Pharmacology Biochemistry & Behavior, Vol. 40, pp. 559–563. © Pergamon Press plc, 1991. Printed in the U.S.A. 0091-3057/91 $3.00 + .00

G-Protein Mediation of Cannabinoid-Induced Phospholipase Activation

CHARLENE A. AUDETTE, SUMNER H. BURSTEIN,[1] SHARON A. DOYLE AND SHEILA A. HUNTER

Department of Biochemistry, University of Massachusetts Medical Center, Worcester, MA 01655

AUDETTE, C. A., S. H. BURSTEIN, S. A. DOYLE AND S. A. HUNTER. *G-protein mediation of cannabinoid-induced phospholipase activation.* PHARMACOL BIOCHEM BEHAV **40**(3) 559–563, 1991.—The release of arachidonic acid from mouse peritoneal and S49 cells induced by Δ^1-tetrahydrocannabinol was found to be altered by prior exposure of the cells to either pertussis toxin or cholera toxin. The stable analogs of GTP and GDP, GTP-γ-S and GDP-β-S, were also effective in changing the extent of arachidonate release in saponin-treated cells. GDP-β-S essentially abolished the THC response, while GTP-γ-S showed effects mainly on vehicle-treated cells. The cataleptic action of THC in intact mice which is mediated by eicosanoids was also attenuated by pertussis toxin pretreatment. It is suggested that the THC receptor is coupled to phospholipases through one or more G-proteins and that adenylate cyclase probably does not have a role in this mechanism.

Δ^1-Tetrahydrocannabinol G-proteins Phospholipases Mouse peritoneal cells S49 lymphocytes Catalepsy Arachidonic acid

ONE of the numerous cellular responses to THC and other cannabinoids is an increase in arachidonic acid metabolism (7). This has been a subject of interest in our laboratory for some time and we have reported evidence which suggests that THC-induced stimulation of eicosanoid production may have significance for some of the in vivo actions of this drug (8). Data from other investigators have supported this hypothesis (14); however, no explanation of the molecular basis for these observations has been advanced.

We reported data that strongly suggested that THC somehow increases the activity of one or more cellular phospholipases (5,13). This is in agreement with the generally held belief that the free arachidonic acid required for eicosanoid synthesis arises by its release from esterified pools occurring in various cellular phospholipid storage sites. The mechanism of THC-induced phospholipase activation is not well understood, which apparently is true for other agonists of this type.

Recently, reports have begun to appear in the literature which support a role for guanine nucleotide binding proteins (G-proteins) in the control of arachidonate release mediated by phospholipases A_2 and C (1,3). A variety of experimental models have been studied indicating that this may be a general type of control mechanism for this process. Several probes were used to establish G-protein involvement in the eicosanoid response, including sensitivity to pertussis and cholera toxins and the use of stable analogs of GTP and GDP.

In the present study, we have examined the possibility that THC-induced activation of cellular phospholipases involves participation by one or more G-proteins. Our approaches were based on those used by other investigators in studying receptor-mediated events leading to the release of free arachidonic acid from cellular phospholipid pools. THC probably operates through its own unique receptor or receptors, and in this report we give evidence suggesting that the signalling mechanism coupled to the receptor involved in THC-induced arachidonate release, involves G-proteins.

ABBREVIATIONS

THC, Δ^1-tetrahydrocannabinol; PL, phospholipase; CT, cholera toxin; PT, pertussis toxin; DMEM, Dulbecco's modified Eagle's medium; MEM, minimum essential medium.

METHOD

Materials

The cannabinoids were obtained from the National Institute on Drug Abuse (Rockville, MD) and their purity was monitored by reversed-phase high pressure liquid chromatography. Pertussis and cholera toxins were purchased from List Biological Labs, Inc. (Campbell, CA). GTP-γ-S and GDP-β-S were purchased from Boehringer Mannheim (Indianapolis, IN). [1-^{14}C] Arachidonic acid (specific activity, 52.7 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, MA). The S49 cells (wild type) were grown from stock originally obtained from Henry Bourne. The cells were incubated until a density of 1×10^6/ml was reached in DMEM-high glucose supplemented with 10% fetal calf serum.

Preparation of Macrophages

Resident cells were obtained by peritoneal lavage following sacrifice by cervical dislocation from 20–25 g female CD-1 mice (Charles River). The cell mixture was suspended in MEM containing 1% penicillin-streptomycin, and the macrophages al-

[1]Requests for reprints should be addressed to Sumner H. Burstein.

lowed to attach overnight to plastic culture dishes in an incubator at 37°C in an atmosphere of 95% O_2:5% CO_2. This procedure yielded approximately 1.0×10^6 cells/ml.

