The cannabinoid agonist WIN55,212-2 increases intracellular calcium via CB₁ receptor coupling to G₉₁₁ G proteins

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Central nervous system responses to cannabis are primarily mediated by CB₁ receptors, which couple preferentially to Gi/o G proteins. Here, we used calcium photometry to monitor the effect of CB₁ activation on intracellular calcium concentration. Perfusion with 5 μM CB₁- aminoalkylindole agonist, WIN55,212-2 (WIN), increased intracellular calcium by several hundred nanomolar in human embryonic kidney 293 cells stably expressing CB₁ and in cultured hippocampal neurons. The increase was blocked by coinoculation with the CB₁ antagonist, SR141716A, and was absent in nontransfected human embryonic kidney 293 cells. The calcium increase was WIN-specific, being essentially absent in cells treated with other classes of cannabinoid agonists, including Δ⁹-tetrahydrocannabinol, HU-210, CP55,940, 2-arachidonoylglycerol, methanandamide, and cannabidiol. The increase in calcium elicited by WIN was independent of Gi/o, because it was present in pertussis toxin-treated cells. Indeed, pertussis toxin pretreatment enhanced the potency and efficacy of WIN to increase intracellular calcium. The calcium increases appeared to be mediated by Gαq G proteins and phospholipase C, because they were markedly attenuated in cells expressing dominant-negative Gαq or treated with the phospholipase C inhibitors U73122 and ET-18-OCH₃ and were accompanied by an increase in inositol phosphates. The calcium increase was blocked by the sarco/endoplasmic reticulum Ca²⁺ pump inhibitor thapsigargin, the inositol trisphosphate receptor inhibitor xestospongin D, and the ryanodine receptor inhibitors dantrolene and 1,1'-dihydroxy-4,4'-bipyridinium dibromide, but not by removal of extracellular calcium, showing that WIN releases calcium from intracellular stores. In summary, these results suggest that WIN stabilizes CB₁ receptors in a conformation that enables Gαq signaling, thus shifting the G protein specificity of the receptor.

The CB₁ cannabinoid receptor (CB₁) mediates the majority of the psychotropic and behavioral effects of cannabis (1, 2). CB₁ is a member of the heptahelial G-protein-coupled receptor (GPCR) superfamily (1). It couples via pertussis toxin (PTX)-sensitive Gi/o G proteins to inhibit adenyl cyclase and L-, N-, and P/Q-type calcium channels and to activate potassium channels and mitogen-activated protein kinase (2). Rarely, CB₁ has also been found to couple to phospholipase C (PLC) in a PTX-sensitive manner involving the βγ subunits from Gi/o (3, 4).

Several distinct agonists activate CB₁. The classical cannabinoids are tricyclic dibenzopyran compounds. The prototype of this group is Δ⁹-tetrahydrocannabinol (THC), the main psychoactive ingredient of cannabis. THC is a low-affinity partial agonist for CB₁ (2), whereas other synthetic classical compounds such as HU-210 are both more potent and efficacious (2). Nonclassical cannabinoids lack the dihydropyran ring yet maintain some similarity to THC’s stereochemistry. The best known, CP55,940 (CP), is a potent and efficacious CB₁ agonist. The third group, the eicosanoids, includes endogenous ligands for CB₁, such as anandamide and 2-arachidonoylglycerol (2-AG), and their analogs (5, 6). The aminoalkylindoles (AAIs) were synthesized as antiinflammatory drugs, with the goal to reduce gastrointestinal side effects. When tested, they were first found to have potent antinociceptive activity and weak antiinflammatory properties (7) and later discovered to bind to cannabinoid receptors (8). AAIs, and in particular WIN55,212-2 (WIN), produce the full spectrum of in vivo effects seen with THC and other cannabinimimetic agonists (9).

