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Cannabinoids inhibit N-type calcium channels in neuroblastoma–glioma cells

(aminoalkylindole/cyclic AMP/guanine nucleotide-binding protein/ω-conotoxin/pertussis toxin)

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ABSTRACT **The psychoactive properties of *Cannabis sativa* and its major biologically active constituent, Δ^9-tetrahydrocannabinol, have been known for years. The recent identification and cloning of a specific cannabinoid receptor suggest that cannabinoids mimic endogenous compounds affecting neural signals for mood, memory, movement, and pain. Using whole-cell voltage clamp and the cannabinomimetic aminoalkylindole WIN 55,212-2, we have found that cannabinoid receptor activation reduces the amplitude of voltage-gated calcium currents in the neuroblastoma–glioma cell line NG108-15. The inhibition is potent, being half-maximal at less than 10 nM, and reversible. The inactive enantiomer, WIN 55,212-3, does not reduce calcium currents even at 1 μM. Of the several types of calcium currents in NG108-15 cells, cannabinoids predominantly inhibit an ω-conotoxin-sensitive, high-voltage-activated calcium current. Inhibition was blocked by incubation with pertussis toxin but was not altered by prior treatment with hydrolysis-resistant cAMP analogues together with a phosphodiesterase inhibitor, suggesting that the transduction pathway between the cannabinoid receptor and calcium channel involves a pertussis toxin-sensitive GTP-binding protein and is independent of cAMP metabolism. However, the development of inhibition is considerably slower than a pharmacologically similar pathway used by an α_2-adrenergic receptor in these cells. Our results suggest that inhibition of N-type calcium channels, which could decrease excitability and neurotransmitter release, may underlie some of the psychoactive effects of cannabinoids.**

The principal cannabinoid and major psychoactive ingredient in extracts of the marijuana plant (*Cannabis sativa*) is Δ^9-tetrahydrocannabinol (1). Because of its widespread recreational use and potential therapeutic applications (1, 2), a search for its cellular site(s) of action has been the focus of considerable attention. However, despite much work, the mechanism(s) of action remain uncertain (3). The only well-established biochemical effect is an inhibition of hormone-stimulated adenylyl cyclase via a pertussis toxin (PTX)-sensitive GTP-binding regulatory protein (4, 5). As many receptors that couple to adenylyl cyclase via the G protein G_i also interact with ion channels (6), we investigated the relationship between cannabinoid receptor activation and calcium current amplitude (I_{Ca}) in neuroblastoma–glioma (NG108-15) cells. The low aqueous solubility of the naturally occurring cannabinoids and their propensity to stick to glass and plastic makes experiments with them difficult and prone to artifacts (7). In our studies we have taken advantage of the recent development of the nonclassical cannabinoids (8) and the aminoalkylindoles (9), two families of cannabinomimetic compounds that we will refer to loosely as "cannabinoids." These compounds appear to mimic the receptor-mediated effects of the naturally occurring cannabinoids in every way tested to date (8–13). Their increased potency and aqueous solubility circumvent many of the problems associated with the study of naturally occurring cannabinoids.

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METHODS

Materials. Minimum essential medium and fetal bovine serum were from GIBCO or Sigma. Prostaglandin E_1, 3-isobutyl-1-methylxanthine, Nystatin, dibutyryl-cAMP, norepinephrine, albumin, and hypoxanthine/aminopterin/thymidine were from Sigma. PTX was from List. 8-Chlorophenylthio-cAMP was from Boehringer Mannheim. Tetrodotoxin was from Calbiochem. Pluronic F127 was from Molecular Probes. ω-Conotoxin GVIA (ωCTX) was from Peninsula Laboratories.

Cell Culture. NG108-15 cells (passages 16–28) were grown by standard techniques on polylysine-coated coverslip fragments (14, 15). Four to seven days prior to recording, differentiation was induced by decreasing the serum content of the medium to 1% and by adding 100 μM IBMX and 10 μM prostaglandin E_1. In the PTX experiments, differentiated NG108-15 cells were cultured for an additional 20–28 hr in medium containing PTX at 500 ng/ml. Control cells were treated similarly, except PTX heated at 95°C for 15 min was substituted for native PTX. As a control for variability in calcium channel expression and cannabinoid response, concurrent controls from the same passage were always used.

