The Journal of Neuroscience, October 1995, 15(10): 6552–6561

Cannabinoids Activate an Inwardly Rectifying Potassium Conductance and Inhibit Q-Type Calcium Currents in AtT20 Cells Transfected with Rat Brain Cannabinoid Receptor

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Rat brain cannabinoid receptor (CB-1) was stably transfected into the murine tumor line AtT-20 to study its coupling to inwardly rectifying potassium currents (K_{ir}) and high voltage-activated calcium currents (I_{Ca}). In cells expressing CB-1 ("A-2" cells), cannabinoid agonist potently and stereospecifically activated K_{ir} via a pertussis toxin-sensitive G protein. I_{Ca} in A-2 cells was sensitive to dihydropyridines and ωCTX MVIIC, less so to ωCgTX GVIA and insensitive to ωAga IVa. In CB-1-expressing cells, cannabinoid agonist inhibited only the ωCTX MVIIC-sensitive component of I_{Ca}. Inhibition of Q-type I_{Ca} was voltage dependent and PTX sensitive, thus similar in character to the well-studied modulation of N-type I_{Ca}. An endogenous cannabinoid, anandamide, activated K_{ir} and inhibited I_{Ca} as efficaciously as potent cannabinoid agonist. Immunocytochemical studies with antibodies specific for class A, B, C, D, and E voltage-dependent calcium channel α_1 subunits revealed that AtT-20 cells express each of these major classes of α_1 subunit.

[Key words: cannabinoid, calcium current, G protein, potassium current, AtT-20, anandamide]

Cannabinoids, the primary psychoactive constituents of marijuana, have profound effects on mood, perception, and memory. Most of these effects appear to be mediated via the brain (CB-1) cannabinoid receptor (Pertwee, 1993). This receptor is a member of the G protein–coupled receptor superfamily (Matsuda et al., 1990) and previously has been shown to couple in an inhibitory fashion to adenylyl cyclase (Howlett, 1985) and N-type calcium channels (Caulfield and Brown, 1992; Mackie and Hille, 1992). In neurons and other excitable cells, several G protein coupled receptors also activate inwardly rectifying potassium channels via a pertussis toxin sensitive G protein (North, 1989; Kubo et al., 1993). These channels often contribute to setting the resting membrane potential and play a major role in determining neuronal excitability. In addition to N-type calcium channels, some neuronal G protein–coupled receptors also appear to be able to modulate P-type I_{Ca} and other, less well characterized, voltage-dependent calcium channels (Mintz and Bean, 1993). We asked the question if the same was true for CB-1 receptor. Lacking a neuronal preparation expressing the appropriate ion channels and cannabinoid receptor, and desiring to establish a system for investigating cannabinoid receptor–effector coupling, we transfected the mouse tumor cell line, AtT20, with rat CB-1 receptor. This cell line was chosen as it expresses an inwardly rectifying potassium current that is activated by somatostatin (Dousmanis and Pennefather, 1992; Pennefather et al., 1988) and m_4 muscarinic receptors (Jones, 1992). In addition it expresses high voltage-activated calcium currents that are inhibited by G protein-coupled receptors (Luini et al., 1986; Surprenant et al., 1992). AtT-20 cells secrete β-endorphin and ACTH and have served as a useful model for studying neuropeptide synthesis, transport and release (Burgess and Kelly, 1987; Matsuuchi and Kelly, 1991). Although they are often assumed to be closely related to pituitary corticotrophs, strong evidence has been presented that these cells have many enzymatic and structural features more in common to neuropeptide secreting neurons (Tooze et al., 1989). Thus, they may be a more appropriate model to study neuronal processes than previously appreciated. By determining how cannabinoid receptors couple to ion channels, we hope to increase our understanding of the mechanisms underlying the behavioral actions of cannabinoids and the endogenous ligands for this receptor.

The indentification and classification of voltage-dependent calcium channels is still evolving (Birnbaumer et al., 1994). In the rat brain, multiple isoforms of the principal α_1 subunit of voltage-dependent calcium channels have been identified using molecular cloning techniques and have been designated class A, B, C, D, and E (Snutch et al., 1990; Snutch and Reiner, 1992; Birnbaumer et al., 1994; Soong et al., 1994). The rat brain class C and D genes encode L-type calcium channel α_1 subunits (α_{1C} and α_{1D}) which have a high affinity for dihydropyridine calcium channel antagonists and are approximately 75% identical in amino acid sequence with rabbit skeletal muscle calcium channels and L-type calcium channels from other tissues (Snutch et al., 1990; Hui et al., 1991; Chin et al., 1992; Dubel et al., 1992; Seino et al., 1992; Williams et al., 1992; Tomlinson et al., 1993). The rat brain class B gene encodes N-type calcium channel α_1 subunits with high affinity for ω-conotoxin-GVIA (Dubel et al.,

Received Mar. 24, 1995; revised May 16, 1995; accepted May 19, 1995.

