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Further evidence for the presence of cannabinoid CB_1 receptors in guinea-pig small intestine

[1]Roger G. Pertwee, Susanthi R. Fernando, Joanne E. Nash & Angela A. Coutts

Department of Biomedical Sciences, Marischal College, University of Aberdeen, Aberdeen, AB9 1AS, Scotland

1 CP 50,556, CP 55,940, nabilone, CP 56,667, Δ^9-tetrahydrocannabinol (THC) and cannabinol each inhibited electrically-evoked contractions of the myenteric plexus-longitudinal muscle preparation of guinea-pig small intestine in a concentration-related manner. The IC_{50} values of these cannabinoids, respectively 3.45, 3.46, 30.61, 162.94, 214.63, and 3913.5 nM, correlate well with previously obtained potency values for displacement of [^{3}H]-CP 55,940 from cannabinoid binding sites.

2 Electrically-evoked contractions of the myenteric plexus-longitudinal muscle preparation were also inhibited by AM 630 (6-iodo-pravadoline) and by WIN 55,212-2 (IC_{50} = 1923.0 and 5.54 nM, respectively). The present finding that AM 630 is an agonist, contrasts with a previous observation that it behaves as a cannabinoid receptor antagonist in the mouse isolated vas deferens.

3 SR141716A produced dose-related parallel rightward shifts in the log concentration-response curves of CP 55,940, WIN 55,212-2, THC and AM 630 for inhibition of electrically-evoked contractions of the myenteric plexus-longitudinal muscle preparation. SR141716A (1 μM) did not reverse the inhibitory effects of normorphine and clonidine on electrically-evoked contractions or potentiate the contractile response to acetylcholine.

4 Doses of naloxone and yohimbine that reversed the inhibitory effects of normorphine or clonidine on electrically-evoked contractions of the myenteric plexus-longitudinal muscle preparation did not affect the inhibitory response to WIN 55,212-2.

5 Electrically-evoked release of acetylcholine from strips of myenteric plexus-longitudinal muscle was decreased by 200 nM CP 55,940 and this inhibitory effect was almost completely reversed by 1 μM SR141716A. Acetylcholine-induced contractions were not affected by 200 nM CP 55,940.

6 These results support the hypothesis that guinea-pig small intestine contains prejunctional cannabinoid CB_1 receptors through which cannabinoids act to inhibit electrically-evoked contractions by reducing release of the contractile transmitter, acetylcholine.

7 THC was found to be more susceptible to antagonism by SR141716A than CP 55,940 or AM 630, raising the possibility that guinea-pig small intestine contains more than one type of cannabinoid receptor.

8 At concentrations of 10 nM and above, SR141716A produced small but significant increases in the amplitude of electrically-evoked contractions of the myenteric plexus-longitudinal muscle preparation suggesting that this tissue may release an endogenous cannabinoid receptor agonist or that some cannabinoid receptors in this tissue are precoupled and that SR141716A can reduce the number of receptors in this state.

Keywords: Myenteric plexus; guinea-pig small intestine; acetylcholine release; cannabinoid receptor agonists; cannabinoid receptor antagonist; Δ^9-tetrahydrocannabinol; AM 630; SR141716A

Introduction

Previous experiments have shown that the myenteric plexus-longitudinal muscle preparation of guinea-pig small intestine contains specific cannabinoid binding sites (Paterson & Pertwee, 1993) and that certain cannabinoids show high potency and remarkable stereoselectivity as inhibitors of electrically-evoked contractions of this preparation (Pertwee *et al.*, 1992). These results make it likely that this effect of cannabinoids on the twitch response is mediated by cannabinoid receptors. We have now explored this hypothesis further by investigating the ability of the selective CB_1 cannabinoid receptor antagonist, SR141716A (Rinaldi-Carmona *et al.*, 1994) to attenuate or reverse the inhibitory effects of certain cannabinoid receptor agonists on electrically-evoked contractions of myenteric plexus-longitudinal muscle preparation. Experiments were also carried out with AM 630 (6-iodo-pravadoline), which, like SR141716A (Rinaldi-Carmona *et al.*, 1994; Pertwee *et al.*, 1995a), behaves as a competitive cannabinoid receptor antagonist in the mouse isolated vas deferens (Pertwee *et al.*, 1995b). The agonists chosen for these experiments were Δ^9-tetrahydrocannabinol (THC), CP 55,940 and WIN 55,212-2, each of which is a representative of a different chemical class of cannabinoid receptor agonist (Pertwee, 1995). Because there is evidence that cannabinoids may inhibit electrically-evoked contractions of the myenteric plexus-longitudinal muscle indirectly, by suppressing prejunctional release of acetylcholine (see Pertwee, 1990), we performed an additional set of experiments to monitor the effect of a cannabinoid receptor agonist on acetylcholine release during electrical stimulation and to establish the ability of SR141716A to reverse this effect. We have also compared the potencies of several cannabinoid receptor agonists as inhibitors of electrically-evoked contractions of the myenteric plexus-longitudinal muscle preparation with their potencies as competitors of a labelled probe for cannabinoid binding sites, by use of binding data already in the literature. These experiments were performed with THC, nabilone, CP 55,940, CP 56,667, CP 50,556 and cannabinol (see Pertwee, 1995) as their binding potencies have all been determined in the same laboratory (Herkenham *et al.*, 1990).

Some of the results described in this paper have been presented to the British Pharmacological Society (Coutts *et al.*, 1995).

[1] Author for correspondence.

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 (310–620 g) by the method of Paton & Zar (1968). Tissues were immersed in Krebs solution that 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. Additional constituents were present when acetylcholine release was being measured (see below).

Evoked isometric contractions

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 2 min. In 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 platinum electrodes attached to the upper and lower ends of each bath. Stimuli were usually 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). Contractions were monitored by computer (Apple Macintosh LC) with a data recording and analysis system (MacLab) that was linked via preamplifiers (Macbridge) to Dynamometer UF1 transducers (Pioden Controls). In some experiments, stimuli were generated by a Grass S88 stimulator and the resulting contractions registered on a polygraph recorder (Grass model 7D) with Pye Ether UF1 transducers.