Here, we are concerned with the G protein specificity of CB₁. Initial dogma was that each GPCR interacts with a certain class of G protein. Subsequently, more promiscuous GPCR–G protein coupling was recognized. Multiplicity in G protein coupling activates multiple second-messenger cascades, each with distinct effects. Such promiscuity should allow a cell greater range in response to activation of a single GPCR. Some examples include D₁ dopamine and α₂-adrenergic receptors, which couple to both Gi, and Gq, and the PACAP type I and II receptor receptors, which activate both Gi, and Gq₁₁ (10–13). CB₁ has been shown to activate both Gi/o and Gq (14). This multiplicity in G protein coupling can be agonist-regulated. The prevailing idea here is that receptors exist in different activated states capable of coupling to different G proteins. Binding of a specific agonist stabilizes a distinct active state, favoring coupling to a certain G protein (15, 16). Examples of this have been reported for A₁ adenosine receptors and the Ca²⁺-sensing receptor (17, 18). In this study, we report the previously undescribed observation that CB₁ couples to Gq₁₁ G proteins in an agonist-specific manner to increase the concentration of intracellular calcium.

Materials and Methods

Cells. Human embryonic kidney (HEK) 293 cells stably expressing rat CB₁ cannabinoid receptor (CB₁-HEK293) were generated by using standard techniques (19). Most experiments were done with cells expressing CB₁ at a density of 2.2 pmol/mg protein. Before an experiment, cells were plated onto poly D-lysine-coated coverslips and, when required, transfected the next day. DNA for additional genes (M₁R and dominant-negative Gαq) were transiently transfected with Lipofectamine 2000. DsRed (0.5 μg) was cotransfected as a transfection marker. Cells were used for photometry experiments 24–36 h after transfection. Hippocampal neurons were cultured according to the method of Brewer (20).

Dye Loading and Photometry. Cytosolic Ca²⁺ was monitored with the ratiometric calcium indicator fura-2. Cells were loaded at

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Abbreviations: CB₁, CB₁ cannabinoid receptor; AAIs, aminoalkylindoles; THC, Δ⁹-tetrahydrocannabinol; WIN, WIN55,212-2; CP, CP55,940; 2-AG, 2-arachidonoylglycerol; HEC, human embryonic kidney; PTX, pertussis toxin; PLC, phospholipase C; IP, inositol phosphate; IP₃, inositol 1,4,5-triphosphate; GPCR, G protein-coupled receptor; [Ca²⁺]i, intracellular calcium concentration; ER, endoplasmic reticulum; SERCA, sarco/ER Ca²⁺-ATPase; M₁R, M₁ muscarinic receptor; Tg, thapsigargin; XeD, xestospongin D; SR, SR141716A; Oxo-M, oxotremorine-M; RyR, ryanodine receptor.

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room temperature for 15–20 min with fura-2 acetoxyethyl ester (AM) dissolved in DMSO, dispersed in 20% pluronic 127, and diluted to 8 µM in 0.5 ml of Ringer’s solution. Test solutions were applied to the incubation chamber with 5 of solenoid-controlled gravity-fed multibarreled local perfusion device.

During [Ca^{2+}]i measurements, the dye was excited by 340 and 380 nm light and a photodiode collected emission above 520 nm (Polychrome IV TILL Photonics, Planegg, Germany). The standard calibration parameters 1, 2, 3, and 4 were obtained 5, 6, and 7 from HEK293 cells equilibrated in KCl-based internal solutions containing ionomycin (10 µM) and either 20 mM EGTA, 15 mM CaCl2, or 20 mM EGTA + 15 mM CaCl2 (149 mM free Ca^{2+}). For experiments with hippocampal neurons, the relative [Ca^{2+}]i was given as the fluorescence ratio F_{380}/F_{360}. Photometric measurements were analyzed in Igor (WaveMetrics, Lake Oswego, OR) and statistical analyses performed in EXCEL (Microsoft), except ANOVAs, which were performed on the statistical methods web site, developed by Tom Kirkman at the College of St. Benedict/St. John’s University (St. Joseph and Collegeville, MN). All results are presented as mean ± SEM. Each drug was tested at least on 2 different days, with concurrent interleaved controls. Each calcium measurement represents a single cell from an individual coverslip. The measurement “rise in [Ca^{2+}]i” was calculated as maximum [Ca^{2+}]i during drug application — basal [Ca^{2+}]i, where basal [Ca^{2+}]i was the mean [Ca^{2+}]i for 47 s before drug application.

