pertwee et al. [39]. Hence testing was initiated 10 min after a challenge dose of ANA, or 30 min after Δ^9-THC.

A series of four consecutive observations was performed on each mouse based on a standard procedure employed to evaluate cannabinoid-induced effects in mice [29], with similar time intervals as described previously [20]. Briefly, each mouse was observed in an open field (20 × 30 cm divided into 12 squares of equal size). Horizontal (ambulation) and vertical (rearing) activity were measured for 8 min. Activity was recorded every 2 min. Since no differential drug effects were noted between 2, 4, 6 and 8 min intervals, activity is presented only for the 8 min period. Inhibition of intestinal motility has been observed after THC administration [9]. Hence, after the animal was removed from the open field, the number of fecal pellets was recorded as a measure of intestinal motility. Immediately after the open field test, catalepsy (immobility on a ring of 5.5 cm diameter [38]) was assessed for 4 min and expressed as % immobility. Body temperature (BT) was measured immediately before injection (BT1) and again after the ring test (BT2) with a telethermometer (Yellow Springs Instruments Co., Yellow Spring, OH). The difference was designated BT2-BT1. Finally, analgesia on a hot plate [16] maintained at 55°C was measured as the latency (in s) until the first hind paw lick or jump from the plate (the latter response was rarely observed) with a maximum of 45 s. It should be noted that Ankier [5] did not observe any histopathological damage when mice were kept for up to 60 s on a 59°C hot plate. Hot plate latency was expressed as % Maximum Possible Effect, %MPE = 100 × ((test latency − baseline latency)/(45 − baseline latency)), where test latency is the number of seconds of the experimental animal and baseline latency, the average score of the control group in the same experiment.

2.5. Statistical analyses

Data were analyzed (STATVIEW) by one way analyses-of-variance (ANOVA) or two-way ANOVA (repeated: treatment × time, where mice were retested 1 week after cessation of chronic ANA treatment) or by unpaired *t*-tests, after testing for homogeneity of variance with Box's test. Post-hoc comparisons were performed using Fisher's protected least significant difference test ($P < 0.05$).

3. Results

3.1. Tolerance to anandamide (20:4, n-6) or anandamide (20:3, n-6)

Mice were divided into 4 chronic (2 weeks of daily injections) treatment groups. Two groups received vehicle.