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 AM 630) or 30 min. Once a cannabinoid receptor agonist or antagonist had been added, tissues were incubated for several hours without replacing the fluid in the bath.

Acetylcholine release

Acetylcholine release from strips of myenteric plexus-longitudinal muscle was measured by bioassay by use of a method modified from Waterfield (1973). A donor strip of myenteric plexus-longitudinal muscle was set up in a 3 ml organ bath and bathed in Krebs-Henseleit solution containing choline chloride (20 μM) (Sigma). The preparation was allowed to equilibrate for 10 min then stimulated as described above until the contractile responses were constant. Physostigmine sulphate (Sigma) was added to the bathing medium to give a final bath concentration of 7.7 μM physostigmine and the preparation left unstimulated for 1 h before the collection of samples for bioassay. The donor bath fluid was refreshed at the beginning of each 4-min collection period during which the preparation was stimulated electrically at 0.1 Hz (24 pulses). At the end of the collection period, a sample of bath fluid was withdrawn by syringe for assay and the bath fluid replaced. Preparations were not stimulated between collection periods. Samples (50 to 350 μl) were collected from the donor bath, firstly in the absence of any drug, secondly in the presence of 200 nM CP 55,940 and finally in the presence of 1 μM SR141716A or of Tween 80. Under each of these conditions, acetylcholine output was measured until it was fairly constant for three consecutive collection periods. As there was no significant difference between mean output values obtained in the final three collection periods before administration of CP55,940 (analysis of variance followed by Scheffé's test; data not shown), these were pooled to yield a single value of mean output. Similarly, there was no significant difference between mean output values obtained in the final three collection periods after administration of CP55,940 and these data too were pooled to yield a single value. Values for mean acetylcholine output were also determined approximately 20 min after the addition of 1 μM SR141716A or of Tween 80. Neither CP 55,940, SR141716A nor Tween 80 had any effect on the sensitivity of myenteric plexus-longitudinal muscle preparations to acetylcholine (see Results). Consequently, these drugs were omitted from standard solutions of acetylcholine. Standard solutions and test drugs were diluted in Krebs-Henseleit solution identical to that bathing the donor tissue. Two or three aliquots of each unknown sample were assayed against standard acetylcholine solutions. At the end of each experiment, the donor preparation was blotted dry and weighed.

A second myenteric plexus-longitudinal muscle preparation from the guinea-pig small intestine was used for the bioassay of acetylcholine. The tissue was mounted in a 3 ml organ bath and immersed in Krebs-Henseleit solution containing 1.3 μM morphine HCl (MacFarlan Smith) in order to reduce spontaneous activity due to the release of endogenous acetylcholine from the recipient preparation. Responses of the longitudinal muscle were recorded isotonically (Harvard Apparatus Ltd.) under a resting tension of 0.5 g and displayed on a pen oscillograph. Contractions due to aliquots of unknown sample were bracketed between smaller and larger contractions due to standard solutions of acetylcholine and the corresponding concentrations of acetylcholine in the unknown sample calculated by interpolation. Volumes of standard solutions were as close as possible to the volumes of unknown samples. Responses both to unknown solutions and to standard solutions of acetylcholine were abolished by atropine (100 nM).

Drugs

AM 630 was synthesized in the laboratory of Professor Alexandros Makriyannis, University of Connecticut, U.S.A., Δ^9-tetrahydrocannabinol (THC) was obtained from the National Institute on Drug Abuse, CP 55,940 ((–)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol), CP 56,667 ((+)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol, and CP50,556 ((–)-(6S, 6aR,9R,10aR)-5, 6, 6a, 7, 8,9,10a-octahydro-6-methyl-3-[(R)-1-meth-yl-4-phenylbutoxyl]-1,9-phenanthridinediol 1-acetate hydrochloride) from 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) from Sanofi Winthrop nabilone from Lilly, cannabinol from Sigma and SR141716A ((N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride) from Sanofi. These drugs were 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, acetylcholine chloride, yohimbine HCl and atropine sulphate were supplied by Sigma, naloxone HCl by Endo Laboratories Inc. and normorphine by Burroughs Wellcome. These were all dissolved in saline. Drug additions were made in a volume of 10 μl. Interactions between agonists and antagonists were investigated either by administering the antagonist at least 15 min before the agonist or by adding it after the agonist response had been fully elicited. In control experiments, Tween 80 was added instead of cannabinoid receptor agonists and antagonists.

Analysis of data

Values are expressed as means and limits of error as standard errors. The degree of inhibition of the twitch response by

cannabinoid receptor agonists 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. The concentration of a cannabinoid that produces 50% of its maximum inhibitory effect on the twitch response (IC_{50}) was used to characterize its potency. Output of acetylcholine is expressed as nmol g^{-1} per pulse.

To determine the dissociation constant (K_D) for the interaction between SR141716A and cannabinoid receptors, dose-response curves of cannabinoid receptor agonists were constructed in the presence of SR141716A or its vehicle. In most sets of experiments several concentrations of the antagonist were used so that K_D values could be calculated from the slope ($1/K_D$) of the best-fit straight line of a plot of $(x-1)$ against B, constrained to pass through the origin (Tallarida *et al.*, 1979). The equation for this graph is $(x-1)=B/K_D$, where x (the 'concentration-ratio') is the concentration of an agonist that produces a particular degree of inhibition in the presence of SR141716A at a concentration, B, divided by the dose of the same agonist that produces an identical degree of inhibition in the absence of SR141716A. Concentration-ratio values and their 95% confidence limits were determined by symmetrical (2+2) dose parallel line assays (Colquhoun, 1971), using responses to pairs of agonist concentrations located on the steepest part of each log concentration-response curve. In none of these assays did pairs of log concentration-response curves show a significant deviation from parallelism ($P>0.05$). The Schild slope for the interaction between cannabinoid receptor agonists and SR141716A was obtained from the best-fit straight line of a plot of log $(x-1)$ against log B (Arunlakshana & Schild, 1959). The equation for this graph, log $(x-1)=(\log B)-\log K_D$, predicts a slope of unity for receptor-mediated interactions between agonists and antagonists that are competitive and reversible. The K_D value of SR141716A determined from experiments with AM 630 was calculated by substituting a single dose-ratio value into the above equation.