Receptor Expression Studies and Measurement of [3H]Inositol Phosphate (IP) Formation. To determine the level of CB1 expression, CB1-HEK293 cells were grown in 10-cm plates and membrane binding performed with [3H]CP, as described (22). [3H]Inositol phosphate production was measured in cells loaded with myo-[2-3H]inositol by using established techniques (23).

Solutions and Materials. Fura-2-AM and pluronic 127 were from Molecular Probes, and thapsigargin (TG), xestospongin D (XeD), and ET-18-OCH3 were from Calbiochem. PTX was from List Biological Laboratories (Campbell, CA); and HU210, U73122, and 1,2-diheptyl-4,4′-bipyridinium dibromide were from Tocris Cookson (Ellisville, MO). SR141716A (SR), THC, and CP were supplied by the National Institute on Drug Abuse from Tocris Cookson (Ellisville, MO). AM356 was a gift of A. Makriyannis (Northeastern University, Boston). Cannabidiol, [3H]CP, and myo-[2-3H]inositol were gifts from N. Stella (University of Washington, Seattle). All other chemicals were from Sigma.

The control mammalian Ringer’s bath solution contained 160 mM NaCl, 2.5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM Hepes, and 8 mM glucose adjusted to pH 7.4 with NaOH. Ca^{2+}-free Ringer’s solution was the same as control solution without CaCl2 and with 0.5 mM EGTA. PTX and oxotremorine-M (Oxo-M) were dissolved in water. All other compounds were dissolved in DMSO. Final dilutions were made with Ringer’s bath solution (DMSO <0.05%). One milligram per milliliter BSA was added as a carrier to all cannabinoïd-containing solutions except those used in the receptor expression and IP measurements. After experiments, perfusion lines and tips were flushed with 100% ethanol followed by dH2O. For the receptor expression and IP formation assays, siliconized tubes and pipette tips were used for drug preparation and delivery.

Results

WIN Evokes a Large Slow CB1-Specific Increase in Intracellular Calcium Concentration. Perfusion of 5 µM of the cannabimimetic AAI WIN raised intracellular calcium in CB1-HEK293 cells by >500 nM (Fig. 1A). Perfusion of 5 µM WIN also increased intracellular calcium in HEK293 cells transiently expressing rCB1 (790 ± 120 nM, n = 9). The CB1 specificity of this response was shown in three ways. First, coapplication of 1 µM of the CB1 antagonist SR with WIN markedly attenuated the calcium increase (Fig. 1A). Second, perfusion of 5 µM WIN55,212-3, the inactive enantiomer of WIN (2), did not increase intracellular calcium (data not shown). Third, the transient was absent in nontransfected HEK293 cells perfused with 5 µM WIN (Fig. 1A). WIN caused a calcium transient in 100% of cells expressing CB1 (121,121), although the amplitude and speed of the response varied among cells (Fig. 1B). Overall, the WIN-induced calcium increase was markedly slower than that caused by 10 µM of the muscarinic receptor agonist, Oxo-M, in HEK293 cells expressing M1R, the M1R-mediated calcium increase had a 10–90% rise time of 4 ± 1 s, significantly faster than the WIN-induced calcium rise time of 40 ± 8 s (P <0.01) (Fig. 1C and D).

The effect of WIN is not restricted to expression systems. Five micromolar WIN caused a calcium rise in cultured mouse hippocampal neurons like that in CB1-HEK293 (the F340/F380 ratio increased to 0.9, n = 9). This response also depended on CB1, because 1 µM of the antagonist SR attenuated WIN-induced F340/F380 increase by 76% (P <0.001). WIN had no effect on calcium levels in astrocytes, cells generally regarded to not express CB1 (data not shown).