Current Recording. To minimize alterations in the cytoplasm, we recorded currents by using the Pluronic modification (16) of the Nystatin perforated-patch technique (17) of whole-cell GΩ-seal clamp (18). Pipettes were pulled from hematocrit microcapillary glass (VWR). The tip contained 100 mM CsCl/10 mM CsEGTA/5 mM $MgCl_2$/40 mM Hepes, pH 7.30, with CsOH. The rest of the pipette was backfilled with the same solution to which was added Pluronic F127 (5 μg/ml) and Nystatin (5 μg/ml), both in dimethyl sulfoxide (final concentration of dimethyl sulfoxide was 30 μl/ml). Three to 10 min after a GΩ seal was established, the access resistance had typically fallen to 7 to 15 MΩ, and recording was begun. The 200-μl recording chamber was perfused at 1–2 ml/min with 140 mM NaCl/10 mM tetraethylammonium chloride/8 mM glucose/5 mM $CaCl_2$/4 mM KCl/1 mM $MgCl_2$/10 mM Hepes, pH 7.35 with NaOH. Tetrodotoxin (200 nM) and bovine serum albumin (3 μM) were added to block voltage-gated sodium currents and to decrease adsorption of the cannabinoids, respectively. Voltage protocols were generated and data were digitized, recorded, analyzed, and plotted using BASIC-FASTLAB (Indec Systems, Capitola, CA). Currents were filtered at 2 kHz and sampled at 4 kHz. Unless otherwise indicated, current records have been corrected by leak subtraction. Junction potentials are uncor-

Abbreviations: I_{Ca}, calcium current; IBMX, 3-isobutyl-1-methylxanthine; ωCTX, ω-conotoxin GVIA; PTX, pertussis toxin.

rected. Where appropriate, data are expressed as mean ± SE.

Drug Application. Cannabinoid stocks were dissolved at 10 mM in dimethyl sulfoxide and diluted into the perfusion solution. The maximum final concentration of dimethyl sulfoxide, 0.1% with 10 μM cannabinoid, had no consistent effect on I_{Ca}. In most experiments, drug-containing solution was applied with a system of reservoirs connected by multiple inlet valves. However, in one set of experiments we were concerned that the slow onset of action of the synthetic cannabinoids might be secondary to their adsorption to the tubing of the perfusion system, resulting in a ramp, rather than a step, application of agonist to the cell. We sought to circumvent this problem by constructing a U-tube apparatus using a rapid perfusion system (19). Drug-containing solution flowed constantly from one side of a theta tubing, while the other side, draining by gravity, immediately aspirated the drug back again. The cell was positioned 250 μm from the tubing. (By suitable selection of the reservoir and drain heights, no drug action could be detected until the tip of the theta tubing was less than 100 μm from the cell.) Then a computer-controlled valve on the drain tubing was suddenly closed, causing the drug-containing solution to flow out over the cell. As determined visually with phenol red and quantitatively by changes in holding current, during application of 25 mM KCl-containing Ringer's, solution change was com-