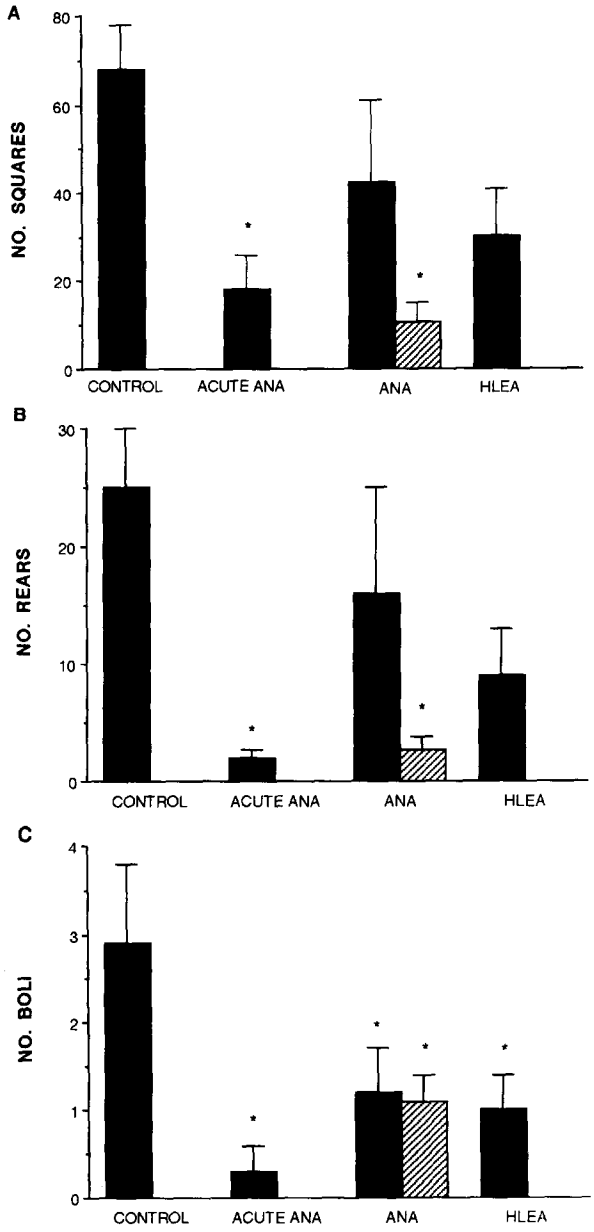

Fig. 1. Four groups of female Sabra mice were injected (i.p.) daily for 2 weeks with 20 mg/kg of ANA, HLEA, or vehicle (2 groups) respectively. Twenty four h after the last injection, one vehicle-treated group received another dose of vehicle (Control), the other vehicle-treated group was injected with 20 mg/kg ANA (Acute ANA), as were the other two groups (ANA and HLEA). Ten min later ambulation (no. squares, (A)), rearing (no. rears (B)), and defecation (no. boli (C)) in an open field were measured. An additional 3 groups of mice, injected daily with ANA (20 mg/kg) or vehicle (2 groups), were also re-challenged one week after cessation of drug treatment (striped bars). Data were analyzed with one-way ANOVAs and are presented ± S.E.M. $F = 2.6$, df3,22, $P < 0.08$ (A), $F = 2.9$, df3,22, $P < 0.06$ (B), $F = 4.0$, df3,23, $P < 0.05$ (C). (ANOVAs for repeated measures for the re-challenged mice: $F = 3.9$, df2,15, $P < 0.05$ (A), $F = 4.9$, df2,15, $P < 0.02$ (B), $F = 5.6$, df2,15, $P < 0.02$ (C). * Different from control, $P < 0.05$, Fisher's protected least significant difference test.