WIN Is the Only CB1 Agonist to Increase Intracellular Calcium Robustly. We tested several classes of CB1 agonists at concentrations at least 200 times the CB1-binding Ki value (with the exception of

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2-AG, which was used at 20 times its $K_i$ (2)], THC (10 $\mu$M) raised calcium by only $40 \pm 15$ nM (Fig. 2), which is well within the range seen for resting cells and was only 5% of the WIN-induced increase. Agonists structurally similar to THC, such as cannabidiol (CBD, 3 $\mu$M), also showed no significant effects, with the exception of 1 $\mu$M HU-210, which increased calcium slightly in CB$_1$-HEK293 cells (Fig. 2), but not in hippocampal neurons. Neither the nonclassical cannabinoid, CP55,940 (CP, 1 $\mu$M), nor the endogenous cannabinoid, 2-AG (1 $\mu$M), nor the metabolically stable anandamide analog, methanandamide (AM356, 10 $\mu$M), caused a significant calcium increase (Fig. 2). The increase in intracellular calcium by WIN was significantly more than for other agonists (ANOVA, 95% confidence interval).

**WIN Increases Intracellular Calcium via PTX-Insensitive G Proteins from the G$_{q11}$ Family.**

To determine whether the WIN-evoked calcium increase was mediated by members of the G$_{q10}$ protein family, cells were pretreated for 16 h with 500 ng/ml PTX. Local perfusion of these cells with increasing concentrations of WIN revealed a concentration-dependent increase in intracellular calcium. Unexpectedly, the increase was augmented by PTX at all WIN concentrations (Fig. 3A). This augmentation was particularly dramatic for 1 $\mu$M WIN, where PTX increased the calcium rise by 1,200%. PTX increased the 3 $\mu$M WIN-evoked calcium rise by 220%, and the 5 $\mu$M WIN-evoked calcium rise by 120% (Fig. 3A). PTX pretreatment did not affect the 10–90% rise times for the WIN-evoked calcium increase (Fig. 3B). The calcium increase in PTX-treated cells was mediated by CB$_1$ receptors, because it was blocked by 1 $\mu$M SR (data not shown).

In cells pretreated with heat-inactivated PTX, the WIN-induced calcium rise was like that in untreated cells (data not shown). The WIN-induced calcium rise in cultured hippocampal neurons was also PTX-insensitive. WIN increased the F$_{340/380}$ ratio to 0.5 $\pm$ 0.1 (n = 4). After PTX treatment, WIN increased the F$_{340/380}$ ratio to 0.4 $\pm$ 0.1 (n = 4). Activity of the PTX toward G$_{q10}$ was confirmed by its ability to block CP-stimulated activation of mitogen-activated protein kinase (data not shown). Together, these data show that the pathway we describe here is not inhibited by PTX, so it does not involve G$_{q10}$ G proteins.

PTX pretreatment enhanced the calcium rise in response to 1 $\mu$M CP to a much lesser extent. CP alone caused a negligible calcium increase of 10 $\pm$ 1 nM (n = 6). Overnight treatment with PTX increased the calcium rise in response to CP to 28 $\pm$ 5 nM (n = 5, P < 0.05). Unlike PTX, heat-inactivated PTX did not augment the response to CP (8 $\pm$ 3 nM, n = 5). PTX pretreatment had small and nonsignificant effects on the calcium rise following perfusion of 10 $\mu$M THC and 10 $\mu$M AM356 (data not shown).

Because inhibition of G$_{q10}$ G proteins did not reduce the WIN-induced calcium increase, we investigated the importance of the PTX-resistant G$_{q11}$ family of G proteins. Transient expression of a dominant-negative G$_{q11}$ (DN G$_{q11}$, Q209L/Q277N) (24) suppressed the WIN-induced transient by 70% ($P < 0.001$, Fig. 4A). M$_1$R activation increases intracellular calcium by G$_q$ G proteins (25). Thus, as a positive control for DN G$_{q11}$, we checked its action on the M$_1$R-mediated calcium transient. In cells transiently expressing M$_1$R, overexpression of DN G$_{q11}$ reduced the 10 $\mu$M Oxo-M calcium increase by 40% ($P < 0.05$, Fig. 4A). Thus, G$_{q11}$ G proteins are involved in the WIN-induced calcium increase.