We thank L. Matsuda for providing the CB-1 receptor cDNA; M. Watson, S. Bounds, S. Brown, D. Anderson, and L. Miller for technical help; B. Hille for helpful discussions and facilities; W. Catterall, J. Harrington, B. Nair, and M. Shapiro for reading the manuscript; W. Devane and R. Mechoulam for providing anandamide; Neurex for providing SNX 230; and Sterling Winthrop for providing WIN 55,212-2 and WIN 55,212-3. This work was supported by Foundation for Anesthesia Education and Research, the Keck Foundation, a McKnight Neuroscience Research Award, and the NIH Grants NS01588 and NS08174.

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 0270-6474/95/156552-10$05.00/0

1992; Williams et al., 1992). The class A gene encodes α_1 subunits which form a high-voltage-activated calcium channel with novel physiological and pharmacological properties and is highly expressed in the cerebellum (Mori et al., 1991; Starr et al., 1991; Sather et al., 1993). The class A channels are insensitive to dihydropyridines and ω-conotoxin-GVIA, weakly blocked by ω-agatoxin-IVa, and completely blocked by ω-conotoxin MVIIC. The functional properties of class A α_1 subunits are distinct from the physiologically defined N-type and P-type calcium channels, but closely resemble those of calcium channels in cerebellar granule cells which have been designated Q-type (Zhang et al., 1993; Randall and Tsien, in press). Functional expression of class E (α_{1E}) in *Xenopus* oocytes produced rapidly inactivating calcium channels insensitive to high affinity organic and peptide calcium channel blockers (Williams et al., 1992; Soong et al., 1994; Randall and Tsien, in press). The currents conducted by α_{1E} are similar in some respects to T-type calcium currents as well as the recently identified R-type and G1-3 calcium channels in cerebellar granule cells (Forti et al., 1994; Randall and Tsien, in press). However, the precise relationship between α_{1E} and native calcium currents remains to be defined. In this study, using antibodies and channel blockers, we have characterized the calcium channels present in AtT-20 cells.

Materials and Methods

Materials. Tissue culture reagents were from GIBCO, BSA, leupeptin, IBMX, and PMSF from Sigma, PTX from List, ωAgaIVa from Peptides International, leupeptin and ωCgTX GVIA from Bachem, ^{3}H WIN 55,212-2 was from New England Nuclear, ωCTX MVIIC (SNX 230) from Neurex, somatostain and ωCgTX GVIA from Peninsula Labs and TTX and GTP from Calbiochem. Synthetic anandamide was provided by both William Devane and Raphael Mechoulam and its purity verified as previously described (Mackie et al., 1993). WIN 55,212-2 and WIN 55,212-3 were gifts from Sterling Research Group.

Cell culture. For electrophysiological recording, A-2 cells were plated on polylysine-coated coverslips and grown in DMEM + 10% heat-inactivated horse serum + 1:200 penicillin/streptomycin + 400 μg/ml G418 in a humidified environment with 10% CO_2 at 35°C. For binding and adenylyl cyclase studies, A-2 cells were grown on 500 cm^2 tissue culture plates (NUNC) using the above conditions. Cells were passaged using 0.05 mg/ml trypsin in phosphate-buffered saline (PBS) and used within 10 passages after the initial clone was isolated.

Cannabinoid receptor expression. PCR amplification was used to introduce restriction sites on each side of the cannabinoid receptor cDNA to facilitate subcloning of the gene from SKR6 (Matsuda et al., 1990) into the pcDNA3 mammalian expression plasmid (Invitrogen). Primers (5′-CGGGATCCATGAAGTCGATCCTAGATGGCTTG-3′ and 5′-CGGGATCCATGGCACAAAAGCAGCAGCTCACAGAGC-3′) and *pfu* polymerase were used to amplify the CB-1 receptor cDNA from the SKR6 plasmid. The resulting PCR product was first subcloned into the *Bam*HI site of pUC19 and then subcloned into the *Bam*HI site of pcDNA3 (CB-1-pcDNA3). Colonies containing CB-1-pcDNA3 were identified by hybridization with a ^{32}P-labeled cannabinoid receptor oligonucleotide (PCR sense primer) and the correct orientation was verified by restriction digests with *Hind*III. AtT20 cells were transfected by the calcium phosphate technique with purified (ProMega) CB-1-pcDNA3. Selection was carried out for 10 d in G418, several positive colonies expanded and screened by ^{3}H-WIN 55,212-2 binding.