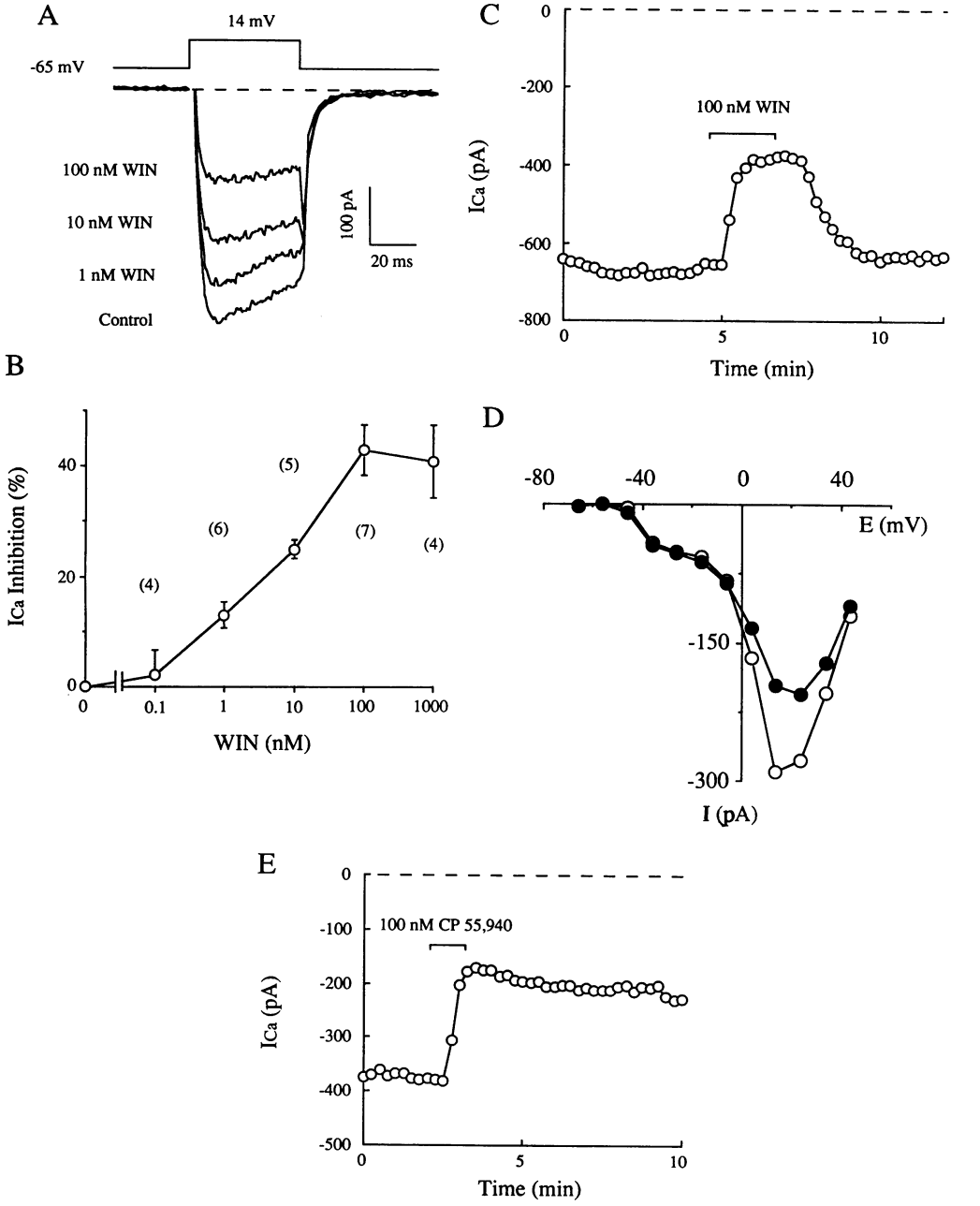

FIG. 1. Inhibition of I_{Ca} by synthetic cannabinoids. (*A*) Increasing concentrations of WIN 55,212-2 (WIN) decrease the inward calcium current evoked during a depolarizing step. The dashed line represents zero current. (*B*) Concentration–response curve of the inhibition of I_{Ca}. Numbers in parentheses represent the number of cells tested. (*C*) Reversible inhibition of I_{Ca} by WIN. I_{Ca} was measured near the end of 25-ms depolarizing steps to +14 mV applied every 15 s from a holding potential of −65 mV. (*D*) Current–voltage relationship of I_{Ca} as measured near the end of 50-ms voltage steps to the indicated potential. Despite a 32% decrease in high-voltage-activated current with 100 nM WIN (●), the low-voltage-activated current was unaffected. Data are representative of 11 cells. Holding voltage was −65 mV. (*E*) The nonclassical cannabinoid CP 55,940 also inhibits I_{Ca}, but the inhibition with this less hydrophilic compound is only slightly reversible.

plete within 1000 ms when the tip of the theta tube was 250 μm from the cell. Inhibition was normalized to the mean inhibition occurring 30–60 s after drug application.