**The WIN-Induced Calcium Rise Depends on PLC Activation.**

The major downstream effector for G$_{q10}$ is PLC$\beta$ (26). PLC$\beta$ is a likely candidate for the pathway identified in this study, because it
cleaves the membrane lipid phosphatidylinositol-4,5-bisphosphate into the second messengers inositol 1,4,5-trisphosphate (IP3) and diacylglycerol, both of which modulate intracellular calcium. To test for the involvement of PLC, CB1-HEK293 cells were pretreated for 160 s with 3 μM of the PLC inhibitor U73122 followed immediately by 5 μM WIN. This concentration of U73122 has been shown to inhibit PLC (27) yet not increase intracellular calcium on its own (Fig. 5B). The latter is an important point, because U73122 at concentrations >3 μM can increase intracellular calcium concentration by releasing calcium from intracellular stores (28). U73122 (3 μM) suppressed the 5 μM WIN-evoked calcium transient by 80% (P < 0.01, Fig. 5A). The same concentration of its inactive analog, U73343, affected neither the calcium rise in response to 5 μM WIN (n = 4, Fig. 5A) nor intracellular calcium by itself (Fig. 5B). As a positive control for U73122, we verified that it inhibited the M1R-induced calcium rise, which requires PLCβ2 (29). In cells transiently expressing M1R, 3 μM U73122 decreased the calcium rise in response to 5 μM Oxo-M by 70%. The phosphatidylinositol-specific PLC inhibitor, ET-18-OCH3 (30), also attenuated the WIN-induced calcium rise. A 20-min pretreatment with 15 μM ET-18-OCH3 reduced the calcium increase by 70% (180 ± 60 nM; n = 6) compared with that in control cells treated with 5 μM WIN (650 ± 145 nM; n = 12). P < 0.001; t test).

Accumulation of IP derivatives is a measure of PLC activity. In CB1-HEK293 cells, 5 μM WIN (n = 6) increased IP levels by 90% over basal amounts (measured in untreated cells, n = 9, P < 0.01; t test), as well as 80% over amounts measured in nontransfected cells (n = 6, P < 0.01; t test) stimulated with the same concentration of WIN (Fig. 6). Incubation with 3 μM WIN also increased IP levels (Fig. 6). As a positive control, 10 μM Oxo-M increased IP accumulation by 210% over basal amounts in HEK293 cells transiently expressing M1R (n = 9, P < 0.001; t test), and by 150% over levels in nontransfected cells also treated with 10 μM Oxo-M (n = 6, P = 0.006; t test, Fig. 6). IP production was PLC-specific: 3 μM U73122 suppressed both the 3 μM and 5 μM WIN-mediated increases in IP accumulation by 70%, and the 10 μM Oxo-M-mediated increase by 80% (data not shown).

**The WIN-Evoked Calcium Rise Comes from IP3- and Ryanodine-Sensitive Intracellular Calcium Stores**

Two main candidate calcium sources could contribute to the intracellular calcium increase. First, extracellular calcium might enter through plasmalemmal ion channels. Second, calcium might be released from the endoplasmic reticulum (ER) or another intracellular pool. A role for extracellular calcium was assessed by using a calcium-free medium (0 Ca Ringer’s). External calcium entry did not appear to be important, because the calcium transient was unaffected when WIN was delivered in 0 Ca Ringer’s (Fig. 7). The latency to onset and the time course of the calcium increase were unchanged (Fig. 7 Inset).

Because CB1 activation did not promote calcium entry, it was likely that calcium was being released from intracellular stores. The sarco/endoplasmic reticulum Ca2+ (SERCA) pump is an ATP-dependent pump located on the ER surface that maintains and replenishes agonist-sensitive calcium stores. If the SERCA pump is blocked, the stores empty as calcium leaks out and is not replaced. CB1-HEK cells transiently transfected with M1R were incubated with 1 μM of the SERCA pump inhibitor thapsigargin (TG) in 0 Ca Ringer’s for 20 min before the experiment, a treatment sufficient to empty TG-sensitive intracellular stores (31). TG pretreatment reduced the 5 μM WIN calcium transient by 90% (P < 0.05 by ANOVA, Fig. 7). As a positive control, the same TG treatment almost abolished the calcium increase from M1R activation, known to be mediated by TG- and IP3-sensitive stores (29) (data not shown). These
observations suggest that either the calcium is released from TG-sensitive intracellular calcium stores or that WIN inhibits the SERCA pump. In the latter case, the rise in intracellular calcium concentration would be due to leakage of calcium from SERCA pump-dependent stores. To test this possibility, we applied 100 μM 2,5-di-tert-buty-1,4-benzohydroquinone (BHQ), a fast and reversible SERCA pump inhibitor, and measured the change in intracellular calcium. We reasoned that if BHQ perfusion increased calcium to a lesser extent than WIN, we could discount the possibility that the actions of WIN were solely due to SERCA pump inhibition. BHQ treatment elicited a calcium increase only 10% of that seen with WIN (P < 0.05 by ANOVA, Fig. 7). This suggests that inhibition of SERCA pump activity is not the major cause of the WIN-evoked calcium transient.