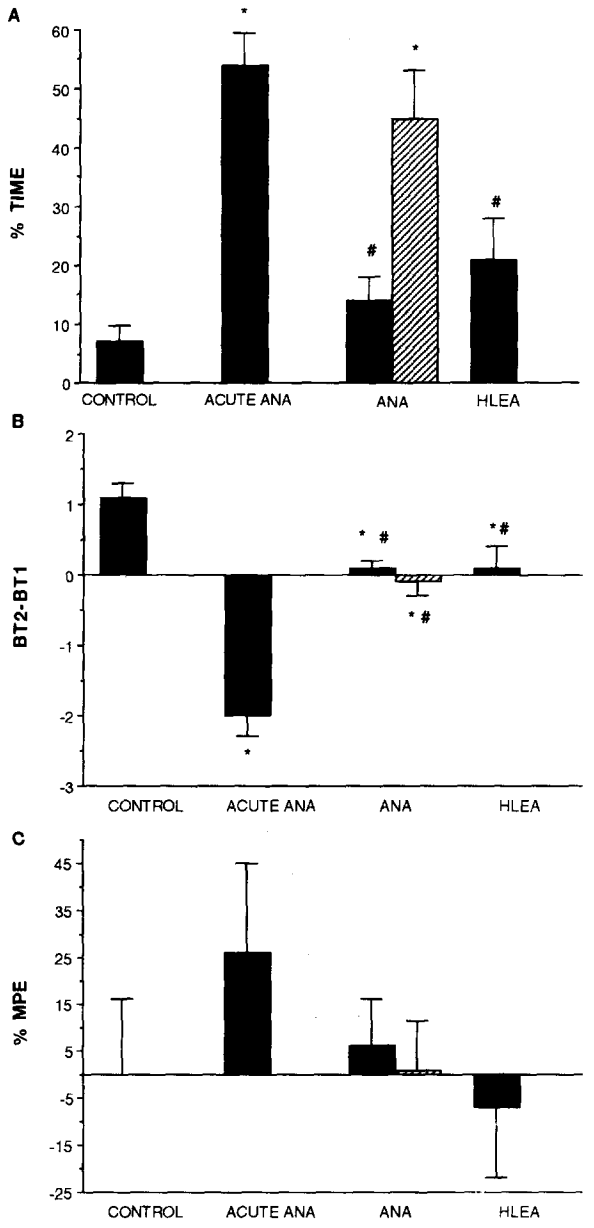

Fig. 2. Four groups of female Sabra mice were injected daily (i.p.) for 2 weeks with 20 mg/kg of ANA, HLEA, or vehicle (2 groups), respectively. Twenty four h after the last injection, one vehicle-treated group received another dose of vehicle (Control), the other vehicle-treated group was injected with 20 mg/kg ANA (Acute ANA), as were the other two groups (ANA and HLEA). Ten min later immobility on a ring of 5.5 cm diameter ('catalepsy' (A)), the change in body temperature (BT2-BT1 (B)), and analgesia on a hot plate (%MPE, see Methods (C)) were assessed. Data were analyzed with one-way ANOVAs and are presented ± S.E.M. $F = 18.9$ df3,23, $P < 0.0001$ (A), $F = 28.2$, df3,23, $P < 0.0001$ (B), $F = 1.5$, df3,22, $P < 0.1$ (C). An additional three groups of mice were injected daily with ANA (20 mg/kg) or vehicle (2 groups) and tested as described in legend to Fig. 1, ANOVAs for repeated measures for the re-challenged mice: $F = 3.9$, df2,15, $P < 0.05$ (A), $F = 4.9$, df2,15, $P < 0.02$ (B), $F = 5.6$, df2,15, $P < 0.02$ (C). * Different from Control, $P < 0.05$, Fisher's protected least significant difference test. # Different from Acute ANA, $P < 0.05$, Fisher's protected least significant difference test.

One group received a dose of 20 mg/kg of ANA (anandamide 20:4, n-6) and one group received 20 mg/kg HLEA (anandamide 20:3, n-6). For both compounds such a dose induces significant cannabinoid effects when administered acutely [7,20].