^{14}C-Arachidonate Labelling and Analysis

The peritoneal cells, approximately 1×10^6/18 mm culture well, were incubated at 37°C for 2–3 h with [1-^{14}C] arachidonic acid ($1 \times -2 \times 10^5$ dpm/ml MEM). The media were decanted and the cells washed twice with MEM (1.0 ml) containing 0.1% bovine serum albumin (BSA) to remove unincorporated fatty acid. The cells in fresh MEM (1.0 ml) were given appropriate treatments and incubated for 30 min at 37°C. For arachidonic acid release determinations, the media were collected, centrifuged at $3000 \times g$ for 5 min, and duplicate aliquots of the supernatant were assayed for radioactivity by liquid scintillation counting. S49 cells were treated similarly except the procedures were done on suspensions in 12×72 mm culture tubes. All experiments were repeated at least three times and a representive result is presented here.

Catalepsy Measurements

The cataleptic response was measured by the "ring test" method as described by Pertwee (16). In a stable environment at 30°C, devoid of auditory stimuli or bright lights, mice are placed on a wire ring 5.5 cm in diameter held by a 16-cm vertical rod. The fraction of time the mouse is immobile over a 5-min period is recorded as a measure of catalepsy. More detailed information regarding the criteria for immobility is given in reference (16).

RESULTS

Pertussis toxin had significant effects on the release of arachidonate from macrophages which had been labelled with ^{14}C-arachidonic acid (Fig. 1). In the absence of THC, the toxin had a stimulatory effect which also was observed at lower concentrations of THC. A reversal of the effect was seen when the level of THC was increased to 16 μM where the toxin had a marked inhibitory influence on THC-induced release. In the S49 cell, pertussis toxin increased the release response to 16 μM THC and decreased the extent of arachidonate release in the vehicle controls (Table 1). The effects were significant when compared to the appropriate vehicle-treated controls. In permeabilized S49 cells, however, PT which had been activated by DTT produced a small (13%) decrease in the THC response (Table 2).

A somewhat different pattern was seen when arachidonate-labelled peritoneal cells were exposed to cholera toxin followed by a challenge with THC. Over the entire range from 3.2 to 16 μM THC, toxin-treated cells were less responsive to cannabinoid treatment in terms of arachidonate release (Fig. 2). It is interesting to note that at low levels of THC, the toxin-treated cells gave a weaker response than was seen in the vehicle-treated controls. Cholera toxin also effectively reduced arachidonate release in S49 cells (Table 1) when exposed to 16 μM THC. An inhibitory effect for CT was also observed in permeabilized S49 cells (Table 2).

Macrophages which were permeabilized by treatment with saponin, were treated with the stable guanine nucleotide analogs GTP-γ-S and GDP-β-S (Table 3). The GTP analog caused a large increase in arachidonate release in vehicle-treated cells; however, there were no significant changes in THC-treated cells. The GDP analog, on the other hand, completely abolished the THC response when both high and low doses of the drug were used. In permeabilized S49 cells, GDP-β-S again reduced THC-induced arachidonate release although, in this case, the effect was greater at 3.2 μM THC than at 16 μM THC (Table 4). In contrast to the macrophages, GTP-γ-S reduced the vehicle response in S49 cells which gives an increased THC response when compared to the appropriate controls. Table 4 also gives data showing that the vehicle, 1% ethanol, does not stimulate release; in fact, a small reduction in free arachidonate was seen.

The cataleptic action of THC in intact mice was also observed to be sensitive to pertussis toxin (Table 5). Mice which were injected with toxin IP and subsequently given THC were almost 50% less responsive than mice receiving only THC.