IP3-sensitive ER calcium stores were a likely source contributing to our calcium increase because IP3 is produced by PLC, which is central in the pathway (Figs. 5 and 6). A 30-s pretreatment with 1 μM of the IP3-R inhibitor xestospongin D (XeD) in 0 Ca Ringer’s immediately followed by perfusion of 5 μM WIN reduced the calcium rise by 75% (P < 0.05 by ANOVA, Fig. 7). The ryanodine receptor (RyR) also plays a role in calcium release, because pretreatment for 20–25 min with 10 μM of the RyR inhibitor dantrolene in 0 Ca Ringer’s reduced the calcium rise by 50% (P < 0.05 by ANOVA, Fig. 7). Further evidence for RyR involvement, treatment with its inhibitor, 1,1′-dihetyl-4,4′-bipyridinium dibromide (DHBP, 50 μM) attenuated the WIN-evoked calcium increase by 70% (P < 0.05 by ANOVA, Fig. 7). Pretreatment with both XeD and dantrolene decreased calcium transients to 15% of that seen in untreated cells perfused with WIN (P < 0.05 by ANOVA, Fig. 7). These data suggest that the calcium transient is the result of calcium release from IP3- and Ry-sensitive intracellular stores.

Discussion

We have found that WIN, a cannabinoid A1 agonist, can increase intracellular calcium in CB1-HEK293 cells, as well as in cultured hippocampal neurons. Because the WIN-induced calcium increase was absent in untransfected HEK293 cells and was blocked by a CB1 antagonist, it is specific. The large slow WIN-induced rise in intracellular calcium was PTX-insensitive and required Gq/11 G proteins and PLC. WIN increased phosphatidylinositol turnover, as measured by IP production, and the IP3 released calcium from IP3-sensitive ER stores. RyRs also contributed to the calcium release.

Other groups have reported instances where CB1 activation increases intracellular calcium (3, 4, 32–35). Generally, the reported calcium rises were more modest than those we report here. Unlike our results, the calcium rise in the earlier studies was mediated by PTX-sensitive Gq/11 β3-ε acting via PLC to release calcium from TG-sensitive intracellular stores (3, 4, 32, 33, 35). The one exception was a report that the nonclassical cannabinoid agonist, DALN, increases intracellular calcium by enhancing the entry of calcium ions through 1-type calcium channels by a PTX-insensitive mechanism (34). Because HEK293 cells do not have 1-type voltage-sensitive calcium channels, this could not be the mechanism for the calcium increase we report here. Thus, our study provides evidence that CB1 functionally couples to G proteins from the Gq/11 family to increase intracellular calcium. The WIN-induced calcium increase identified here is agonist-specific. For example, WIN was more potent and efficacious in increasing intracellular calcium than CP. In contrast, WIN is less potent than CP at inhibiting cAMP production, a G1-coupled pathway (36). As a working hypothesis, we propose that CB1 exists in several active states; the relative ratio of each is a function of the ligand. WIN likely stabilizes a conformation of CB1 that couples more readily to Gq/11. The reversal of agonist potency in the inhibition of cAMP production (36) supports this hypothesis. Similarly, accumulating evidence suggests that the binding site for WIN only partially overlaps that for other CB1 agonists. Although WIN displaces CP from CB1 in radioligand-binding assays (2), mutagenesis studies uncovered an amino acid residue (K192) in helix three of CB1 that, when mutated to alanine, abolished CP binding with little effect on binding or receptor activation by WIN (37). A second mutation, where V282 in helix five was mutated to phenylalanine, increased the affinity of CB1 for WIN with no effect on affinity for CP (38). Favorable interactions with distinct residues may result in WIN preferentially stabilizing a specific active conformation of CB1 that couples not only to Gq/11 but also to Gq/12. Other structurally related AAs may also enhance CB1 coupling to Gq/11.