Values with their 95% confidence limits for the slope, $1/K_D$, for the Schild slope and for agonist concentrations producing 50% inhibition of the twitch response (IC_{50}s), were calculated by nonlinear regression analysis by GraphPAD InPlot (GraphPAD Software, San Diego). Mean values were compared by Student's *t* test for unpaired data or by analysis of variance followed by Scheffé's test (Super Anova, Abacus Concepts Inc., Berkeley). A *P* value <0.05 was considered to be significant.

Results

Effects of cannabinoids on contractions or acetylcholine release evoked by electrical stimulation

Electrically-evoked contractions of the myenteric plexus-longitudinal muscle preparation were inhibited by THC, nabilone, CP 55,940, CP 56,667, CP 50,556 WIN 55,212-2 and cannabinol in a concentration-related manner (Figure 1). AM 630 was also inhibitory. The log concentration-response curves are all sigmoid in nature ($r^2=0.926$ to 1.0) and the IC_{50} values, calculated by nonlinear regression analysis, are listed in Table 1. At a concentration of 200 nM, CP 55,940 also significantly decreased electrically-evoked release of acetylcholine from strips of myenteric plexus-longitudinal muscle (Figure 2). Acetylcholine release was not significantly affected by Tween 80 (data not shown).

The binding potencies of several of the compounds used in the present experiments, as measured by their ability to compete with [^{3}H]-CP 55,940 for cannabinoid binding sites, have been determined previously (Herkenham *et al.*, 1990). These binding potency values correlate closely with those for inhibition of the twitch response determined in the present investigation (Figure 3).

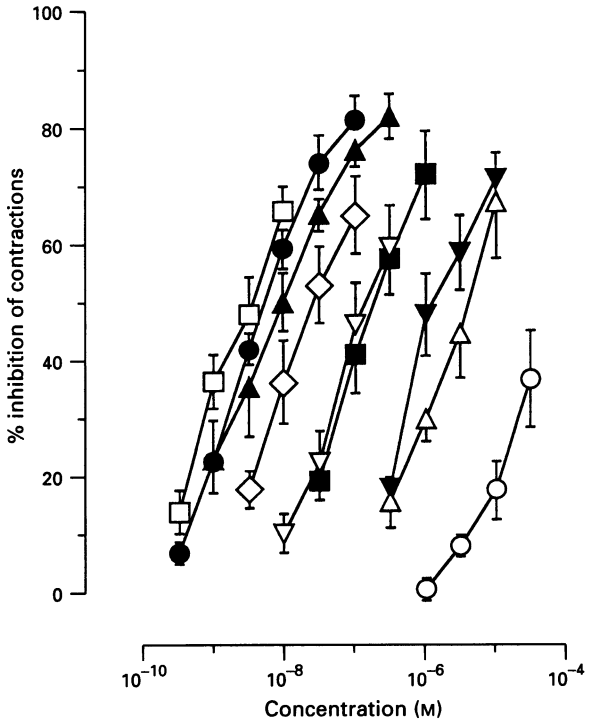

Figure 1 Mean concentration-response curves for CP 50,556 (□), CP 55,940 (●), WIN 55,212-2 (▲), nabilone (◇), CP 56,667 (▽), THC (■), AM 630 (▼) and cannabinol (△). (○) Responses to doses of Tween 80 that are required to achieve cannabinoid concentrations in the organ bath of 1, 3, 10 or 31 μM. Each 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 compound to the organ bath ($n=5$ to 7 different myenteric plexus-longitudinal muscle preparations for each agonist); vertical lines indicate s.e.mean.

Table 1 IC_{50} values of certain cannabinoids for inhibition of electrically-evoked contractions of the myenteric plexus-longitudinal muscle preparation of the guinea-pig small intestine

Agonist	*IC_{50} mean (nM)*	*IC_{50} 95% confidence limits (nM)*
CP50556	3.45	1.55 & 7.65
CP55940	3.46	2.30 & 5.21
WIN 55212-2	5.54	4.35 & 7.08
Nabilone	30.61	16.96 & 55.25
CP56667	162.94	83.4 & 318.3
THC	214.63	125.0 & 368.4
AM 630	1923.0	586.8 & 6301.8
Cannabinol	3913.5	2901.7 & 5278.1

Effects of SR141716A pretreatment on cannabinoid-induced inhibition of the twitch response

SR141716A behaved as a competitive surmountable antagonist. Thus it produced parallel rightward shifts in the log concentration-response curves of CP 55,940, WIN 55,212-2, THC and AM 630 (Figure 4) and the Schild slope, which was determined for the interaction between SR141716A and three of these agonists (Table 2), was found to be close to unity. The susceptibility of CP 55,940, WIN 55,212-2, THC and AM 630 to antagonism by SR141716A is summarized in Table 2. Pretreatment with SR141716A did not decrease the ability of normorphine or clonidine to inhibit the twitch response (Figure 5) or enhance the ability of acetylcholine to induce contractions in the absence of electrical stimulation (Figure 6a). Acetylcholine-induced contractions of the myenteric plexus-

