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Evidence that methyl arachidonyl fluorophosphonate is an irreversible cannabinoid receptor antagonist

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1 Methyl arachidonyl fluorophosphonate (MAFP) (1 μM) significantly attenuated the ability of WIN 55,212-2, CP 55,940, (−)-Δ^9-tetrahydrocannabinol (THC), nabilone and (*R*)-(+)-arachidonoyl-1′-hydroxy-2′-propylamide (methanandamide) to inhibit electrically-evoked isometric contractions of the myenteric plexus-longitudinal muscle preparation of guinea-pig small intestine.

2 The sizes of the maximal responses to WIN 55,212-2 and CP 55,940 decreased significantly in the presence of 1 μM MAFP.

3 MAFP (1 μM) essentially abolished the inhibitory effects on the twitch response of the highest concentration of methanandamide used (3.162 μM). The dextral shift it induced in the log concentration-response curve of nabilone was non-parallel. In contrast, the dextral shift in the log concentration-response curve of THC produced by MAFP did not deviate significantly from parallelism and was relatively small with a mean value of 3.45 and 95% confidence limits of 1.19 and 13.08.

4 MAFP (1 μM) did not attenuate the effects of normorphine or clonidine on the twitch response of the myenteric plexus-longitudinal muscle preparation or affect the contractile response of this preparation to acetylcholine.

5 When administered by itself at concentrations of 1 to 1000 nM, MAFP had no detectable effect on the twitch response of the myenteric plexus-longitudinal muscle preparation.

6 These results support the hypothesis that MAFP is an irreversible cannabinoid CB_1 receptor antagonist that possesses some degree of selectivity.

Keywords: Methyl arachidonyl fluorophosphonate; myenteric plexus; guinea-pig small intestine; cannabinoid receptor agonists; cannabinoid receptor antagonist; Δ^9-tetrahydrocannabinol

Introduction

The discovery of two types of cannabinoid receptor, CB_1 and CB_2, has prompted a search for selective agonists and antagonists for these receptors (Pertwee, 1996). As a result, a competitive, reversible CB_1 receptor antagonist, SR141716A, has been developed (Rinaldi-Carmona *et al*., 1994). CB_2 receptor antagonists still remain to be developed as do irreversible antagonists of cannabinoid receptors. Recently, it was shown that the phospholipase A_2 inhibitor, methyl arachidonyl fluorophosphonate (MAFP), induces irreversible inhibition of the enzymic hydrolysis of the endogenous cannabinoid receptor agonist, arachidonoyl ethanolamide (anandamide) and undergoes irreversible binding to cannabinoid CB_1 receptors (Deutsch *et al*., 1997). The present investigation has followed up the second of these findings by exploring the possibility that MAFP is an irreversible cannabinoid receptor antagonist.

Experiments were carried out with the myenteric plexus-longitudinal muscle preparation of guinea-pig small intestine which contains cannabinoid CB_1 receptors that can mediate inhibition of electrically-evoked contractions (Pertwee *et al*., 1992; 1996; Paterson & Pertwee, 1993). Our strategy was to establish whether MAFP possesses cannabimimetic activity and, if not, whether it can produce insurmountable antagonism of cannabinoid receptor agonists as measured by reductions in the size of the maximal responses of these agonists or in the slopes of their log concentration-response curves. The cannabinoid receptor agonists used in these experiments were (−)-Δ^9-tetrahydrocannabinol (THC), nabilone, WIN 55,212-2, CP 55,940 and methanandamide which is less susceptible than anandamide to enzymic hydrolysis (Abadji *et al*., 1994). Each of these compounds has already been shown to produce a concentration-related inhibition of electrically-evoked contractions of the guinea-pig myenteric plexus-longitudinal muscle preparation (Pertwee *et al*., 1995;1996). The selectivity of MAFP was investigated by determining its ability to attenuate inhibition of electrically-evoked contractions mediated by opioid receptors or by α_2-adrenoceptors, both of which resemble cannabinoid receptors in that they are members of the superfamily of $G_{i/o}$ protein coupled receptors (Alexander & Peters, 1997). Also addressed was the question of whether MAFP has a direct effect on the contractile response to acetylcholine, the neurotransmitter that is primarily responsible for contractions elicited by electrical stimulation of prejunctional neurones of the guinea-pig myenteric plexus-longitudinal muscle preparation (Cowie *et al*., 1978).