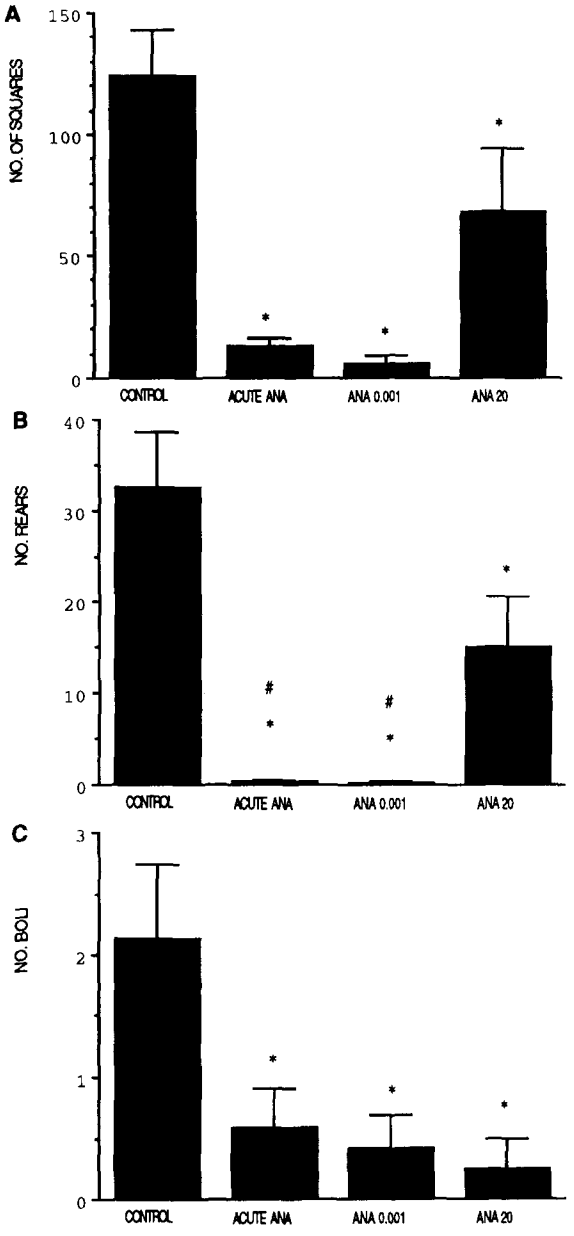

Fig. 3. Four groups of female C57BL/6 mice were injected (i.p.) daily for 2 weeks with ANA (0.001 or 20 mg/kg), or vehicle (2 groups) respectively. Twenty four h after the last injection, one vehicle-treated group received another dose of vehicle (Control), the other vehicle-treated group was injected with 20 mg/kg ANA (i.p., Acute ANA), as were the other two groups (ANA0.001, ANA20). Ten min later ambulation (no. squares, (A)), rearing (no. rears (B)), and defecation (no. boli (C)) in an open field were measured. Data were analyzed with one-way ANOVAs and are presented ±S.E.M. $F = 15.2$ df3,23, $P < 0.0001$ (A), $F = 14.2$, df3,23, $P < 0.0001$ (B), $F = 3.8$, df3,23, $P < 0.05$ (C). * Different from Control, $P < 0.05$, Fisher's protected least significant difference test. # Different from Chronic ANA20, $P < 0.05$, Fisher's protected least significant difference test.