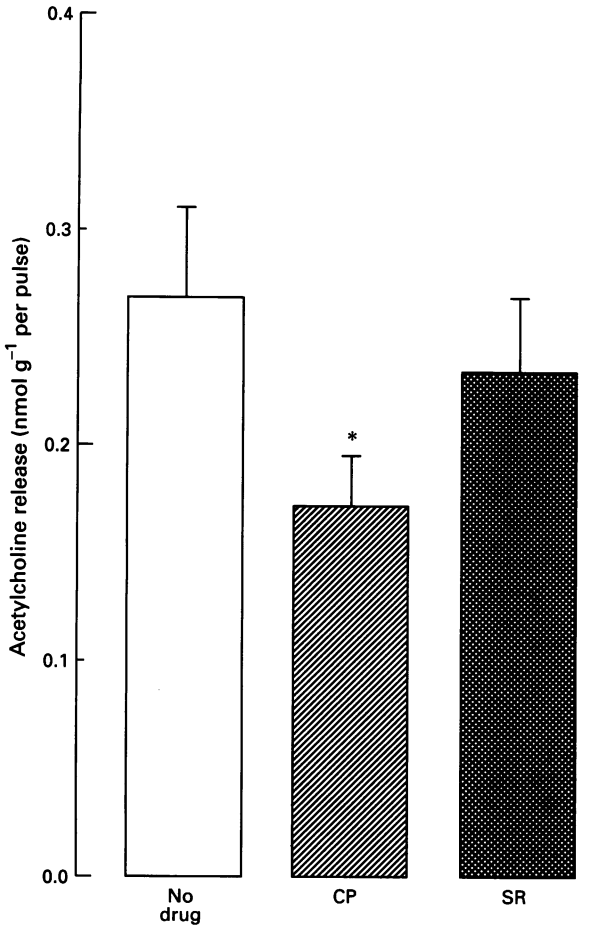

Figure 2 Release of acetylcholine from electrically stimulated strips of myenteric plexus-longitudinal muscle before drug administration (no drug), in the presence of 200 nM CP 55,940 (CP) alone and in the presence of 200 nM CP 55,940 plus 1 μM SR141716A (SR). Each column represents the mean value of acetylcholine release expressed as nmol g^{-1} per pulse ($n=6$ different donor preparations); vertical lines indicate s.e. mean. The asterisk denotes a significant difference between release before and after drug administration (analysis of variance followed by Scheffés test).

longitudinal muscle preparation were also unaffected by pretreatment with CP 55,940 at a concentration of 200 nM (Figure 6b).

The interaction between SR141716A and AM 630 was studied with only one concentration of antagonist (Figure 4). This is because the production of larger rightward shifts in the log concentration-response curve of AM 630 than the one shown in Figure 4 would have required the use of concentrations of AM 630 above 10 μM and these would have produced Tween 80 concentrations in the organ bath that are themselves inhibitory (Figure 1). For the same reason, the log concentration-response curve of AM 630 constructed in the presence of the antagonist is incomplete.

By itself, SR141716A caused the amplitude of electrically-evoked contractions to rise (Figure 7). Additions of twitch inhibitors in the presence of SR141716A were made only after the amplitude of the twitch response had reached a new steady level. This occurred 10 to 15 min after administration of SR141716A.

Further experiments with SR141716A

These experiments showed that CP 55,940-induced inhibition of electrically-evoked release of acetylcholine from strips of myenteric plexus-longitudinal muscle could be largely reversed by SR141716A (Figure 2). No significant change in electrically-evoked release of acetylcholine occurred when Tween 80 was added instead of SR141716A (data not shown). SR141716A

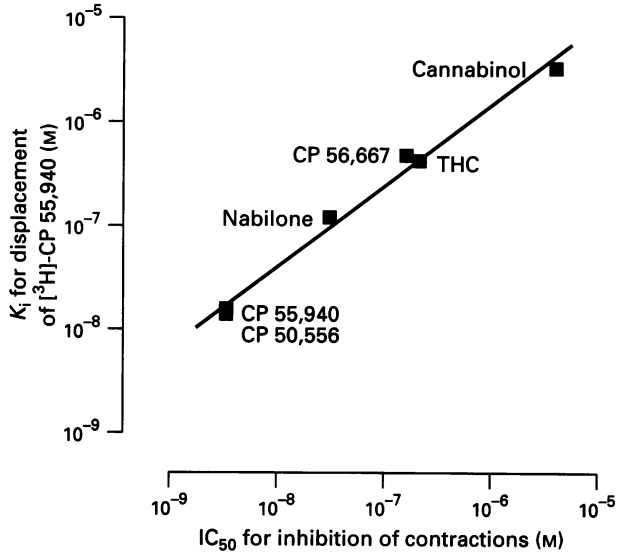

Figure 3 Correlation between the potencies of certain cannabinoids as inhibitors of the twitch response and their potencies for displacement of [^{3}H]-CP 55,940 from cannabinoid binding sites ($r^2=0.988$). Linear regression analysis (GraphPAD InPlot) was used to plot IC_{50} values for inhibition of the twitch response of the myenteric plexus-longitudinal muscle preparation against K_i values, calculated by Herkenham *et al.* (1990) from data they obtained in a competitive binding assay.