Methods

In vitro preparation

Experiments were carried out with strips of myenteric plexus-longitudinal muscle, dissected from the small intestine of male albino Dunkin-Hartley guinea-pigs (271–602 g) using the method of Paton & Zar (1968). Tissues were immersed in Krebs solution which was kept at 37°C and bubbled with 95% O_2 and 5% CO_2. The composition of the Krebs solution was (mM): NaCl 118.2, KCl 4.75, $MgSO_4.7H_2O$ 1.29, KH_2PO_4 1.19, $NaHCO_3$ 25.0, glucose 11.0 and $CaCl_2.6H_2O$ 2.54. Each tissue was mounted in a 4 ml organ bath under an initial tension of 0.5 g. In some experiments, contractions were induced by acetylcholine, concentration-response curves being constructed non-cumulatively with a dose-cycle of 5 min. In all other experiments, contractions were evoked by electrical field stimulation, single bipolar rectangular pulses of 110% maximal voltage, 0.5 ms duration and 0.1 Hz frequency being applied through a platinum electrode attached to the upper end and a stainless steel electrode

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attached to the lower end of each bath. Stimuli were generated by a Grass S48 stimulator, then amplified (Med-Lab channel attenuator) and divided to yield separate outputs to four organ baths (Med-Lab StimuSplitter). All contractions were recorded isometrically. They were monitored by computer (Apple Macintosh Performa 475) with a data recording and analysis system (MacLab) that was linked via preamplifiers (Macbridge) to Dynamometer UF1 transducers (Pioden Controls).

Concentration-response curves of normorphine and clonidine were constructed cumulatively with a dose-cycle of 2 min. In experiments with cannabinoid receptor agonists, only one concentration-response curve was constructed per tissue, previous experiments having shown that it is impossible to reverse the effects of cannabinoids by perfusing the organ baths with drug-free Krebs-Henseleit solution (Pertwee *et al.*, 1992). Cannabinoids were added cumulatively at intervals of 15 min (WIN 55,212-2 and CP 55,940) or 30 min. MAFP was administered 30 min before the first addition of any other drug. Once a twitch inhibitor had been added, tissues were incubated for several hours without replacing the fluid in the bath.

Drugs

Methanandamide ((*R*)-(+)-arachidonoyl-1′-hydroxy-2′-propylamide) was obtained from Dr H. Seltzman (Research Triangle Institute, U.S.A.). THC was supplied by the National Institute on Drug Abuse, CP 55,940 ((−)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol) by Pfizer, WIN 55,212-2 ((*R*)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholino)methyl]pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl](1-naphthyl)methanone) by Sanofi Winthrop, nabilone by Lilly and MAFP by Biomol. Each of these drugs was mixed with 2 parts of Tween 80 by weight and dispersed in a 0.9% aqueous solution of NaCl (saline) as described previously for THC (Pertwee *et al.*, 1992). Clonidine HCl and acetylcholine chloride were supplied by Sigma and normorphine HCl by MacFarlan Smith. These were all dissolved in saline. Drug additions were made in a volume of 10 μl. In control experiments, Tween 80 was added instead of MAFP.