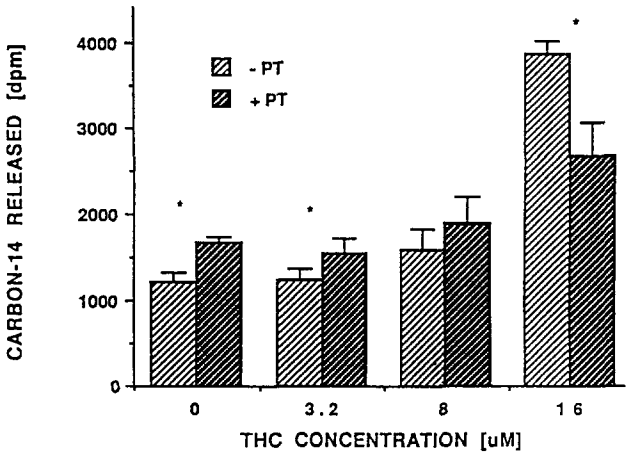

FIG. 1. Effects of pertussis toxin on arachidonic acid release in mouse peritoneal cells. The cells were prepared and labelled with ^{14}C-arachidonate as described in the Method section. Pertussis toxin (100 ng/ml) was added to the cells 2 h prior to labelling and was found to have no effect on arachidonate uptake. THC (in 10 μl EtOH) was added after washing the cells with 2×1.0 ml MEM containing 0.1% BSA and incubated for 30 min. ^{14}C release was measured as described in the Method section. Values shown are the means of four replicates ± S.D. *Indicates a difference between toxin treated and untreated cells which is significant at the 95% level by ANOVA at the dose of THC indicated.

TABLE 1

EFFECTS OF TOXINS ON THC-INDUCED ARACHIDONATE RELEASE IN S49 CELLS

Treatment	^{14}C-Released (dpm) ± S.D.	Change vs. EtOH (%)	Change vs. Toxin (%)
EtOH	2456 ± 80	—	—
THC	5616 ± 444	128*	—
CT; EtOH	2713 ± 279	—	11†
CT; THC	5130 ± 649	89*	−8.7*
PT; EtOH	2025 ± 314	—	−18*
PT; THC	5344 ± 484	164*	−4.8†

*Indicates a significance level of 95% by ANOVA.

†Not significantly different from control.

The cells were grown and labelled as described in the Method section. Cholera toxin (5 μg/ml) and pertussis toxin (100 ng/ml) were added 18 h prior to labelling with ^{14}C-arachidonate (123,000 dpm/ml/10^6 cells). The cells were washed after 3 h of labelling, challenged with THC (16 μM) in ethanol (10 μl) for 30 min and released arachidonate measured as described in the Method section. The values are the means of six replicates ± S.D.

TABLE 2

EFFECTS OF TOXINS ON THC-INDUCED ARACHIDONATE RELEASE IN PERMEABILIZED S49 CELLS

Treatment	^{14}C-Released (dpm) ± S.D.	Change vs. EtOH (%)	Change vs. Toxin (%)
EtOH	37,600 ± 1260	—	—
THC	42,700 ± 1060	14*	—
CT; EtOH	22,700 ± 522	—	−40*
CT; THC	23,900 ± 788	5.5†	−44*
PT; EtOH	33,800 ± 1370	—	−10*
PT; THC	37,400 ± 1100	11*	−13*

*†As in Table 1.

The cells (10^6/ml) were grown as in the Method section and labelled with ^{14}C-arachidonate (225,000 dpm/10^6 cells) as in Table 1. The cells were then permeabilized in the following medium: phosphate buffer (pH 7.4), 100 mM KCl, 20 mM NaCl, 2 mM $MgCl_2$, 0.2 mM NAD, 1.5 mM ATP, saponin (20 μg/ml) and 0.1% BSA. Cholera toxin (5 μg/ml) and pertussis toxin (100 ng/ml) were activated by treatment with 20 μM DTT in phosphate buffer (pH 7.4) for 18 h and included in the permeabilization medium where indicated. After 30 min, the cells were exposed to THC (16 μM) in ethanol (10 μl) for an additional 30 mins. Values represent the means of six replicates ± S.D.

TABLE 3

EFFECTS OF GUANINE NUCLEOTIDE ANALOGS ON THC-INDUCED ARACHIDONATE RELEASE IN PERMEABILIZED PERITONEAL CELLS

Treatment	^{14}C-Released (dpm) ± S.D.	Change vs. EtOH (%)	Change vs. Analog (%)
EtOH	4685 ± 1040	—	—
THC (3.2 μM)	5715 ± 381	22*	—
THC (16 μM)	6075 ± 670	30*	—
GDP-β-S + EtOH	4305 ± 557	—	− 8.0†
GDP-β-S + THC (3.2 μM)	4500 ± 326	4.5†	−16*
GDP-β-S + THC (16 μM)	4210 ± 282	−2.3†	−12*
GTP-γ-S + EtOH	5755 ± 1086	—	23*
GTP-γ-S + THC (3.2 μM)	5875 ± 598	2.1†	2.6†
GTP-γ-S + THC (16 μM)	5455 ± 876	−5.2†	−10†

*†As in Table 1.