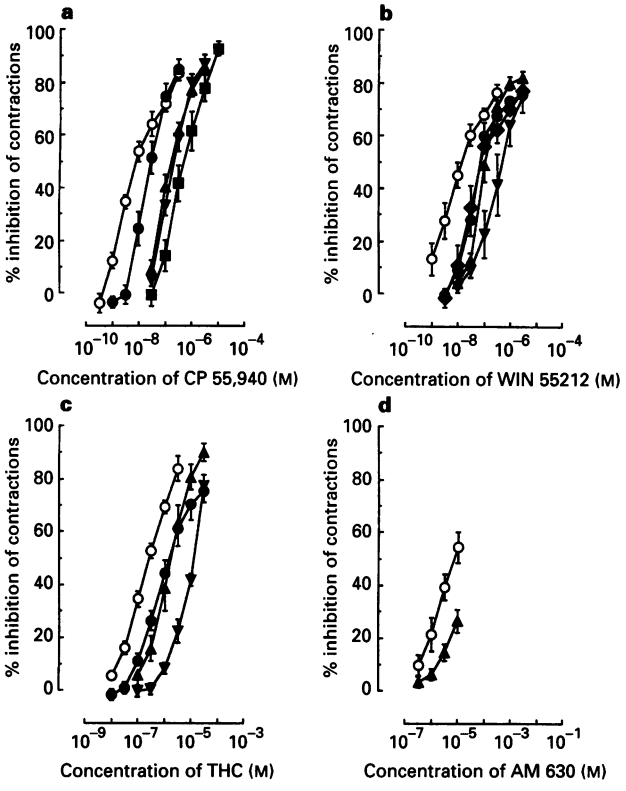

Figure 4 Mean concentration-response curves for (a) CP 55,940, (b) WIN 55,212-2, (c) THC and (d) AM 630 constructed in the presence of SR141716A at concentrations of 10 nM (◆), 31.62 nM (●), 100 nM (▲) 316.2 nM (▼) or 1000 nM (■) or in the presence of Tween 80 (○). Each symbol represents the mean value 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 ($n=5$ to 8 different myenteric plexus-longitudinal muscle preparations); vertical lines indicate s.e.mean. SR141716A and Tween 80 were added 15 min before the first addition of agonist.

Table 2 Dissociation constants (K_D) of SR141716A determined in the presence of various cannabinoid receptor agonists in the myenteric plexus-longitudinal muscle preparation of guinea-pig small intestine

Agonist	K_D mean (nM)	K_D 95% confidence limits (nM)	Schild plot Slope[1]	r^2
THC	5.47	3.99 & 8.69	1.11±0.48	0.843
WIN 55212-2	9.65	7.45 & 13.67	0.71±0.10	0.981
CP 55940	12.07	9.17 & 17.66	0.78±0.20	0.887
AM 630	21.20*	12.12 & 55.82*	–	–

*Determined with a single concentration of SR141716A (100 nM). Other K_D values were determined by Schild analysis with at least three of the following concentrations of SR141716A:31.62, 100, 316.2 and 1000 nM. [1]Mean values± s.e.mean are presented.

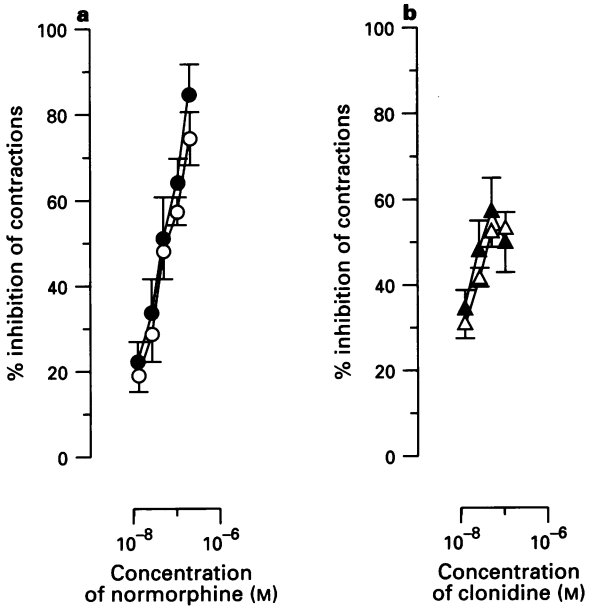

Figure 5 Mean concentration-response curves for (a) normorphine and (b) clonidine constructed in the presence of 1 μM SR141716A (●, ▲) or in the presence of Tween 80 (○, △). Each symbol represents the mean value of inhibition of electrically-evoked contractions expressed as a percentage of the amplitude of the twitch response measured immediately before the first addition of a twitch inhibitor to the organ bath ($n=4$ to 7 different myenteric plexus-longitudinal muscle preparations); vertical lines indicate s.e.mean. SR141716A and Tween 80 were added at least 15 min before the first addition of normorphine or clonidine.

was also found to reverse the inhibitory effect of WIN55,212-2 on the twitch response of this preparation but not the inhibitory effects of normorphine or clonidine (Figure 8). Naloxone and yohimbine did not reverse the inhibitory effect of WIN55,212-2 on the twitch response when administered at concentrations that did attenuate the effects of normorphine or clonidine (Figure 8).

Discussion

The results obtained confirmed previous findings that cannabinoids can inhibit electrically-evoked contractions of the myenteric plexus-longitudinal muscle preparation of guinea-pig small intestine (Pertwee *et al.*, 1992; see also Pertwee, 1990). They also showed that when this preparation was exposed to the cannabinoid receptor agonist, CP 55,940, at a

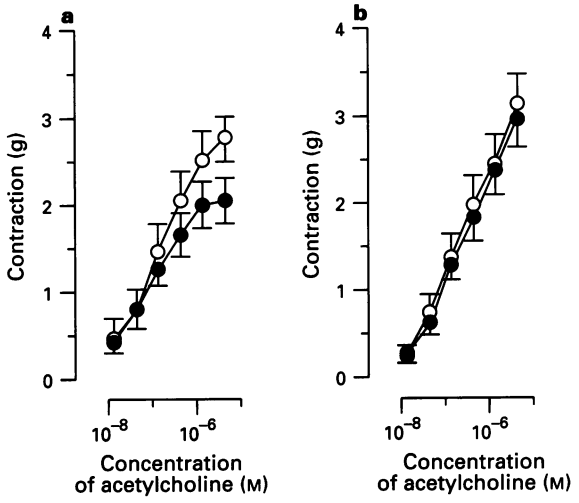

Figure 6 Mean concentration-response curves for acetylcholine constructed (a) in the presence of 1 μM SR141716A (●) or Tween 80 (○) and (b) in the presence of 200 nM CP 55,940 (●) or Tween 80 (○). Each symbol represents the mean value of the amplitude of contractions produced by acetylcholine ($n=4$ to 6 different myenteric plexus-longitudinal muscle preparations); vertical lines indicate s.e.mean. SR141716A, CP 55,940 and Tween 80 were added at least 15 min before the first addition of acetylcholine.