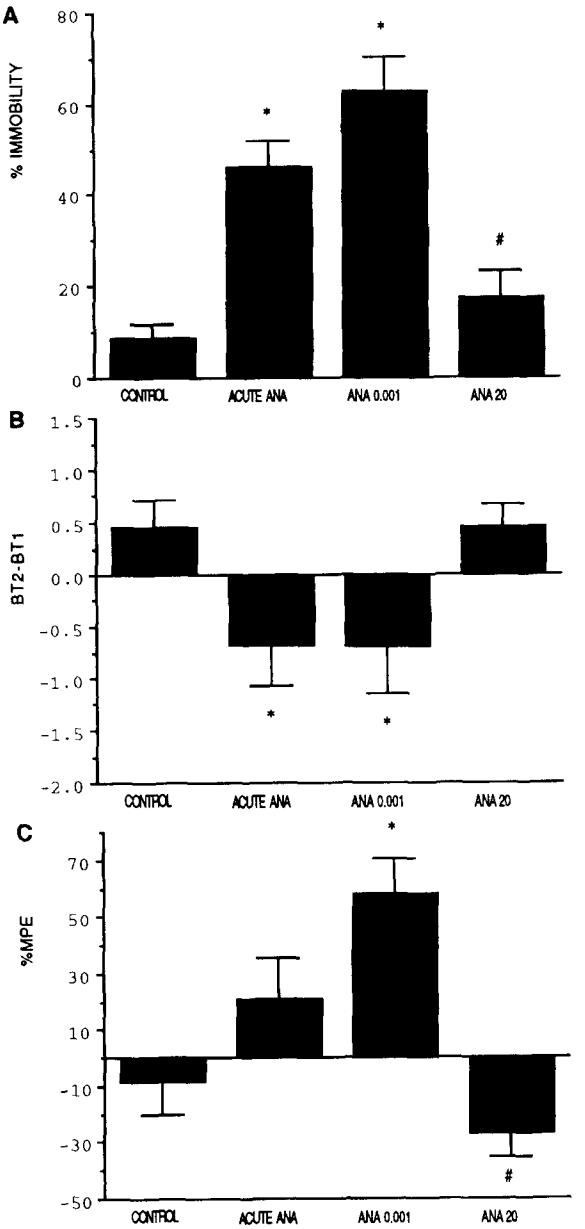

Fig. 4. Four groups of female C57BL/6 mice were injected daily (i.p.) for 2 weeks with ANA (0.001 or 20 mg/kg), or vehicle (2 groups) respectively. Twenty four h after the last injection, one vehicle-treated group received another dose of vehicle (Control), the other vehicle-treated group was injected with 20 mg/kg ANA (i.p., Acute ANA), as were the other two groups (ANA0.001, ANA20). Ten min later immobility on a ring of 5.5 cm diameter ('catalepsy' (A)), change in body temperature (BT2-BT1 (B)), and analgesia on a hot plate (%MPE, see Methods) (C) were assessed. Data were analyzed with one-way ANOVAs and are presented ±S.E.M. $F = 20.6$ df3,23, $P < 0.0001$ (A), $F = 3.5$, df3,23, $P < 0.05$ (B), $F = 7.2$, df3,23, $P < 0.01$ (C). * Different from Control, $P < 0.05$, Fisher's protected least significant difference test. # Different from Acute ANA and Chronic ANA0.001, $P < 0.05$, Fisher's protected least significant difference test.

Twenty four h after the last injection, the chronic ANA and chronic HLEA groups received a single dose of 20 mg/kg ANA. One vehicle group also received 20 mg/kg

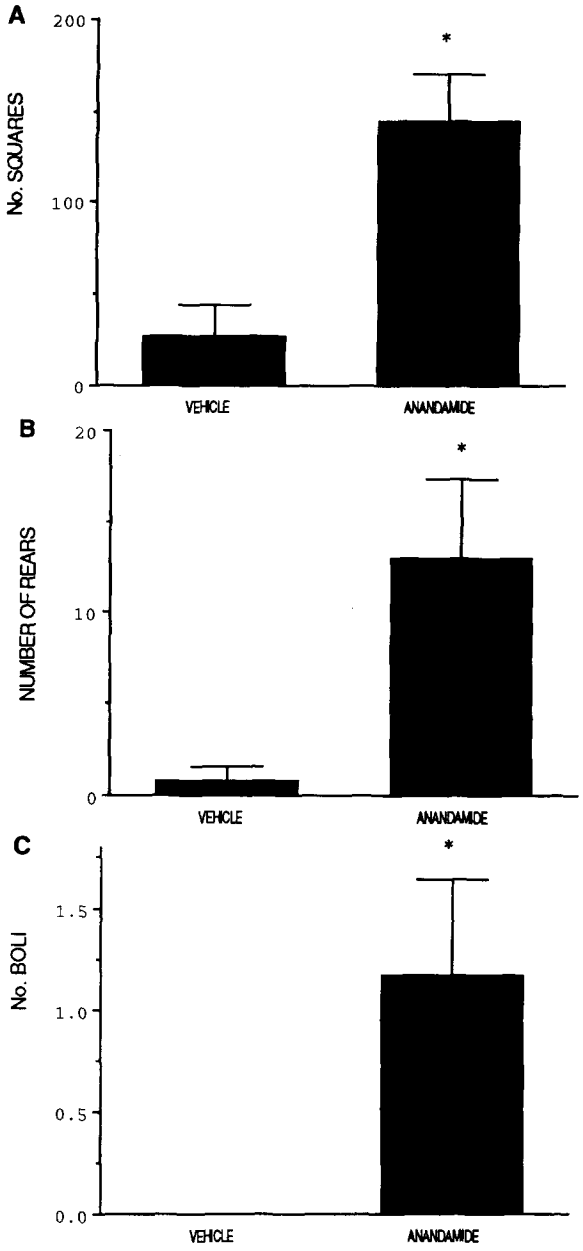

Fig. 5. Two groups of female C57BL/6 mice were injected daily (i.p.) for 2 weeks with ANA (20 mg/kg) or vehicle. Twenty four h after the last injection, all mice were injected with 20 mg/kg Δ^9-THC. Thirty min later, ambulation (no. squares, (A)), rearing (no. rears (B)), and defecation (no. boli (C)) in an open field. Data were analyzed by unpaired t-tests. * $P < 0.05$, $^{\#}$ $P < 0.1$.