Cell monolayers were prepared as described in the Method section and labelled with ^{14}C-arachidonate (123,000 dpm/ml) for one hour. They were then permeabilized with saponin (20 μg/ml) and exposed to the analogs (100 μM) for 5 min followed by a 30-min exposure to THC. The values are the means of four replicates ± S.D.

DISCUSSION

Recent reports in the literature suggesting that phospholipase activities may be mediated by G-proteins (1,3) prompted us to investigate this as a component of the mechanism for THC-induced release of arachidonic acid. The model which we have previously used to study this effect was the cultured mouse peritoneal cell where the arachidonate pools were carbon-14-labelled (6). Treatment of these cells with THC, or one of its metabolites, resulted in the release of free arachidonic acid into the culture medium that contained 0.1% BSA as a trapping agent. An interesting observation resulting from these experiments was that the potency of the cannabinoid in the model seemed to show some relevance to several of its biological activities such as the "high" in humans and behavioral responses in monkeys.

A cell type which has been used for studying G-protein-mediated signal transduction mechanisms is the S49 lymphocyte (11). We investigated the possibility of utilizing this model for our studies on the mechanism of action of THC. The cells readily incorporated carbon-14-labelled arachidonate into their phospholipid pools and, like the macrophages, released significant amounts of arachidonate upon exposure to THC in the presence of 0.1% BSA (Table 1). An interesting property of various lymphocyte lines is their inability to convert arachidonic acid into either cyclooxygenase or lipoxygenase products (9). We also observed no metabolism of arachidonate either in ex-

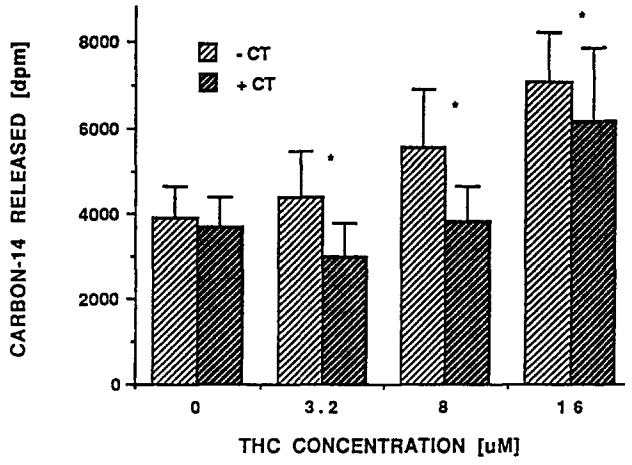

FIG. 2. Inhibition of arachidonate release by cholera toxin. Conditions were as in Fig. 1 except that cholera toxin (5 μg/ml) was used instead of pertussis toxin.

TABLE 4

EFFECTS OF GUANINE NUCLEOTIDE ANALOGS ON THC-INDUCED ARACHIDONATE RELEASE IN PERMEABILIZED S49 CELLS

Treatment	^{14}C-Released (dpm) ± S.D.	Change vs. EtOH (%)	Change vs. Analog (%)
None	1760 ± 35	—	—
EtOH (1%)	1519 ± 99	—	—
THC (3.2 μM)	2073 ± 137	37*	—
THC (16 μM)	2721 ± 115	79*	—
GDP-β-S + EtOH	1505 ± 107	—	0
GDP—β-S + THC (3.2 μM)	1752 ± 182	16†	−15*
GDP-β-S + THC (16 μM)	2585 ± 194	72*	−4.9†
GTP-γ-S + EtOH	1080 ± 79	—	−29*
GTP-γ-S + THC (3.2 μM)	1544 ± 417	43*	−26*
GTP-γ-S + THC (16 μM)	2691 ± 386	149*	0

*†As in Table 1.

Conditions as in Table 3. Values are the means of five replicates ± S.D.

TABLE 5

INHIBITION OF THC-INDUCED CATALEPTIC RESPONSE BY PERTUSSIS TOXIN

	Control	Pertussis Toxin-Treated
Experiment 1	23 ± 13	12 ± 6.8
Experiment 2	41 ± 11	23 ± 15
Mean	32 ± 12	18 ± 12*

*t-Test (2-tail): $p = 0.03$.