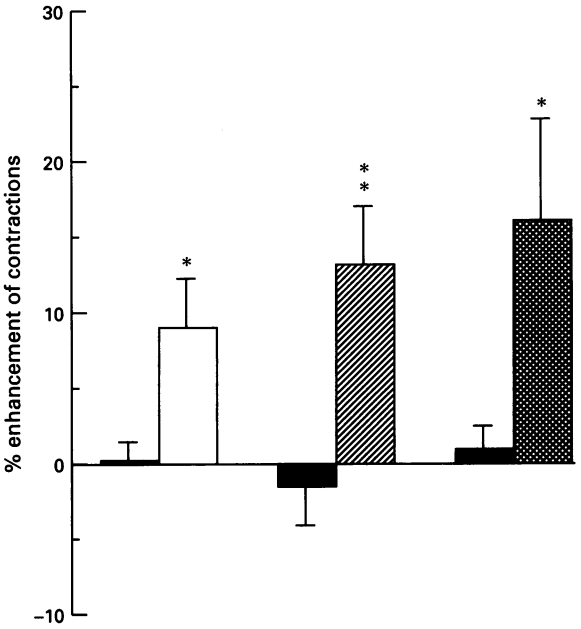

Figure 7 Effect of SR141716A on electrically-evoked contractions of strips of myenteric plexus-longitudinal muscle. Preparations were exposed at 10 min intervals either to SR141716A concentrations of 10 nM (open column), 40 nM (hatched column) and 100 nM (cross-hatched column) or to corresponding concentrations of Tween 80 (solid columns). Each column represents the mean value±s.e.mean of the change in the amplitude of contractions expressed as a percentage of the amplitude measured immediately before the addition of SR141716A or Tween 80 to the organ bath ($n=11$ or 12 different myenteric plexus-longitudinal muscle preparations). For each pair of columns, the significance of the difference between the effect of Tween 80 and of SR141716A has been calculated by Student's t test for unpaired data (*$P<0.05$; **$P<0.01$).

concentration that markedly inhibits the twitch response, electrically-evoked release of acetylcholine fell significantly whilst the contractile response to acetylcholine remained unchanged. These observations are in line with findings from previous experiments with resting strips of myenteric plexus-longitudinal muscle (Paton *et al.*, 1972) or guinea-pig whole

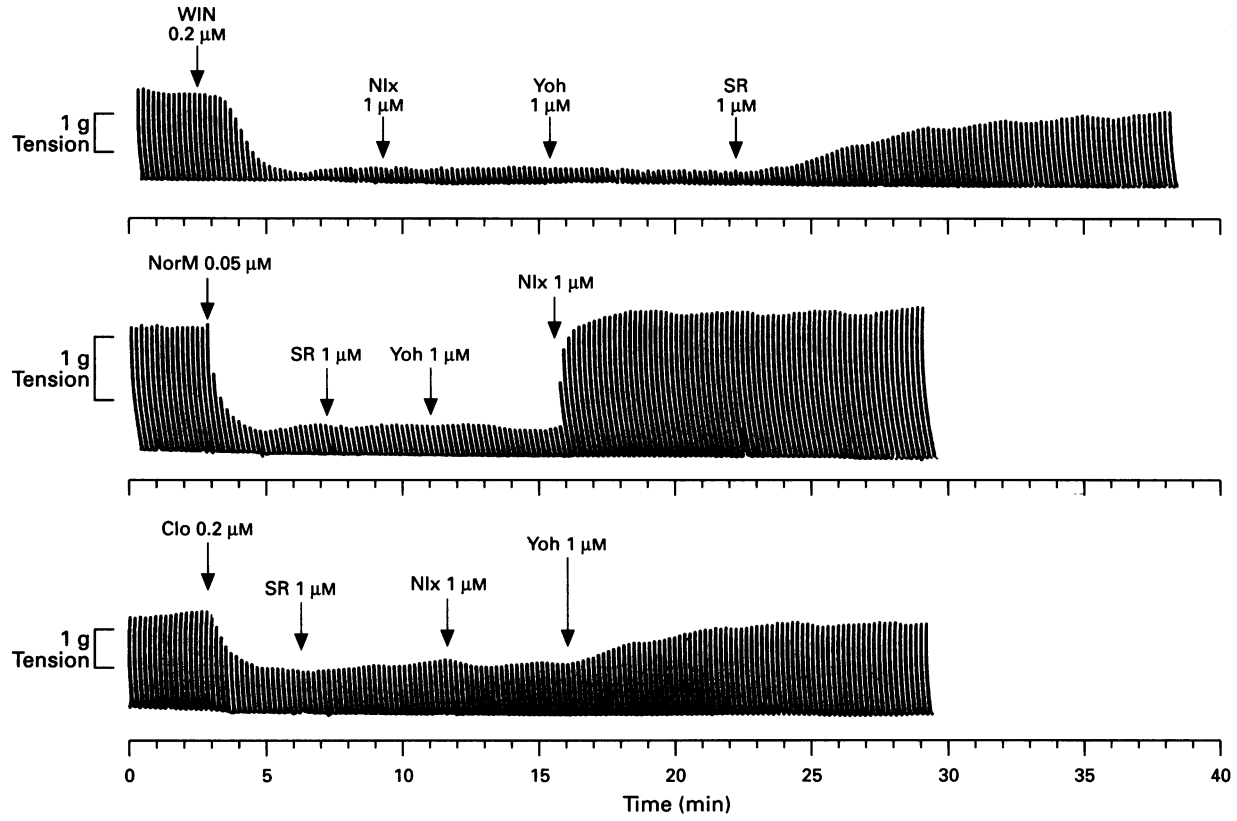

Figure 8 Typical effects of WIN 55,212-2 (WIN) normorphine (NorM) and clonidine (Clo) on the amplitude of electrically-evoked contractions of strips of myenteric plexus-longitudinal muscle before and after administration of 1 μM naloxone (Nlx), yohimbine (Yoh) or SR141716A (SR).

intestine (Layman & Milton, 1971) and support the hypothesis that cannabinoids inhibit the twitch response by acting prejunctionally rather than through a direct action on intestinal smooth muscle.