Analysis of data

Values are expressed as means and limits of error as s.e.mean. The degree of drug-induced inhibition of the twitch response is expressed in percentage terms. This was calculated by comparing the amplitude of the electrically-evoked twitch response immediately before agonist administration with the amplitude of the twitch response at various times after agonist administration. Dose-ratio values and their 95% confidence limits were determined by symmetrical (2+2) dose parallel line assays (Colquhoun, 1971), by measuring responses to pairs of agonist concentrations located on the steepest part of each log concentration-response curve. This method was also used to establish whether any pairs of log concentration-response curves showed significant deviation from parallelism. Values, with their 95% confidence limits for the sizes of mean maximal responses, were calculated by nonlinear regression analysis with GraphPad InPlot (GraphPad Software, San Diego). A P value <0.05 was considered to be significant.

Results

When administered by itself at concentrations of 1, 10, 100, 316.2 or 1000 nM, MAFP had no detectable effect on the twitch response of the myenteric plexus-longitudinal muscle preparation. However, it did produce concentration-related dextral shifts in the log concentration-response curves of WIN 55,212-2 and CP 55,940 (Figure 1). The maximal responses to both agonists decreased significantly in the presence of the highest concentration of MAFP used (1 μM). This concentration of MAFP also significantly attenuated the ability of THC, nabilone and methanandamide to inhibit electrically-evoked