Radioligand binding and adenylyl cyclase activity. For binding studies, AtT20 cells transfected with pcDNA3 (control) or CB-1 were grown to confluency in 500 cm^2 plates. The monolayers were washed twice with TEM (in mM: 25 Tris-HCl, 6 $MgCl_2$, 1 EDTA, 10 μM phenylmethylsulfonyl fluride (PMSF), and 1 μg/ml leupeptin, pH 7.4) and homogenized in TEM (1 ml/plate). The homogenate was centrifuged at 800 × *g* for 10 min, the resulting pellet homogenized in TEM and spun at 800 × *g* for 10 min. The combined supernatants were resuspended, centrifuged at 100,000 × *g* for 1 hr and the pellets were homogenized in TEM at a final protein concentration of 1-5 mg/ml. Membranes (50 μg) were incubated in 20 mM Hepes and 1 mg/ml bovine serum albumin (BSA), pH 7.5 (final volume 200 ml) in 0.325-6 nM ^{3}H WIN 55,212-2. After 90 min at 30°C, the membranes were filtered on a Tomtec Harvester 96 programmed to rapidly filter and wash captured membranes with 3 × 5 ml Hepes/BSA (Kuster et al., 1993). Specific binding was defined as the fraction of binding displaced by 1 μM of unlabeled WIN 55,212-2. Saturation studies were analyzed by computer using the INPLOT program.

For adenylyl cyclase studies, A-2 cells grown on 24 well plates were rinsed twice with PBS and preincubated first with DMEM for 60 min, and then the DMEM, 1 mM isobutylmethylxanthine, and 3 μM BSA for 10 min. When appropriate, 100 nM WIN 55,212-2 or WIN 55,212-3 was added and the sample incubated for 5 min. The samples were next incubated for 10 min at 37°C with or without 10 μM forskolin, lysed with 0.1 *N* HCl and cAMP measured using a scintillation proximity assay kit (Amersham).

Electrophysiological recording. Currents were recorded using the whole-cell voltage-clamp technique (Hamill et al., 1980). Pipettes were pulled from microhematocrit glass (VWR) and fire polished. For recording, a coverslip containing cells was transferred to a 200 μl chamber that was constantly perfused (1–2 ml/min) with the appropriate external solution. Solution reservoirs were selected by means of a series of solenoid valves, and solution changes were accomplished in <1 min. Voltage protocols were generated and data were digitized, recorded, and analyzed using BASIC-FASTLAB (Indec Systems, Capitola, CA). Junction potentials are uncorrected.

For measuring potassium currents, the pipette solution contained (mM): 130 KCl, 20 Hepes, 10 EGTA, 5 $MgCl_2$, 3 Na_2ATP, 0.6 GTP, 0.08 leupeptin, pH 7.25 with KOH, while the external solution contained (mM): 20 KCl, 130 NaCl (or *N*-methylglucamine–NMG), 1 $CaCl_2$, 25 Hepes, 10 glucose, pH 7.35 with NaOH. Fatty acid-free bovine serum albumin (BSA) (3 μM) was added to decrease adsorbtion of cannabinoids. In some experiments to increase the amplitude of the potassium currents the concentration of KCl was increased to 30 or 40 mM and the concentration of NaCl or NMG decreased correspondingly, to take advantage of the relationship between $[K]_o$ and the voltage dependence of K_{ir} current gating. The K_{ir} current was defined as that component of the current sensitive to 1 mM Ba^{2+} elicited during the final 150 msec of a 250 msec hyperpolarizing pulse to −100 mV from a holding potential of −45 mV. Currents were sampled at 1 kHz. As the magnitude of the K_{ir} current was dependent on cell size, aggregate current data are presented as current densities normalized to cell capacitance.

For measuring calcium currents, the pipette solution contained (mM): 100 CsCl, 40 Hepes, 10 EGTA, 5 $MgCl_2$, 3 Na_2ATP, 0.2 GTP, 0.08 leupeptin, pH 7.30 with CsOH, while the external solution contained (mM): 160 NaCl, 5 $CaCl_2$, 4 KCl, 1 $MgCl_2$, 10 Hepes, 8 glucose, pH 7.35 with NaOH. Tetrodotoxin (200 nM) was added to block voltage-dependent sodium currents and BSA was added to decrease adsorbtion of the cannabinoids, as described above. In experiments using peptide calcium channel blockers, 8 μM cytochrome c was substituted for 3 μM BSA. This substitution had no effect on I_{Ca} or its modulation by WIN 55,212-2 (see Fig. 5*A–C*). I_{Ca} was measured near the end of a −25 ms depolarizing pulse to 0 mV from a holding potential of −90 mV and was defined as that component of the current sensitive to 100 μM $CdCl_2$. Currents were sampled at 4 kHz.

To control for possible response variations with passage number and to avoid one source of systematic bias, experimental and control measurements were alternated whenever possible and concurrent controls were always performed. Where appropriate, data are expressed as mean ± standard error.