Our results also provide further evidence for the presence of cannabinoid CB_1 receptors in the guinea-pig small intestine and for the hypothesis that cannabinoid-induced inhibition of intestinal contractions is mediated by these receptors. Thus we found firstly, that the potency of cannabinoids as inhibitors of the twitch response correlates well with their potency for the displacement of a radiolabelled probe from cannabinoid binding sites in brain tissue as determined by Herkenham *et al.*, (1990) and secondly, that the selective cannabinoid CB_1 receptor antagonist, SR141716A, can markedly attenuate cannabinoid-induced inhibition both of the twitch response and of acetylcholine release. It is unlikely that this inhibitory effect of cannabinoids is also mediated by opioid receptors or α_2-adrenoceptors as inhibition of the twitch response by WIN 55,212-2 was reversed by SR141716A but not by naloxone or yohimbine and the inhibitory effects of normorphine and clonidine were not reversed by SR141716A. The finding that a dose of SR141716A that antagonized the inhibitory effect of cannabinoids on the twitch response could also reverse cannabinoid-induced inhibition of acetylcholine release strengthens the notion that cannabinoids inhibit the twitch response by acting prejunctionally.

SR141716A produced parallel rightward shifts in the log concentration-response curves of all the cannabinoid receptor agonists investigated and the Schild slopes for its interactions with THC, CP 55,940 and WIN 55,212-2 were all reasonably close to unity. These data support the classification of SR141716A as a competitive antagonist (Tallarida *et al.*, 1979; Rinaldi-Carmona *et al.*, 1994). An alternative possibility, that SR141716A attenuates cannabinoid-induced inhibition of the twitch response of the myenteric plexus-longitudinal muscle preparation by inhibiting acetylcholine metabolism or augmenting the contractile effect of this transmitter is unlikely, as SR141716A did not increase the amplitude of contractions induced by the addition of acetylcholine and it reversed the inhibitory effect of CP 55,940 on acetylcholine release in the presence of 7.7 μM physostigmine and therefore after acetylcholinesterase had already been inhibited (Waterfield, 1973).

According to the K_D values calculated by Schild analysis and listed in Table 2, THC is more susceptible to antagonism by SR141716A than CP 55,940 or AM 630. Inspection of these K_D values also suggests that the susceptibility of WIN 55,212-2 and CP 55,940 to antagonism by SR141716A is respectively 5 and 33 times less in the myenteric plexus preparation than in the mouse vas deferens, whereas the susceptibility of THC to SR141716A is about the same in the two preparations (Pertwee *et al.*, 1995a). Whether these data indicate the presence of more than one type of cannabinoid receptor in these tissues remains to be established.

In the absence of other drugs, SR141716A produced small but significant increases in the amplitude of electrically-evoked contractions, suggesting that cannabinoid receptors can inhibit the twitch response even when no cannabinoid agonist has been administered. This may be because guinea-pig small intestine can itself produce a cannabinoid receptor agonist that is capable of inhibiting the twitch response. Possible candidates are anandamide, although this has so far been detected only in the brain (Devane *et al.*, 1992), and 2-arachidonylglycerol which has already been found in the intestinal tract of dogs (Mechoulam *et al.*, 1995). The effect of SR141716A on twitch amplitude can also be explained in terms of the 2-state model of drug receptors (see Leff, 1995) providing that certain assumptions are made. These are that cannabinoid receptors in guinea-pig small intestine can exist either in an active state that can couple to the signal transduction mechanism or in an inactive state that cannot, that these two states are normally in equilibrium such that even in the absence of cannabinoid agonists at least some receptors are in the active state (precoupled receptors) and that SR141716A binds preferentially to the inactive state. If these assumptions are valid, then

SR141716A would be expected to produce a shift away from the active state thereby causing a reduction in the number of precoupled receptors and hence a disinhibition of the twitch amplitude. Whilst the first of these hypotheses allows SR141716A to remain classified as a pure antagonist, the second requires it to be reclassified as an inverse agonist (see Leff, 1995). The K_D values of this agent listed in Table 2 should, therefore, be interpreted with extra caution as their calculation is based on the assumption that SR141716A is a pure antagonist (see Kenakin *et al.*, 1992).

Although SR141716A increased the amplitude of electrically-evoked contractions in the absence of other drugs, it did not do so in the presence of inhibitory concentrations of normorphine or clonidine. This might be because any ongoing release of cannabinoid(s) by the myenteric plexus-longitudinal muscle preparation can be blocked by normorphine and clonidine. It could also be because cannabinoid receptors, opioid receptors and α_2-adrenoceptors may converge on common effector systems such that in the presence of normorphine or clonidine there can be no further inhibition of these effector systems by precoupled cannabinoid receptors. There is already evidence firstly, that cannabinoid receptors, opioid receptors and α_2-adrenoceptors are all negatively coupled through G proteins to adenylate cyclase and to calcium channels (Watson and Girdlestone, 1995) and secondly, that cannabinoid receptors are negatively coupled to the same adenylate cyclase units as at least one other type of receptor: the γ-aminobutyric acid $(GABA)_B$ receptor (Deadwyler *et al.*, 1995).

Previous experiments have shown AM 630 to behave as a competitive surmountable cannabinoid receptor antagonist in the mouse isolated vas deferens (Pertwee *et al.*, 1995b). However, in our present experiments with the myenteric plexus-longitudinal muscle preparation, it was found to behave as a weak cannabinoid receptor agonist, inducing a concentration-related inhibition of electrically-evoked contractions of this preparation which could be antagonized by SR141716A. The question of why AM 630 should be an antagonist in one tissue and an agonist in another remains to be resolved. One possibility is that there are different types of cannabinoid receptors in these two tissues (see above). Another possibility is that AM 630 interacts with the same type of receptor in both tissues but that the AM 630-receptor complex couples more efficiently to its signal transduction mechanism in the guinea-pig small intestine than in the mouse vas deferens. There is already some evidence that the coupling efficiency of different populations of cannabinoid receptors does vary, at least in the brain (Sim *et al.*, 1995). It could also be that there is an allosteric site associated with the cannabinoid receptor in mice but not in guinea-pigs and that AM 630 can interact with this site to prevent access of ligands to the recognition sites of cannabinoid receptors or to reduce the affinity or efficacy of these receptors.