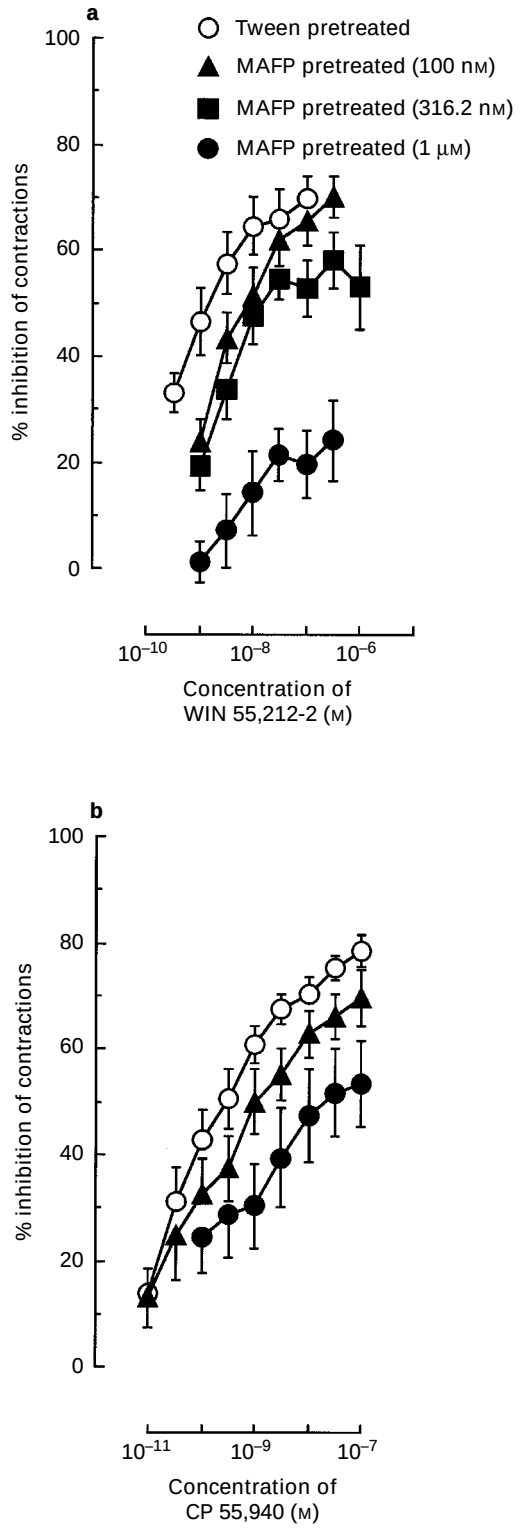

Figure 1 Mean concentration-response curves for (a) WIN 55,212-2 and (b) CP 55,940 constructed in the presence of MAFP or Tween 80. Each symbol represents the mean value of inhibition of electrically-evoked contractions expressed as a percentage of the amplitude of the twitch response measured immediately before the first addition of an agonist to the organ bath (n = 6 or 7 different myenteric plexus-longitudinal muscle preparations); vertical lines show s.e.mean. MAFP and Tween 80 were added 30 min before the first addition of agonist. The estimated mean maximal effect of WIN 55,212-2 with its 95% confidence limits shown in parentheses was 70.6% (66.8 and 74.4%) in the presence of Tween 80 and 22.9% (18.1 and 27.8%) in the presence of 1 μM MAFP (GraphPad InPlot). The corresponding values for CP 55,940 in the presence of Tween 80 and 1 μM MAFP were 78.1% (71.8 and 84.4%) and 55.8% (42.5 and 69.1%), respectively.

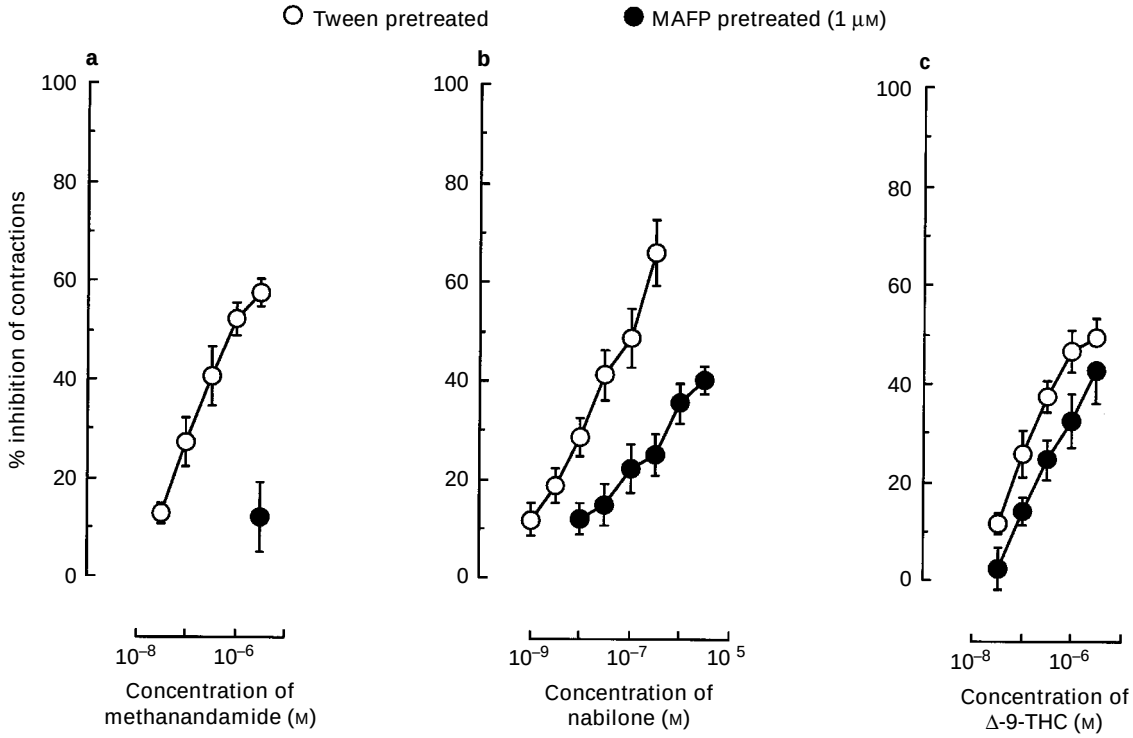

Figure 2 Mean concentration-response curves for (a) methanandamide, (b) nabilone and (c) THC constructed in the presence of MAFP or Tween 80. Each symbol represents the mean value of inhibition of electrically-evoked contractions expressed as a percentage of the amplitude of the twitch response measured immediately before the first addition of an agonist to the organ bath (n = 6 or 8 different myenteric plexus-longitudinal muscle preparations); vertical lines show s.e.mean. MAFP and Tween 80 were added 30 min before the first addition of agonist. The dextral shifts in log concentration-response curves were parallel and statistically significant for THC and non-parallel for nabilone. In the presence of 1 μM MAFP, methanandamide concentrations of 31.62, 100, 316.2 and 1000 nM had no inhibitory effect on electrically-evoked contractions (data not shown; n = 6).