ANA (hence the 'acute ANA' group), while the other vehicle-treated group received an additional injection of vehicle (hence, the control group).

The results from this experiment are presented in Figs. 1 and 2. In contrast to the significant depression in open field behavior seen in acutely treated mice, chronically treated animals were not significantly different from controls (Fig. 1A,B). Catalepsy in the 'ring test' (Fig. 2A), and analgesia on the hot plate (Fig. 2C) were almost absent after chronic ANA or HLEA. Neither hypothermia nor the typical increase in body temperature as seen in control mice, were seen in the chronic treatment groups (Fig. 2B).

In contrast to these parameters, significant inhibition of defecation was evident in the chronically treated groups (Fig. 1C). Thus tolerance to ANA developed within 2 weeks of daily injections with 20 mg/kg of ANA or HLEA for all measures except defecation in the open field.

In a separate experiment, the ability of a 20-fold lower dose of ANA to induce tolerance was examined. Sabra

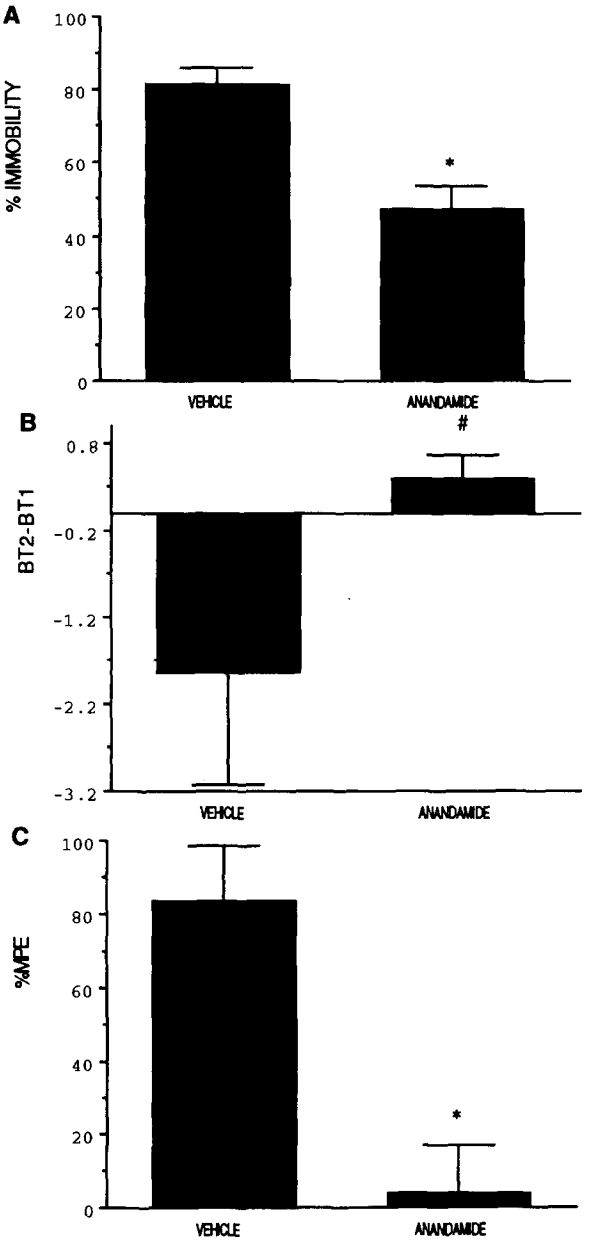

Fig. 6. Two groups of female C57BL/6 mice were injected daily (i.p.) for 2 weeks with ANA (20 mg/kg) or vehicle. Twenty four h after the last injection, all mice were injected with 20 mg/kg Δ^9-THC. Thirty min later immobility on a ring of 5.5 cm diameter ('catalepsy' (A)), change in body temperature (BT2-BT1 (B)), and analgesia on a hot plate (%MPE, see Methods (C)) were assessed. Data were analyzed with unpaired t-tests. * $P < 0.05$, $^{\#}$ $P < 0.1$.

mice were treated for 2 weeks with 1 mg/kg ANA daily which is just below the threshold dose for the production of an observable effect [20,33] and challenged with 20 mg/kg ANA. No differences were observed between the chronically and acutely treated mice (data not shown), i.e., no tolerance to ANA was observed after chronic treatment with 1 mg/kg of ANA.