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References

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol.*, **14**, 48–58.

COLQUHOUN, D. (1971). *Lectures on Biostatistics*. Oxford: Oxford University Press.

COUTTS, A.A., FERNANDO, S.R., GRIFFIN, G., NASH, J.E. & PERTWEE, R.G. (1995). Evidence that guinea-pig small intestine contains CB_1 cannabinoid receptors and possibly also a cannabinoid receptor ligand. *Br. J. Pharmacol.*, **116**, 46p.

DEADWYLER, S.A., HAMPSON, R.E. & CHILDERS, S.R. (1995). Functional significance of cannabinoid receptors in brain. In *Cannabinoid Receptors*, ed. Pertwee, R.G., pp. 205–231. London: Academic Press.

DEVANE, W.A., HANUS, L., BREUER, A., PERTWEE, R.G., STEVENSON, L.A., GRIFFIN, G., GIBSON, D., MANDELBAUM, A., ETINGER, A. & MECHOULAM, R. (1992). Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science*, **258**, 1946–1949.

HERKENHAM, M., LYNN, A.B., LITTLE, M.D., JOHNSON, M.R., MELVIN, L.S., DE COSTA, B.R. & RICE, K.C. (1990). Cannabinoid receptor localization in brain. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 1932–1936.

KENAKIN, T.P., BOND, R.A. & BONNER, T.I. (1992). Definition of pharmacological receptors. *Pharmacol. Rev.*, **44**, 351–362.

LAYMAN, J.M. & MILTON, A.S. (1971). Some actions of Δ^1-tetrahydrocannabinol and cannabidiol at cholinergic junctions. *Br. J. Pharmacol.*, **41**, 379P.

LEFF, P. (1995). The two-state model of receptor activation. *Trends Pharmacol. Sci.*, **16**, 89–97.

MECHOULAM, R., BEN-SHABAT, S., HANUS, L., LIGUMSKY, M., KAMINSKI, N.E., SCHATZ, A.R., GOPHER, A., ALMOG, S., MARTIN, B.R., COMPTON, D.R., PERTWEE, R.G., GRIFFIN, G., BAYEWITCH, M., BARG, J. & VOGEL, Z. (1995). Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem. Pharmacol.*, **50**, 83–90.

PATERSON, S.J. & PERTWEE, R.G. (1993). Characterization of the cannabinoid binding site in the guinea-pig. *Br. J. Pharmacol. (Proc. suppl.)*, **109**, 82P.

PATON, W.D.M. & ZAR, M.A. (1968). The origin of acetylcholine released from guinea-pig intestine and longitudinal muscle strips. *J. Physiol.*, **194**, 13–33.

PATON, W.D.M., PERTWEE, R.G. & TEMPLE, D. (1972). The general pharmacology of cannabinoids. In *Cannabis and its Derivatives*, ed. Paton, W.D.M. & Crown, J., pp. 50–75. London: Oxford University Press.

PERTWEE, R.G. (1990). The central neuropharmacology of psychotropic cannabinoids. In *Psychotropic Drugs of Abuse.*, ed. Balfour, D.J.K., pp. 355–429. New York: Pergamon Press.

PERTWEE, R.G. (1995). Pharmacological, physiological and clinical implications of the discovery of cannabinoid receptors: an overview. In *Cannabinoid Receptors*, ed. Pertwee, R.G. pp. 1–34. London: Academic Press.

PERTWEE, R.G., GRIFFIN, G., LAINTON, J.A.H. & HUFFMAN, J.W. (1995a). Pharmacological characterization of three novel cannabinoid receptor agonists in the mouse isolated vas deferens. *Eur. J. Pharmacol.*, **284**, 241–247.

PERTWEE, R., GRIFFIN, G., FERNANDO, S., LI, X., HILL, A. & MAKRIYANNIS, A. (1995b). AM630, a competitive cannabinoid receptor antagonist. *Life Sci.*, **56**, 1949–1955.

PERTWEE, R.G., STEVENSON, L.A., ELRICK, D.B., MECHOULAM, R. & CORBETT, A.D. (1992). Inhibitory effects of certain enantiomeric cannabinoids in the mouse vas deferens and the myenteric plexus preparation of guinea-pig small intestine. *Br. J. Pharmacol.*, **105**, 980–984.

RINALDI-CARMONA, M., BARTH, F., HEAULME, M., SHIRE, D., CALANDRA, B., CONGY, C., MARTINEZ, S., MARUANI, J., NELIAT, G., CAPUT, D., FERRARA, P., SOUBRIE, P., BRELIERE, J.C. & LEFUR, G. (1994). SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. *FEBS Letts.*, **350**, 240–244.

SIM, L.J., SELLEY, D.E. & CHILDERS, S.R. (1995). *In vitro* autoradiography of receptor-activated G proteins in rat brain by agonist-stimulated guanylyl 5′-[γ-[^{35}S]thio]triphosphate binding. *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 7242–7246.

TALLARIDA, R.J., COWAN, A. & ADLER, A.W. (1979). pA_2 and receptor differentiation: a statistical analysis of competitive antagonism. *Life. Sci.*, **25**, 637–654.

WATERFIELD, A.A. (1973). Factors affecting acetylcholine release from the myenteric plexus of the ileum. *PhD Thesis*, University of Aberdeen.

WATSON, S. & GIRDLESTONE, D. (1995). 1995 receptor and ion channel nomenclature supplement. *Trends Pharmacol. Sci.*, 6th Edition, Cambridge: Elsevier.

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