Groups of five female CD-1 mice were injected with pertussis toxin (80 μg/kg IP); controls were injected with saline (0.1 ml). Three hours later, both groups were given THC (20 mg/kg PO) in peanut oil (50 μl) and the degree of catalepsy measured after one hour (see the Method section). The values shown are the mean immobilities (%) ± S.D.

periments using endogenous or exogenous precursor. This actually could be considered an advantage for the present studies since we would not have to be concerned with feedback or other effects due to the production of eicosanoids.

Agents such as pertussis toxin and cholera toxin have been shown to react with G-proteins by an ADP ribosylation process. This results in a change in their abilities to transduce signals between cellular receptors and their effector molecules. When our mouse peritoneal cell model was subjected to pretreatment with either of these toxins, significant changes were found in the THC-induced release of arachidonate (Figs. 1 and 2). This suggests that one or more G-proteins are important components in this type of cannabinoid action.

Pertussis toxin was initially reported to inhibit receptor-mediated release of arachidonic acid in neutrophils which was independent of cyclic AMP levels (2). Recently, in similar experiments, Morgan and DuBourdieu (15) found a biphasic response with respect to the dose of pertussis toxin and suggested that more than one G-protein might be involved in the release reaction. Such an explanation may also apply to our results (Fig. 1) where pertussis toxin only inhibited the highest dose of THC. At this point, we cannot say whether there are multiple receptor G-protein combinations or several G-proteins associated with a single receptor. In any case, this biphasic effect is reminiscent of other dose response data for THC.

In the S49 lymphocyte model, cholera toxin reduces the THC response (Table 1) as it does in the peritoneal cells (Fig. 2). Pertussis toxin treatment results in an 18% reduction in arachidonate release from vehicle-treated S49 cells which may have masked its effect on the THC response. The amount of released arachidonate (5344 dpm) is lower than in the nontoxin-treated control (5616 dpm); however, when compared with toxin-treated vehicle control there is an apparent 164% stimulation. Vehicle effects have always been a problem in studying cannabinoids which are highly lipophyllic and cannot be administered in aqueous formulations.

Toxin treatment of S49 cells which had been permeabilized with saponin gave similar results (Table 2). Both pertussis and cholera toxin reduced the release effects of vehicle and THC; however, the control THC response was smaller than we have generally observed with intact cells. This could be due to an increased vehicle effect in this model or some other change due to permeabilization. We also modified the toxins by treatment with DTT prior to addition to the cells which activates the toxins and allows entry into the cells. While these modifications seemed to have enhanced the ethanol response relative to THC, we were still able to observe a significant reduction of release following toxin treatment.

Mechanisms involving G-proteins are expected to be sensitive to the intracellular levels of GDP and GTP. Metabolically resistant analogs of these nucleotides are more effective in modulating PLA_2 activity; however, the cells must be permeabilized to allow their entry into the cytoplasm. The effects of these analogs in the S49 model (Table 4) are what would have been predicted from the current understanding of G-protein action, i.e., inhibition by GDP-β-S and stimulation by GTP-γ-S. Peritoneal cells showed a slightly different response (Table 3) in that GTP-γ-S stimulated only the vehicle-treated cells. It may be that under these conditions the cells are maximally stimulated making further response when treated with THC difficult to attain.

The cataleptic response to THC in mice has been extensively used as a model for THC action in vivo (16). The data in Table 5 suggest that this effect may possibly be mediated by G-proteins as well. Although pertussis toxin could inhibit THC-induced catalepsy by a variety of mechanisms, our previous report on the role of eicosanoids in this response (8) supports the possibility that the in vitro and in vivo data reported here are related processes. We have also shown that THC can produce a cataleptic response in mice where its action on arachidonate metabolism is largely peripheral (8). Thus it would not be required for pertussis toxin to cross the blood-brain barrier to inhibit THC action.

Earlier observations from other laboratories hinted at a possible role for G-proteins in cannabinoid action. Hillard and Bloom (10) found that THC stimulated adenylate cyclase activity in mouse cerebral cortical homogenates and the effect was strongly dependent on the presence of GTP. Interestingly, they also gave evidence for a role for prostaglandins in the action of THC in their system. Using a cultured neuroblastoma cell model, Howlett et al. (12) showed that cannabinoid inhibition of adenylate cyclase activity involved an inhibitory G-protein. This conclusion was based primarily on the fact that pertussis toxin abolished the THC effect in the cells and membranes derived from them. They also showed that the toxin actively promoted ADP ribosylation of membrane protein suggesting that this was how the THC effect was neutralized. It is interesting to note that they were unable to find a cannabinoid effect on either receptor-stimulated or forskolin-activated adenylate cyclase activity in membranes from S49 cells. This would suggest that the THC-induced release of arachidonate in S49 cells which we report here is not mediated by cyclic AMP.