3.2. Duration of the tolerance effect

One week after the last ANA injection (20 mg/kg), the animals were re-challenged with 20 mg/kg ANA. A full response to ANA was observed similar to that of the acutely treated mice, for ambulation and rearing in the open field and for immobility on the ring (Fig. 1A,B and Fig. 2A, striped bars). In contrast, only a beginning of a reversal of the hypothermic response was seen (Fig. 2B). The analgesic response had also not returned yet, but the differences in this test were not significant (Fig. 2C).

3.3. Tolerance to a very low dose of anandamide, 20:4, n-6

We recently showed that a very low dose of ANA inhibits Δ^9-THC-induced effects [18]. We hypothesized that one of the routes through which this effect may be explained was by activation of a G_s protein, thus exerting opposite effects to those of a high dose of anandamide (which acts through a G_i protein [17,46]. Here we wanted to see if chronic treatment with a very low dose of ANA could desensitize this component, thereby potentiating the response to a challenge dose of ANA. We also wanted to examine whether induction of tolerance can be generalized to a more widely used strain of mice than the Sabra mice used thusfar. Thus in this experiment, C57BL/6 mice were chronically treated with ANA (0.001 mg/kg or 20 mg/kg) or vehicle. Twenty-four hours after the last injection, they were challenged with 20 mg/kg ANA and tested for cannabinoid effects 10 min later. The results from this experiment are presented in Figs. 3 and 4. Thus chronic treatment with the high dose of ANA (20 mg/kg) induced tolerance, at least as profound as that seen in Sabra mice (Figs. 1 and 2). In contrast, a total lack of tolerance was seen for the inhibition of defecation in the open field (Fig. 3C) even more clearly than what was found in Sabra mice (see Fig. 1C). Further, chronic treatment with the low dose of ANA did not induce tolerance to an ANA challenge. On the contrary, a trend toward more profound effects of the challenge dose compared to the acutely treated mice was observed. However, this trend was only significant for analgesia on the hot plate (Fig. 4C).

3.4. Cross-tolerance between ANA and Δ^9-THC

C57BL/6 mice were injected for 2 weeks with 20 mg/kg ANA or with vehicle. Twenty four h after the last injection all animals were challenged with Δ^9-THC (20 mg/kg) and tested 30 min later.

The chronically treated mice were tolerant to Δ^9-THC in all parameters measured: motor activity (Fig. 5A,B), catalepsy on the ring (Fig. 6A), hypothermia (Fig. 6B) and analgesia on the hot plate (Fig. 6C). Contrary to the lack of tolerance in defecation seen after a challenge dose of ANA (see Fig. 1C and Fig. 3C), tolerance was present when the chronic ANA mice were challenged with Δ^9-THC (Fig. 5C).

4. Discussion

In the present study we demonstrate for the first time that chronic treatment for 2 weeks with the anandamides (20:4, n-6) (ANA) or (20:3, n-6) (HLEA), two members of a family of endogenous cannabinoid receptor ligands [13,21], induces tolerance to a challenge dose of ANA and cross-tolerance to Δ^9-THC in 2 strains of mice, C57BL/6 and Sabra.

Tolerance was observed in a series of tests based on an experimental design used to assess cannabinoid-induced activity [29] including ambulation and rearing in an open field, immobility on a ring ('catalepsy'), hypothermia and analgesia on a hot plate.

These data are in agreement with the tolerance induced by chronic Δ^9-THC treatment [1,3,4,24–26,37,40,41,44].