contractions (Figure 2). The dextral shift induced by MAFP in the log concentration-response curve of methanandamide was at least 100 fold, the response to the highest agonist concentration (3.162 μM) being essentially abolished. Cannabinoid concentrations above 3.162 μM were not used in any of our experiments as these would have produced Tween 80 concentrations that can themselves inhibit the twitch response of the myenteric plexus-longitudinal muscle preparation (Pertwee *et al.*, 1996). MAFP also induced a substantial dextral shift in the log concentration-response curve of nabilone that deviated significantly from parallelism. In contrast, the dextral shift in the log concentration-response curve of THC produced by MAFP did not deviate significantly from parallelism and was relatively small. The mean value of this shift was calculated to be 3.45 with 95% confidence limits of 1.19 and 13.08.

A MAFP concentration of 1 μM did not alter the positions of the log concentration-response curves of normorphine or clonidine (Figure 3). Nor did it affect the contractile potency of acetylcholine (Figure 4). The drug vehicle, Tween 80, had no detectable effect on the dose-response curves of any of the agonists used in this investigation (data not shown; n = 6 for each agonist).

Discussion

The results obtained show that MAFP can significantly attenuate the ability of all the cannabinoid receptor agonists used in our experiments to inhibit electrically-evoked contractions of the myenteric plexus-longitudinal muscle preparation of guinea-pig small intestine. There are two main reasons for believing that this antagonism was irreversible in nature. First, in addition to inducing dextral shifts in the log concentration-response curves of all five cannabinoid receptor agonists investigated, 1 μM MAFP reduced the size of the maximal responses to WIN 55,212-2 and CP 55,940 and decreased the slope of the log concentration-response curve of nabilone. These are the expected consequences of a decrease in receptor population produced by the permanent occupancy of a proportion of this population by an irreversible antagonist (see Kenakin, 1993). Second, Deutsch *et al.* (1997) have found that MAFP binds irreversibly to cannabinoid CB_1 receptors, the same receptor type that seems to mediate cannabinoid-induced inhibition of the twitch response of the myenteric plexus-longitudinal muscle preparation (Pertwee *et al.*, 1996).

THC potency appeared to be less affected by MAFP than the potencies of the other cannabinoids investigated. Why this should be remains to be established. One possible explanation is that the efficacy of THC is higher than that of any of these other cannabinoids so that the ability of THC to inhibit the twitch response is relatively insensitive to partial reductions in the number of available cannabinoid receptors. Another possibility is that THC differs from other cannabinoids in its mode of attachment to CB_1 receptors and that this difference causes it to be less susceptible to antagonism by MAFP. This possibility requires further investigation as there is already evidence that the binding of WIN 55,212-2 to the CB_1 receptor differs subtly from that of (−)-11-hydroxy-Δ^8-tetrahydrocannabinol-dimethylheptyl, CP 55,940 and anandamide (Song & Bonner, 1996). It is also possible that there may be non-CB_1 receptors in the myenteric plexus-longitudinal muscle preparation that interact more readily with THC than with MAFP or with the other cannabinoids investigated.