RESULTS

Depolarizing voltage steps from a holding potential of −65 mV to a test potential of +14 mV open voltage-gated calcium channels, eliciting several hundred picoamperes of I_{Ca} in differentiated NG108-15 cells (Fig. 1*A*). Bath application of the aminoalkylindole WIN 55,212-2 (WIN) inhibited a portion of this current. The inhibition was concentration-dependent with a threshold below 1 nM and a maximal reduction of 43 ± 5% (n = 7, mean ± SE) of I_{Ca} at 100 nM (Fig. 1*B*). The inhibition was stereospecific, as 1 μM of the WIN 55,212-2 enantiomer, WIN 55,212-3, did not inhibit I_{Ca} (0 ± 1%, n = 5). Inhibition by WIN was reversed by removing the drug (Fig. 1*C*). Differentiated NG108-15 cells generally contain both high- and low-voltage-activated calcium currents whose activation is first seen at −40 and 0 mV, respectively (Fig. 1*D*). Only the high-voltage-activated component was inhibited by WIN. The nonclassical cannabinoid CP 55,940 (100 nM) also reduced I_{Ca} (Fig. 1*E*) (36 ± 7%, n = 7); however, in contrast to the aminoalkylindole, complete reversal was not seen upon washout of CP 55,940. The actions of the two drugs occlude each other, and no further inhibition of I_{Ca} was seen by WIN after I_{Ca} had been maximally suppressed by CP 55,940 and vice versa (n = 3).

The cloned cannabinoid receptor has an amino acid sequence typical of G-protein-coupled receptors (20). Like the known inhibition of adenylyl cyclase (4, 5), the cannabinoid-mediated inhibition of I_{Ca} could be blocked by treating the cells with PTX (Fig. 2). Nevertheless, the inhibition of I_{Ca} appears to be independent of adenylyl cyclase activity and reductions in cAMP, as it persisted when hydrolysis-resistant cAMP analogues were combined with inhibitors of phosphodiesterases to keep cellular levels of cAMP high. Thus, in cells preincubated for 5 min with 1 mM dibutyryl-cAMP and 100 μM IBMX, 100 nM WIN reduced I_{Ca} by 36 ± 3% (n = 5), whereas in parallel control cells, 100 nM WIN reduced I_{Ca} by 31 ± 4% (n = 4). Similarly, 100 μM 8-chlorophenylthio-cAMP and 100 μM IBMX did not prevent cannabinoid inhibition of I_{Ca} (n = 6). Furthermore, these treatments with cAMP analogs did not increase I_{Ca}.

Norepinephrine also inhibits I_{Ca} via a PTX-sensitive process that is known to be independent of cAMP metabolism in NG108-15 cells (21). Compared to the inhibition of I_{Ca} by cannabinoids, inhibition of I_{Ca} by 10 μM norepinephrine occurs more quickly and the fraction of I_{Ca} inhibited is slightly, but consistently, greater (Figs. 2 and 3*A*). We confirm that the action of norepinephrine is sensitive to PTX. With supramaximal agonist concentration, the inhibition of I_{Ca} by norepinephrine developed with a time constant of <2.0 s (n = 6, Fig. 3*B*). On the other hand, inhibition by WIN was consistently slower, with time constants of 8.9 s (n = 7) and 8.2 s (n = 6) for 1 and 10 μM WIN, respectively (Fig. 3*B*). This experiment used a method of rapid agonist application that reduced the possibility that delivery was slowed by adsorption of compounds to the perfusion tubing. A similarly slow onset of inhibition was seen with 10 μM CP 55,940 (n = 3).

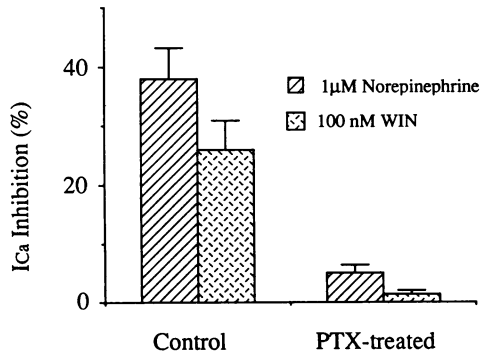

FIG. 2. Treating cells with PTX (500 ng/ml) blocks almost all of the cannabinoid and a substantial fraction of the norepinephrine inhibition of I_{Ca} (n = 9 for control and n = 6 for PTX-treated).