A relatively high concentration of WIN (5 μM) was required for a substantial calcium increase (>400 nM); however, many published studies use concentrations in this range or higher. WIN-activated CB1 appears to couple preferentially to Gq/11, and only when receptor occupancy is very high or when the ratio of Gq/11 to functional Gq/10 increases will coupling to less-favored G proteins, such as Gα11, be evident. This could explain how PTX pretreatment enhanced the potency of WIN. By inhibiting the Gq/10 α subunit, PTX prevents Gq/10 heterotrimeric G proteins from interacting with the receptor (39). This increases the likelihood of CB1 coupling to Gq/11, leading to an enhanced calcium response at the same WIN concentration. PTX pretreatment also unmasked modest coupling between Gq/11 and CB1 activated by CP and THC, but even after PTX, CP and THC were still far less efficacious than WIN.

Our study is not the first example of agonist-driven coupling of CB1 to a less-favored G protein. Bonhaus et al. (40) reported a difference in the ability of cannabinoid agonists to activate GS-coupled pathways. They showed that WIN was more potent and efficacious than CP in increasing forskolin-stimulated cAMP accumulation, yet CP was more potent than WIN in inhibiting adenyl cyclase (40).

There are other examples of promiscuous receptors activating different signal transduction pathways in an agonist-dependent manner that may be explained by different receptor conformations coupling to different G proteins. Rey et al. (18) have studied agonist-specific signal trafficking of the Ca2+ -sensing receptor (CaR) (18). They find that the CaR activated by external calcium stimulates Gq/11, leading to an enhanced calcium response in an oscillatory fashion. However, if the aromatic amino acid ε-phenylalanine activates CaR, the receptor no longer couples to Gq and instead transiently increases [Ca2+]i through the activation of the small GTPase Rho and G proteins of the G12 subfamily. A second example is from Cordeaux et al. (41), who show that the A1 adenosine receptor, traditionally described as G1-coupled, can also couple to Gq and Gq. The efficacy of this coupling depends on the agonist. The two agonists N(6)-cyclopentyladenosine (CPA) and 5′-(N-ethylcarboxamido)adenosine (NECA) are equally efficacious in Gq coupling. After PTX treatment in cells expressing a low level of A1R, NECA, but not CPA, promoted A1R-Gq interactions. After PTX treatment in cells expressing a high level of A1R, receptors activated by NECA coupled to Gq more efficaciously than CPA-activated receptors.

CB1 generally signals more slowly than other GPCRs, even when modulating the same response. For example, Mackie and Hille (42) found that WIN-activated CB1 inhibited N-type calcium channels 15-fold more slowly than the norepinephrine-activated α2-adrenergic receptor, even at supramaximal agonist concentrations. In the current study, the difference was just as pronounced. The WIN-induced calcium increase was 10-fold slower than the Oxo-M-induced calcium increase (Fig. 1D). The reason for this marked kinetic difference is unknown. It may be that CB1 assumes its activated conformation relatively slowly. Another possibility is that CB1 activates G proteins less efficiently compared with other GPCRs (43). In this case, it would
take longer to reach the threshold concentration of downstream effectors necessary to initiate a response.

Our data indicate that unique among CB1 agonists, WIN releases calcium from intracellular stores. WIN does so by coupling to Gi/0,1 and enhancing PLC activity. Because PLCβ initiates the production of the endocannabinoid 2-AG, this suggests the intriguing possibility that WIN activation of CB1 might increase 2-AG levels. These results also suggest that caution should be taken when extending observations made with high levels of WIN to other drugs activating CB1. Although WIN exhibits the full range of pharmacological and behavioral effects seen with THC, there are some differences. Among these, WIN is more potent in drug discrimination trials and in producing antinociception and less potent in the production of hypothermia. The origin of these differences is unknown, but they may be due to high concentrations of WIN increasing intracellular calcium.

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