Production and purification of peptides and antibodies. The antibodies CNB2, CNC1, and CND1 were prepared as described previously (Hell et al., 1993; Westenbroek et al., 1992). Peptides CNA1 (KYPSSPERAPGREGPYGRE), corresponding to residues 865–881 and CNE2 (KYSASQERSLDEGVSIDG), corresponding to residues 896–911, of the α_1 subunit of rat brain class A and E calcium channels, respectively, plus N-terminal lysine and tyrosine extensions, were synthesized by the solid phase method (Merrifield, 1963). The peptides were purified by reverse-phase HPLC on a Vydac 218TP10 column and their identity verified by amino acid analysis.

Anti-peptide antibodies against the CNA1 or CNE2 peptides were generated using the method described previously (Westenbroek et al., 1992). Briefly, the purified peptide CNA1 or CNE2 was coupled through amino groups with glutaraldehyde to bovine serum albumin (Orth, 1979), dialyzed against PBS, and emulsified in an equal volume of Freund's complete (initial injection) or incomplete adjuvant. At 3

week intervals, the coupled peptide was injected into multiple subcutaneous sites on New Zealand White rabbits. Antisera were collected after the second injection and tested by enzyme-linked immunosorbent assay using microtiter plates with wells coated with 0.5 mg of peptide (Posnett et al., 1988). Antibodies against CNA1 or CNE2 peptides were purified by affinity chromatography on CNA1 or CNE2 peptides, respectively, which were coupled to CNBr-activated Sepharose. The antiserum (2.0 ml) was bound to the column at 4°C overnight, washed with TBS, eluted with 5.0 M $MgCl_2$ and then dialyzed against TBS using a Centriprep 30 (Amicon).

Immunocytochemistry. A-2 cells were grown on 35 mm plastic petri dishes for 3–5 d. The cells were rinsed briefly in 0.05 M PB (sodium phosphate, pH 7.4), and fixed for 20 min using 4% paraformaldehyde in 0.1 M PB. The cells were next rinsed with PB briefly followed by 0.05 M PBS (sodium phosphate-buffered saline, pH 7.4) for 30 mins, and then blocked with 10% Avidin (Vector) in PBS for 30 min. The cells were then rinsed in PBS for 20 mins, blocked with 10% Biotin (Vector) in PBS for 30 min, rinsed in PBS for 20 min, and then blocked using 5% normal goat serum in PBS. The cells were incubated overnight at 4°C in affinity purified CNA1 (diluted 1:15), CNB2 (diluted 1:20), CNC1 (diluted 1:15), CND1 (diluted 1:15), or CNE2 (diluted 1:20) antibodies. All antibodies were diluted in PBS containing 10% normal goat serum and 0.025% Triton X-100. The cells were then rinsed with PBS for 45 min and incubated for 2 hr at room temperature in Biotinylated goat anti-rabbit IgG (diluted 1:400,Vector), rinsed in PBS for 45 mins, and then in incubated in Avidin-D-fluorescein (diluted 1:400, Vector). The cells were finally rinsed in PBS for 10 min, PB for 20 min, distilled water for 5 min, and then coverslipped using Vectashield (Vector). All samples were viewed and photographed using a Bio-Rad confocal microscope. Controls included incubating cells without primary antibody or blocking the primary antibody by preincubation for 6 to 8 hr with the corresponding peptide (20–25 μM). The immunocytochemical reaction was then carried out as described above. The staining patterns reported here were abolished in cells treated without primary antibody or with blocked primary antibody.

Results

Establishment of a cell line stably expressing CB-1

After transfection with CB-1-pcDNA3 and selection in 400 μg/ml G418, the clone designated A-2 was chosen for further characterization. Membranes prepared from A-2 cells consistently showed saturable binding for the synthetic cannabinoid ligand ^{3}H WIN 55,212-2 (Fig. 1*A*), with a B_{max} of 864 ± 13 fmol/mg protein and a K_d of 2.6 ± 0.7 nM (n = 6). Specific binding ranged from 60 to 80% of the total binding. In contrast, membranes prepared from AtT20 cells transfected with the vector pcDNA3 alone showed no specific binding to ^{3}H WIN 55,212-2 (n = 3, data not shown). CB-1 expressed in AtT20 cells coupled appropriately to adenylyl cyclase, with 100 nM WIN 55,212-2 inhibiting forskolin-stimulated adenylyl cyclase by about 50% (Fig. 1*B*). Its enantiomer, WIN 55,212-3, was inactive (Fig. 1*B*).

Cannabinoids activate an inwardly rectifying potassium conductance

Exposure of an A-2 cell to 100 nM WIN 55,212-2 activated a prominent inward current when the cell was hyperpolarized to a potential negative to E_K in high potassium external (Fig. 2*A*). This response was seen in more than 90% of the cells tested (data not shown). The current–voltage relationship for the WIN 55,212-2-induced current showed inward rectification over the voltage range of 0 to −90 mV (Fig. 2*B*). This current was also sensitive to low concentrations of barium (Fig. 2*C*) (Hagiwara et al., 1978; Dousmanis and Pennefather, 1992). Both of these are features of the inwardly rectifying potassium current activated by somatostain and muscarinic agonists previously identified in these cells (Pennefather et al., 1988; Jones, 1992). Thus, we will refer to this current as inwardly rectifying potassium current or K_{ir} current. WIN 55,212-2 failed to increase K_{ir} current in AtT20 cells transfected with the pcDNA3 plasmid alone (0.09 ± 0.18 pA/pF, n = 4, data not shown). However, 200 nM somatostatin robustly activated K_{ir} current in these cells as expected (Pennefather et al., 1988) (data not shown).