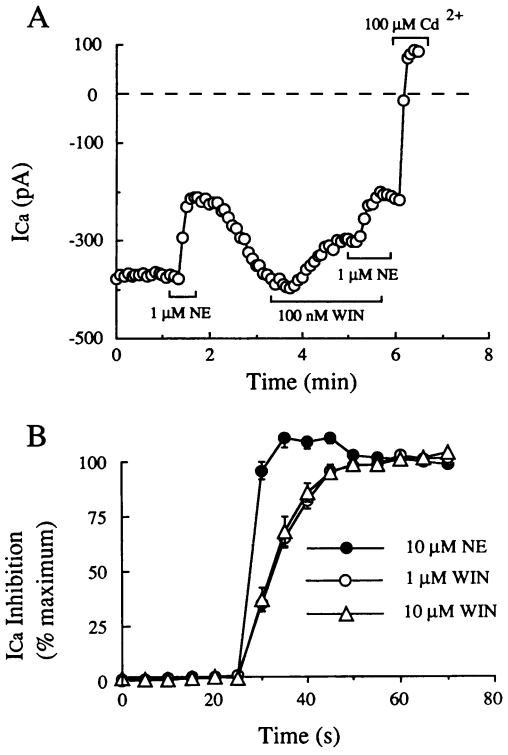

FIG. 3. Inhibition of I_{Ca} by norepinephrine (NE) and WIN. (*A*) Bath-applied NE appears to inhibit I_{Ca} more rapidly than does WIN (points at 5-s intervals). Even after inhibition of I_{Ca} by WIN was maximal, NE still inhibited an additional component of I_{Ca}. Cd^{2+} eliminated the inward current. Record has not been leak-subtracted. (*B*) Time course of I_{Ca} inhibition with rapid application of 10 μM NE and 1 and 10 μM WIN. Drugs were applied by a continuously flowing theta tube as described in *Methods*.

At least two components of high-voltage-activated I_{Ca}, with L-channel and N-channel properties, can be distinguished in NG108-15 cells. Which subtype of calcium channel is being inhibited by cannabinoids? An L-type channel is present, since the dihydropyridine antagonist nitrendipine (1 μM) reduced I_{Ca} by 27 ± 6% (n = 8), and the dihydropyridine agonist S (+)-202,791 (1 μM) increased I_{Ca} by 55 ± 8% (n = 11). However, L-type channels seem insensitive to cannabinoid action, as pretreatment with either dihydropyridine did not alter the portion of I_{Ca} inhibited by 100 nM WIN (normalized to I_{Ca} *prior to* pretreatment; Fig. 4*A*). In contrast, pretreatment with 1 μM ωCTX, which blocks N-type channels, decreased I_{Ca} by 38 ± 12% (n = 10), and in these cells, inhibition by 100 nM WIN was almost abolished (Fig. 4*A*). Furthermore, 100 nM WIN did not inhibit the current remaining after addition of ωCTX mixed with S (+)-202,791, conditions that emphasize L-type I_{Ca} (Fig. 4). Thus the majority of I_{Ca} inhibited by cannabinoids appears to be carried via N-type calcium channels.

DISCUSSION

We have found that cannabinoids act at a specific receptor to inhibit a fraction of the high-voltage-activated I_{Ca} in