MAFP did not affect the abilities of the non-cannabinoids normorphine and clonidine to inhibit electrically-evoked contractions of the myenteric plexus-longitudinal muscle preparation. Nor did it alter the contractile potency of acetylcholine. These results suggest that MAFP possesses at least some degree of selectivity for cannabinoid receptors and that it acts prejunctionally. Further experiments are now required to establish whether this putative cannabinoid re-

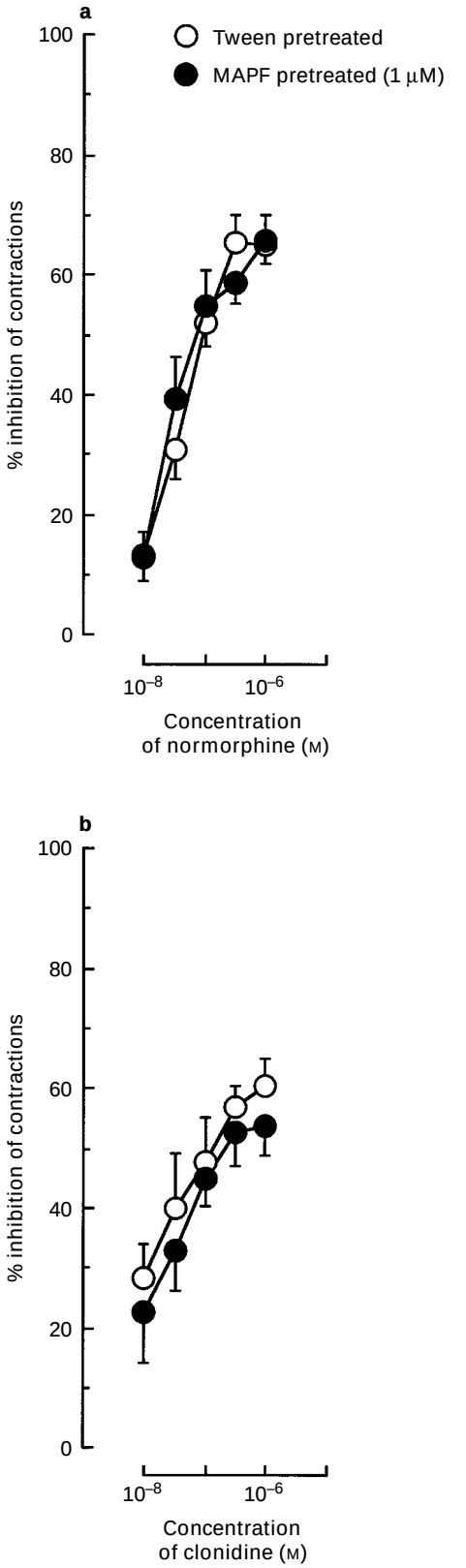

Figure 3 Mean concentration-response curves for (a) normorphine and (b) clonidine constructed cumulatively in the presence of MAFP or Tween 80. Each symbol represents the mean value of inhibition of electrically-evoked contractions expressed as a percentage of the amplitude of the twitch response measured immediately before the first addition of a twitch inhibitor to the organ bath ($n=5$ or 6 different myenteric plexus-longitudinal muscle preparations); vertical lines show s.e.mean. MAFP and Tween 80 were added 30 min before the first addition of normorphine or clonidine.