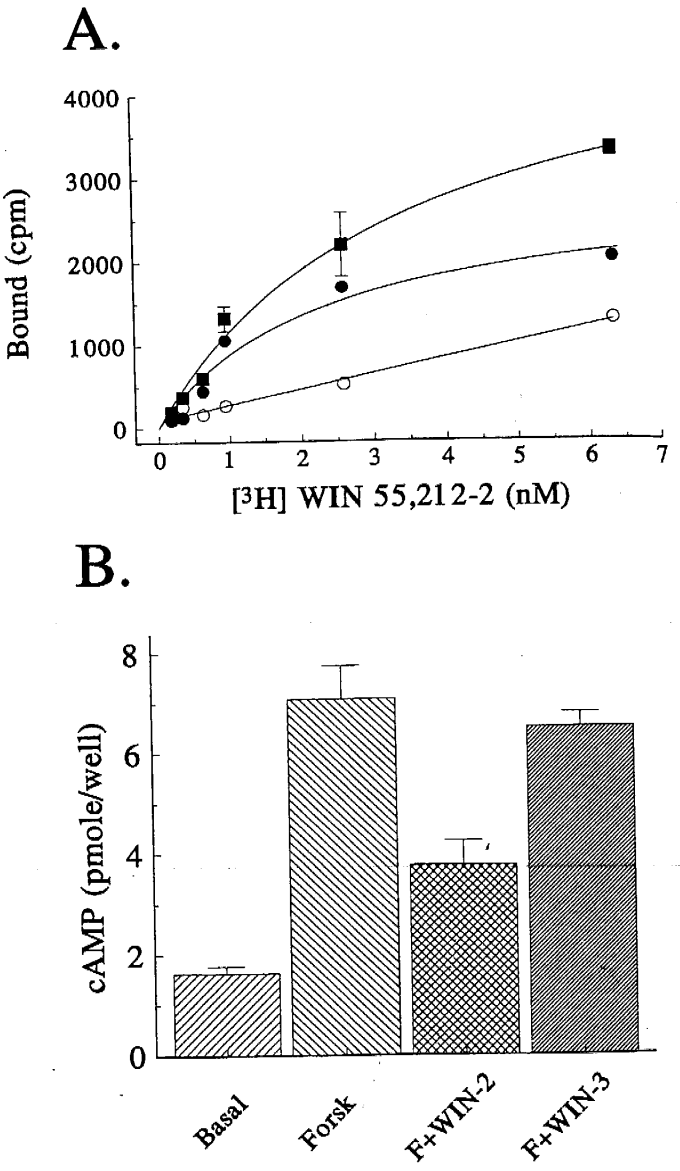

Figure 1. Functional expression of CB-1 cannabinoid receptor in AtT20 cells. *A*, Cannabinoid receptor expressed in AtT20 cells shows saturable binding. Saturation isotherm for binding of ^{3}H-WIN 55,212-2 to membranes prepared from A-2 cells. Nonspecific binding (*open circles*) is ^{3}H-WIN 55,212-2 bound in the presence of 1 μM WIN 55,212-2. *Squares*, Total binding. *Solid circles*, specific binding. *B*, CB-1 expressed in A-2 cells can inhibit adenylyl cyclase. Preincubation with 100 nM WIN 55,212-2 decreased forskolin (*F*)-stimulated synthesis of cAMP ($p < 0.02$ vs forskolin alone), while the inactive enantiomer, WIN 55,212-3 was ineffective ($p < 0.01$ vs forskolin + WIN 55,212-2).

In N18 neuroblastoma cells and NG108-15 neuroblastoma glioma cells WIN 55,212-2-mediated inhibition of N-type I_{Ca} is half-maximal at about 10 nM (Mackie and Hille, 1992; Mackie et al., 1993). Similarly, in A-2 cells WIN 55,212-2 potently activated K_{ir} current with an EC50 of 12 ± 4 nM (Fig. 2*D*). Binding and activation of K_{ir} current did not show marked coopera-