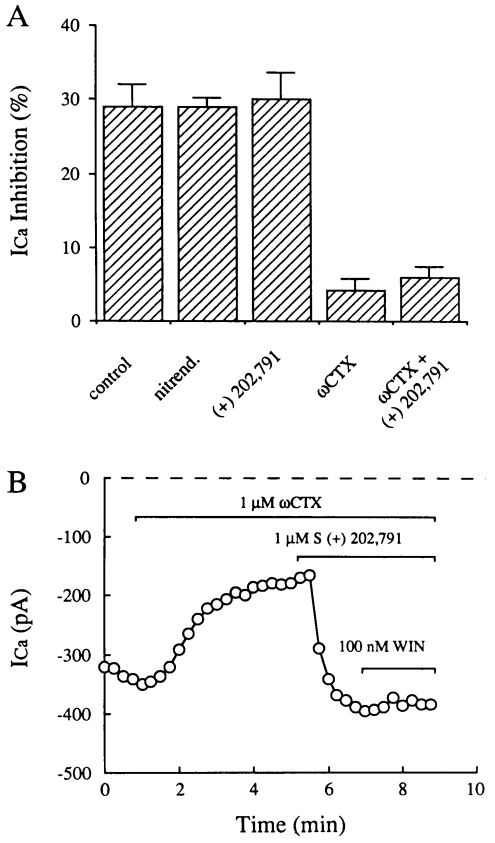

FIG. 4. Identification of the calcium channel subtype inhibited by WIN. (*A*) Inhibition of I_{Ca} by 100 nM WIN after pretreatments with other calcium-channel-active drugs. Pretreatment of cells with 1 μM nitrendipine (nitrend.) (n = 3) or 1 μM S (+)-202,791 (n = 5) had no effect on inhibition of I_{Ca} by WIN (normalized to I_{Ca} *before* any drug application). In cells exposed to 1 μM ωCTX (n = 6), the inhibition of I_{Ca} by WIN was practically abolished. Similarly, in cells exposed to ωCTX and then S (+)-202,791 (n = 4), little inhibition of I_{Ca} by WIN was apparent despite a substantial increase in I_{Ca} by S (+)-202,791. (*B*) One of the experiments summarized in *A*. ωCTX (1 μM) inhibits I_{Ca} during depolarizing steps to +14 mV. Addition of the dihydropyridine agonist S (+)-202,791 (1 μM) increases I_{Ca}. Subsequent addition of 100 nM WIN has no effect on I_{Ca}. The record has not been leak-subtracted and is representative of four experiments.

NG108-15 cells potently and reversibly. As the inhibited current is mostly sensitive to ωCTX (Fig. 4), we classify it as being through N-type calcium channels. How might our findings relate to cannabinoid pharmacology and to the functional role of the as yet undiscovered endogenous cannabinoid? Our results suggest that cannabinoids will blunt processes dependent on calcium entry through N-type channels. As calcium is a versatile second messenger in neurons, many processes will be affected (22, 23). One consequence that could explain some of the psychoactive effects of the cannabinoids is reduction of the release of neurotransmitter-containing small clear vesicles (24, 25)—presynaptic inhibition. Indeed, the cannabinoid receptor is largely located on presynaptic structures (26–28) and cannabinoids inhibit electrically stimulated acetylcholine release in both the ileum (29, 30) and the vas deferens (9).

The signaling pathway between the cannabinoid receptor and N-type calcium channels is PTX-sensitive (Fig. 2), suggesting it is mediated by G protein(s) of the G_o or G_i family. Although both the cannabinoid and norepinephrine inhibition of I_{Ca} are PTX-sensitive, their kinetic differences and the observation that cannabinoids inhibit only a portion of the norepinephrine-inhibited I_{Ca} suggest there are significant differences between their signal-transduction pathways. As has been found with other G-protein-coupled receptors that link to both adenylyl cyclase and ion channels, inhibition of I_{Ca} by occupation of the cannabinoid receptor is not blocked by application of extracellular, cell-permeant cAMP analogues. These results, together with the observation that the EC_{50} for inhibition of I_{Ca} by WIN is about 15-fold lower than the reported IC_{50} values for inhibition of adenylyl cyclase by this compound (9), lead us to speculate that inhibition of N-type calcium channels is a major physiological action of the cannabinoids.

The cannabinoid literature is replete with examples of the nonspecific, probably membrane-disrupting, effects of the cannabinoids (3). Thus experiments using these drugs might initially be viewed with some skepticism. We feel our results are physiologically relevant for the following reasons. (*i*) The compounds we used (WIN 55,212-2 and CP 55,940) appear to bind with high affinity only to the cannabinoid receptor (8, 9, 12). (*ii*) The inhibition of I_{Ca} we observe is potent, stereospecific, and reversible, suggesting that it is receptor-mediated. (*iii*) The inhibition is PTX-sensitive, suggesting that a G protein links the cannabinoid binding site (i.e., receptor) and calcium channel. (*iv*) No inhibition of N-type calcium channels was observed in acutely dissociated or cultured superior cervical or nodose ganglion cells of the rat (K.M. and L. Bernheim, unpublished observations), further arguing against a direct interaction with calcium channels.

We have found a specific inhibition of N-type calcium channels, but further investigation will most likely find cannabinoid receptor-mediated actions with other ion channels, as there is ample evidence that several channel types interact either directly or indirectly with G proteins. In particular, activation of an inwardly rectifying potassium channel is another common direct action of PTX-sensitive G proteins (6). The finding of electrophysiological mechanisms of cannabinoid action makes all the more pressing the need to discover the endogenous ligand for this interesting neurotransmitter pathway.

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