From the results we report, it may be concluded that the release of arachidonic acid by THC is mediated by one or more G-proteins. The signal transduction process probably does not involve cyclic AMP since in one of the models we used, THC was reported to have no effect on adenylate cyclase activity (12). It now remains to identify a specific THC binding site which can couple to this cellular signaling complex. Our findings reported here on the role of G-proteins in THC-induced release of arachidonate should facilitate the search for the receptor responsible for this drug action.

ACKNOWLEDGEMENTS

The authors would like to thank Suzie Hairston for help in growing and maintaining the S49 cells and Annette Dion for preparing the manuscript. This work was supported by the National Institute on Drug Abuse. (Grants DA 02052 and DA 02043 and a Research Scientist Award DA 00043 to S.H.B.)

REFERENCES

1. Axelrod, J.; Burch, R. M.; Jelsema, C. L. Binding proteins: Arachidonic acid and its metabolites as second messengers. Trends Neurosci. 11:117–123; 1988.
2. Bokoch, G. M.; Gilman, A. G. Inhibition of receptor-mediated release of arachidonic acid by pertussis toxin. Cell 39:301–308; 1984.
3. Bourne, H. R. G-protein subunits. Who carries what message? Nature 337:504–505; 1989.
4. Burch, R. M.; Axelrod, J. Dissociation of bradykinin-induced prostaglandin formation from phosphatidylinositol turnover in Swiss 3T3 fibroblasts: Evidence for G-protein regulation of PLA_2. Proc. Natl. Acad. Sci. USA 84:6374-6378; 1987.
5. Burstein, S.; Hunter, S. A. Prostaglandins and cannabis VIII. Elevation of PLA_2 activity by cannabinoids in whole cells and subcellular preparations. J. Clin. Pharmacol. 21:240S–248S; 1981.
6. Burstein, S.; Hunter, S. A.; Ozman, K.; Renzulli, L. Prostaglandins and cannabis XIII. Cannabinoid-induced elevation of lipoxygenase products in mouse peritoneal macrophages. Biochem. Pharmacol. 33:2653–2656; 1984.
7. Burstein, S. Inhibitory and stimulatory effects of cannabinoids on eicosanoid synthesis. Natl. Inst. Drug Abuse Res. Monogr. Ser. 79: 158–172; 1987.
8. Burstein, S. H.; Hull, K.; Hunter, S. A.; Shilstone, J. Immunization against prostaglandins reduces Δ^1-THC-induced catalepsy in mice. Mol. Pharmacol. 35:6–9; 1989.
9. Goldyne, M. E. Lymphocytes and arachidonic acid metabolism. In: Levine, L., ed. Arachidonate metabolism in immunologic systems. Basel: Karger; 1988:140–152.
10. Hillard, C. J.; Bloom, A. S. Possible role of prostaglandins in the effects of the cannabinoids on adenylate cyclase activity. Eur. J. Pharmacol. 91:21–27; 1983.
11. Howlett, A. C.; Van Arsdale, P. M.; Gilman, A. G. Efficiency of coupling between the β adenergic receptor and adenylate cyclase. Mol. Pharmacol. 14:531–539; 1978.
12. Howlett, A. C.; Qually, J. M.; Kachatrian, L. L. Involvement of G_i in the inhibition of adenylate cyclase by cannabimimetic drugs. Mol. Pharmacol. 29:307–313; 1986.
13. Hunter, S. A.; Burstein, S.; Renzulli, L. Effects of cannabinoids on the activities of mouse brain lipases. Neurochem. Res. 11:1273–1288; 1986.
14. Martin, B. Cellular effects of cannabinoids. Pharmacol. Rev. 38: 45–74; 1986.
15. Morgan, D. W.; DuBourdieu, D. J. Multiple pathways involving G-proteins for regulation of arachidonate acid metabolism in rat peritoneal macrophages. In: Vanderhoek, J. Y., ed. Biology of cellular transducing signals '89. abstr. 328; 1989.
16. Pertwee, R. G. The ring test: A quantitative method for assessing the "cataleptic" effect of cannabis in mice. Br. J. Pharmacol. 46: 753–763; 1972.