ANA has been shown to behave as a partial agonist at least under certain experimental conditions [28,33,46]. For example, the maximal hypoactivity and analgetic effects of ANA on body temperature and analgesia were much smaller than those reached by THC [33]. However, in contrast to the incomplete or total lack of tolerance produced by partial agonists such as the benzodiazepine Ro16-6028 [22,34] and the β-adrenergic xamoterol [6], tolerance to ANA for hypothermia and analgesia was complete or almost complete (see Fig. 2B,C and Fig. 4B,C). Thus, at least under the present conditions, the partial agonist properties of ANA do not affect the degree of tolerance.

We observed a reversal of the tolerance effect for behavioral parameters (ambulation and rearing in the open field and 'catalepsy' on the ring) within 1 week after cessation of treatment. Although reversal of tolerance in rats performing on a discriminated-Sidman avoidance schedule has been observed within 9 days [48], duration of tolerance to Δ^9-THC is usually more prolonged [11,14,42]. Thus tolerance to ANA appears to be shorter than that induced by Δ^9-THC. The reason for this observation is not clear. However, tolerance to the short-acting barbiturate pentobarbital [27,47] or the benzodiazepine midazolam [8], develops faster than to their long-acting couterparts. Hence we speculate that the relatively short duration of tolerance to anandamide may be the result of its faster onset and shorter duration of action compared to THC [10,20,43].

Previous data have demonstrated that Δ^9-THC activates the CB_1 receptor through a G_i protein-linked signalling pathway [23]. We have postulated recently [19] that activation of a G_s protein-linked signalling pathway may be involved in the inhibitory effects of very low doses of ANA. Downregulation of G_s and G_i proteins has been demonstrated for several systems such as prostanoid receptors [35]. In the present study we examined the possibility that chronic exposure to a very low dose of ANA would desensitize a G_s protein while leaving the G_i protein intact. As expected, a low dose (0.001 mg/kg) of ANA did not induce tolerance to ANA. Indeed, a trend toward a more pronounced response to ANA, compared to that of the acute ANA group, was evident especially for immobility on the ring and for hot plate analgesia. However, except for the latter test, the trend was not significant. Hence further experiments should be performed to clarify the possible role for a G_s protein-linked receptor in ANA-mediated effects.

For many receptor systems, tolerance is thought to be an expression of desensitization or downregulation of the receptor [1]. Dill and Howlett [15] have shown that prolonged exposure to Δ^9-THC reduces the inhibition of cyclic AMP accumulation in response to cannabinoid drugs. Moreover, two independent studies have shown that behavioral tolerance to THC was accompanied by a decrease in the number of CB_1 receptors in a number of brain nuclei including the nucleus accumbens, caudate-putamen, septum and olfactory tubercle [37,41]. Thus it is likely that chronic anandamide-induced tolerance is mediated by a reduction in cannabinoid receptor numbers and/or desensitization of the cannabinoid receptor.

Tolerance to intestinal hypomotility has been observed after repeated Δ^9-THC injections [4]. In the present study chronic ANA treatment induced tolerance to a challenge dose of Δ^9-THC but not to a challenge with ANA for inhibition of defecation. This was observed in 2 strains of mice. This observation contrasts with the tolerance effect for the other parameters (locomotion, catalepsy, body temperature and analgesia), which was present whether Δ^9-THC or ANA were used as the challenge dose. Recently, an endogenous cannabinoid receptor ligand 2-arachidonylglycerol, has been isolated from canine gut [32]. One may speculate that a CB receptor may be found in the gastro-intestinal tract in addition or similar to 'central' CB_1 or 'peripheral' CB_2 (found in spleen thusfar [36]) receptors. We suggest that inhibition of defecation may be mediated through such putative receptors in the gut. Taken together with the present data, we speculate that the induction of tolerance in the gastro-intestinal system may differ from that of CB_1, which presumably mediates tolerance for the other parameters studied here.

In conclusion, chronic treatment with anandamides in mice produced tolerance to ANA and cross-tolerance to Δ^9-THC, similar to that seen after chronic THC injection. Exceptions to this were an apparently shorter duration of tolerance and a lack of tolerance for intestinal hypomotility when challenged with ANA.

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