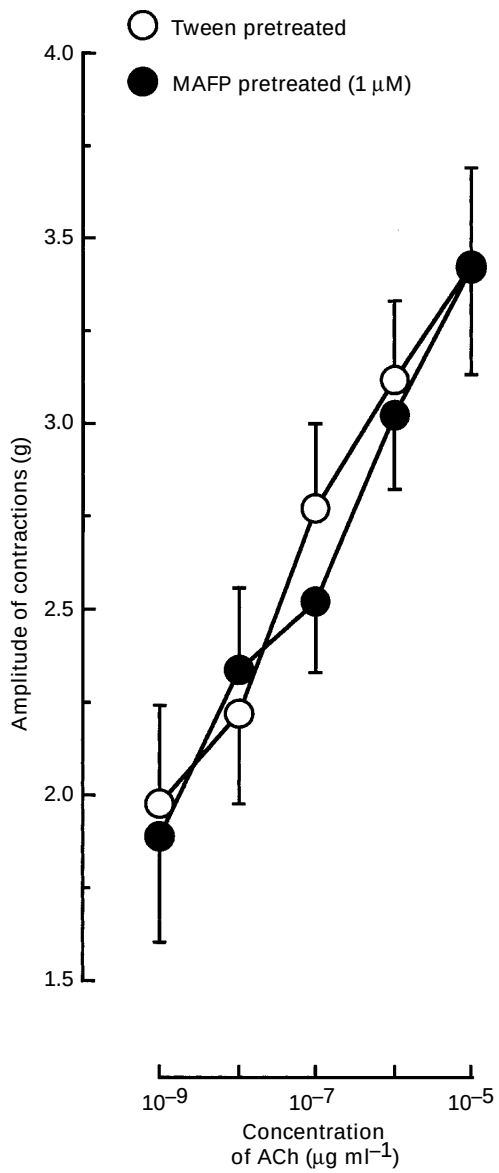

Figure 4 Mean concentration-response curves for acetylcholine constructed non-cumulatively after pretreatment with MAFP or Tween 80. Each symbol represents the mean value of the amplitude of contractions produced by acetylcholine ($n=6$ different myenteric plexus-longitudinal muscle preparations); vertical lines show s.e.mean. MAFP and Tween 80 were added 30 min before the first addition of acetylcholine.

ceptor antagonist has significant affinity for other types of non-cannabinoid receptor. The evidence that MAFP acted on prejunctional sites lends further support to the hypothesis that it antagonizes cannabinoids in the myenteric plexus-longitudinal muscle preparation by binding to cannabinoid receptors, as the location of these receptors in this preparation is also thought to be prejunctional (Pertwee *et al.*, 1996).

The concentrations of MAFP used in the present investigation not only bind irreversibly to cannabinoid CB_1 receptors but also produce irreversible inhibition of phospholipase A_2 and of fatty acid amide hydrolase, an enzyme that catalyses the hydrolysis of anandamide (Cravatt *et al.*, 1996; Huang *et al.*, 1996; Deutsch *et al.*, 1997). The extent to which inhibition of these enzymes contributed to the interactions between MAFP and cannabinoid receptor agonists described here remains to be established. However, it is noteworthy, that if the myenteric plexus-longitudinal muscle preparation does produce anandamide, then inhibition of fatty acid amide hydrolase in this preparation would tend to cause an accumulation of this *N*-

acylethanolamine. Such an accumulation is unlikely to account for the attenuation of responses to added cannabinoids induced by MAFP, as it is already known that in the presence of the general protease inhibitor, phenylmethylsulphonyl fluoride, anandamide is a potent inhibitor of electrically-evoked contractions of the myenteric plexus-longitudinal muscle preparation (Pertwee *et al*., 1995). Indirect evidence that the myenteric plexus-longitudinal muscle preparation may indeed produce a cannabinoid receptor agonist comes from the observation that in the absence of other drugs, the cannabinoid CB_1 receptor antagonist, SR141716A, produces a small but significant increase in the amplitude of electrically-evoked contractions of this preparation (Pertwee *et al*., 1996). However, this evidence is weakened by our finding in the present investigation that MAFP had no such effect on twitch amplitude.

In conclusion, the available data suggest that MAFP is an irreversible cannabinoid CB_1 receptor antagonist that possesses some degree of selectivity. Further experiments are required to investigate whether this compound is a CB_2 receptor antagonist or indeed whether it can serve as an antagonist of any type of non-cannabinoid receptor. It will also be worth investigating whether the chemical structure of MAFP can be modified to reduce or eliminate its ability to inhibit phospholipase A_2 and fatty acid amide hydrolase, without affecting its ability to bind to cannabinoid receptors.

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