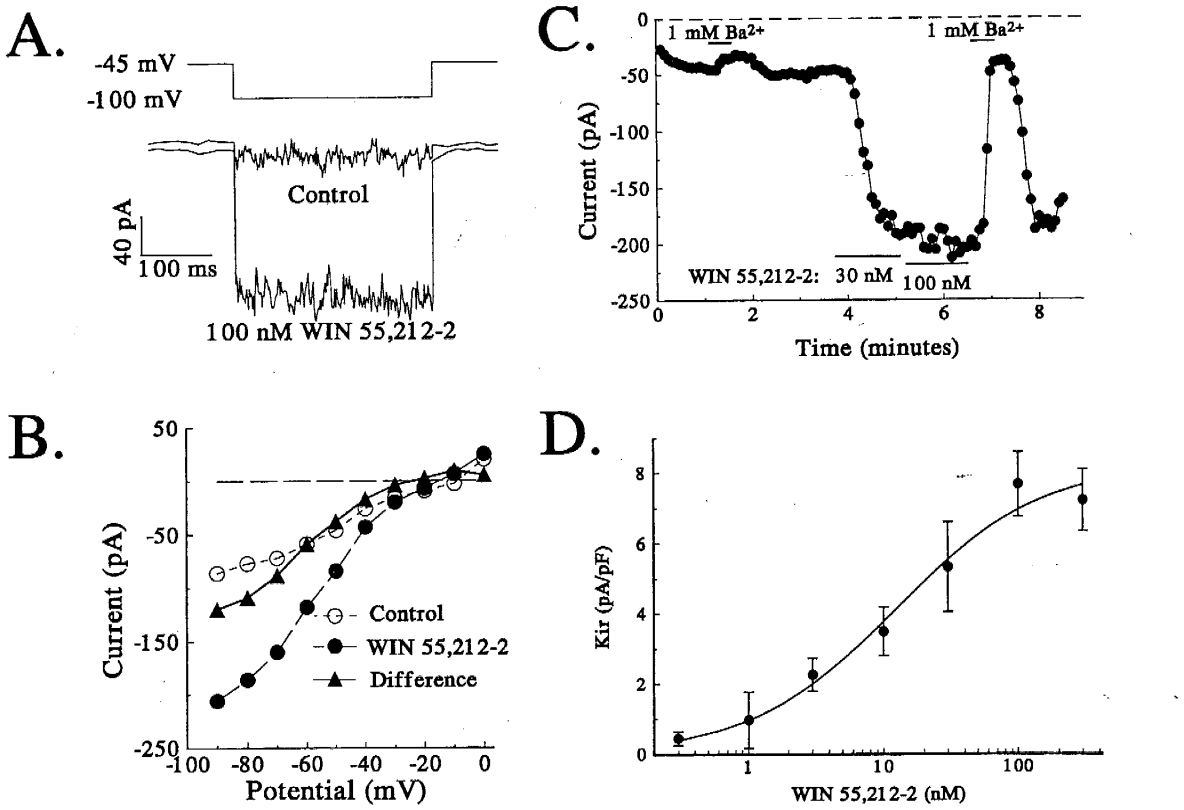

Figure 2. Cannabinoids activate an inward current with the characteristics of an inwardly rectifying potassium current in AtT-20 cells transfected with rat brain cannabinoid receptor in 30 mM K^+ external. *A*, Whole-cell current elicited by a hyperpolarization from −45 mV to −100 mV in control solution or in 100 nM WIN 55,212-2. Sampling rate is a factor of 10 slower before and after the hyperpolarization. *Dashed line* marks the zero current level. *B*, The mean current during a 250 msec hyperpolarization elicited by WIN 55,212-2 shows inward rectification over the voltage range of 0 to −90 mV. The difference curve (*inset*) was obtained by subtracting the current in the absence of WIN 55,212-2 from the current in its presence. *C*, The mean inward current during a 250 msec hyperpolarization applied every 5 sec plotted against time. Barium (1 mM) blocks almost all of the current stimulated by WIN 55,212-2. *D*, WIN 55,212-2 activation of the K_{ir} current is potent. The mean inward current during hyperpolarizations from −45 mV to −100 mV, normalized to cell capacitance was determined for increasing concentrations of WIN 55,212-2 (WIN-2) (n = 3–11). The EC50 is 12 nM, and the Hill coefficient for the fitted line is 0.81.

tivity; the Hill coefficient for activation was 0.81 ± 0.17. As expected for a cannabinoid receptor-mediated process, WIN 55,212-3, the inactive enantiomer of WIN 55,212-2, was ineffective in increasing K_{ir} current in A-2 cells (0.16 ± 0.09 pA/pF, n = 4, data not shown). Cannabinoid activation of K_{ir} current was abolished by overnight incubation with 200 ng/ml pertussis toxin (PTX) (−0.1 ± 0.1 pA/pF, n = 4, data not shown), implicating involvement of G proteins of the G_i or G_o class. In 40 mM external K^+, 200 nM somatostatin, 100 nM WIN 55,212-2, and 10 μM oxotremorine-m activated K_{ir} current by 32.3 ± 4.5 pA/pF, n = 6; 11.5 ± 2.3 pA/pF, n = 9; and 8.6 ± 4.0 pA/pF, n = 7, respectively, at −100 mV (data not shown). Thus somatostatin appears to be a more efficacious activator of K_{ir} in these cells than either WIN 55,212-2 or oxotremorine-m. As AtT-20 cells only express about 200 fmol/mg of somatosatin receptors (about 19,000 receptors/cell) (Thermos and Reisine, 1988), the increased efficacy of somatostatin cannot be due to a higher receptor density and may occur at the level of the G protein.

Anandamide, an endogenous ligand for the brain cannabinoid receptor has been identified, isolated, synthesized and characterized (Devane et al., 1992; Felder et al., 1993; Mackie et al., 1993). In N18 neuroblastoma cells, anandamide has a lower intrinsic efficacy than the potent synthetic cannabinoids CP 55,940 and WIN 55,212-2 (Mackie et al., 1993), acting as a partial agonist for inhibition of N-type I_{Ca}. Thus, it was of interest to see if anandamide acts as a partial agonist in activation of K_{ir} current. This appears not to be the case as K_{ir} current is activated to a similar extent by application of 300 nM anandamide or by application of 100 nM WIN 55,212-2 (Fig. 3*A*,*B*).

Cannabinoids inhibit I_{Ca} via a voltage-dependent and PTX-sensitive pathway

In neuroblastoma cell lines cannabinoids inhibit N-type I_{Ca} (Caulfield and Brown, 1992; Mackie and Hille, 1992; Mackie et al., 1993). We found that cannabinoids also inhibit high voltage-activated I_{Ca} in A-2 cells (Fig. 4*A*,*C*). This response was seen in more than 95% of the cell that expressed I_{Ca} (data not shown). A common form of high voltage-activated I_{Ca} modulation is a voltage-dependent inhibition that can be transiently overcome by application of large depolarizing prepulses (Bean, 1989; Marchetti et al., 1986), that is, prepulse facilitation. In mammalian cells, many receptors coupled to PTX-sensitive G proteins, including the cannabinoid CB-1 receptor, produce such voltage-dependent inhibition (Ikeda, 1991; Kasai, 1992; Mackie et al., 1993). Voltage-dependent inhibition can be assessed by first subjecting the cell to a brief depolarization to 0 mV to obtain the baseline calcium current. After a pause of 2 sec, the cell is

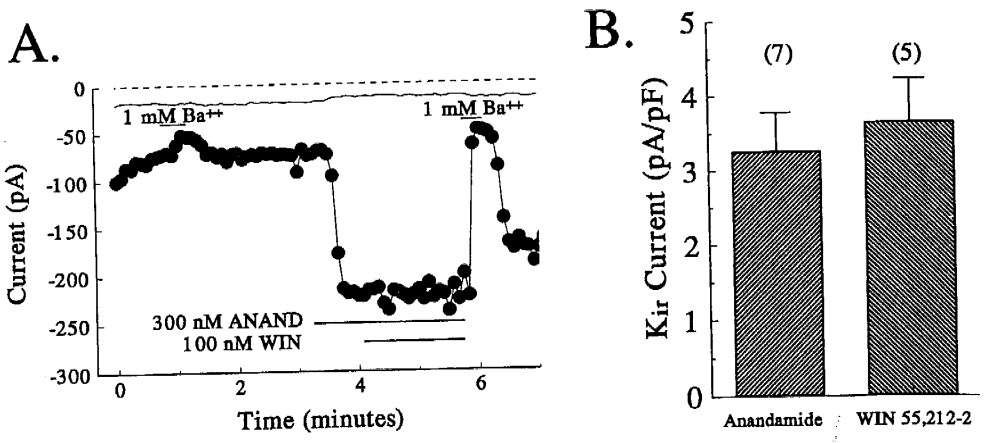

Figure 3. Anandamide, also activates the K_{ir} current. *A*, The mean inward current during a 250 msec hyperpolarization applied every 5 sec from a holding potential of −45 mV to −100 mV plotted against time. 300 nM Anandamide (*ANAND*) elicits a large inward current. Subsequent coapplication of 100 nM WIN 55,212-2 (*WIN*) does not increase this current. The *fine line* corresponds to the holding current at −45 mV. *B*, Comparison of K_{ir} current activation from a number of cells. The number of cells tested for each condition is indicated by the number in parentheses. Anandamide activated K_{ir} current to the same extent as WIN 55,212-2.

strongly depolarized (to ca. 100 mV) to remove any voltage-dependent inhibition, stepped back to the holding potential for a few milliseconds and then stepped to 0 mV to measure the facilitated calcium current (see the voltage protocol in Fig. 4*A* as an example). A comparison of the I_{Ca} during the first and second test pulses will give a measure of the facilitation that occurs during the depolarizing prepulse. During the application of an agonist that shows voltage-dependent inhibition, the reduction of I_{Ca} during the first, baseline pulse will be greater than during the second, facilitated pulse. The degree of relief from agonist-induced inhibition during the facilitated pulse is a measure of the voltage dependence of the inhibition.

The effect of cannabinoids on I_{Ca} in A-2 cells was determined using the double pulse voltage protocol shown in Figure 4*A*. Bath application of 100 nM WIN 55,212-2 inhibited about 25% of the current during the first test pulse (Fig. 4*A*), but only 10% of the current during the second, facilitated pulse. On average, inhibition of I_{Ca} by 100 nM WIN 55,212-2 was 60 ± 7% voltage dependent (n = 6) (data not shown). Application of 100 nM WIN 55,212-2 to AtT20 cells transfected with pcDNA3 alone (mock)

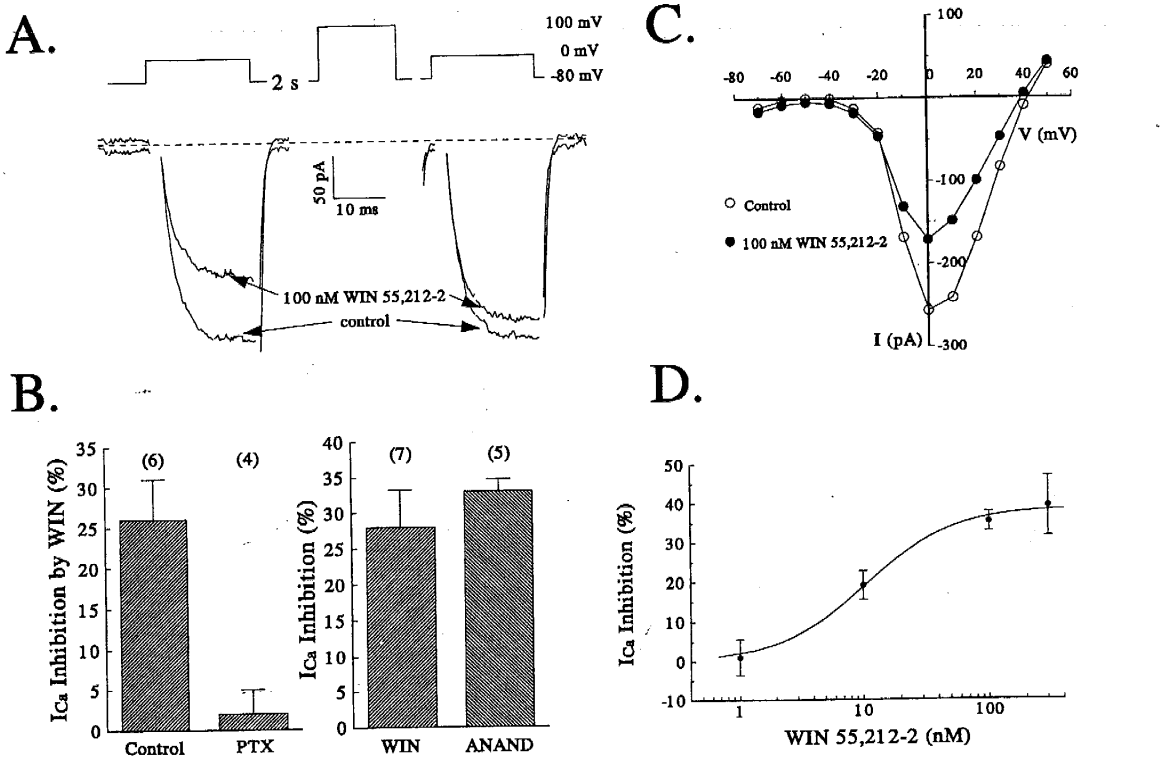

Figure 4. WIN 55,212-2 inhibits a high voltage-activated I_{Ca} in AtT20 cells expressing rat brain cannabinoid receptor in a voltage- and PTX-sensitive fashion. *A*, Calcium currents were measured using a double pulse protocol. After a pause of 2 sec, the first test pulse was followed by a 50 msec depolarization to 100 mV, a 5 msec repolarization to −80 mV and a second test pulse to 0 mV; 100 nM WIN 55,212-2 inhibited approximately 25% of the I_{Ca} during the first pulse, but only 10% of the I_{Ca} during the second pulse indicating voltage dependence. Traces were Cd^{2+} (100 μM) subtracted. *B:Left*, overnight incubation with 500 ng/ml PTX effectively blocked modulation of I_{Ca} in AtT20 cells expressing rat CB-1. *Right*, 300 nM anandamide was as effective as 100 nM WIN 55,212-2 in inhibiting I_{Ca}. *C*, Current–voltage relationship of I_{Ca} in AtT20 cells does not reveal a low voltage-activated I_{Ca}. *D*, Concentration response for WIN 55,212-2 inhibition of I_{Ca} in AtT20 cells expressing CB-1. Inhibition was half-maximal at 11 nM.

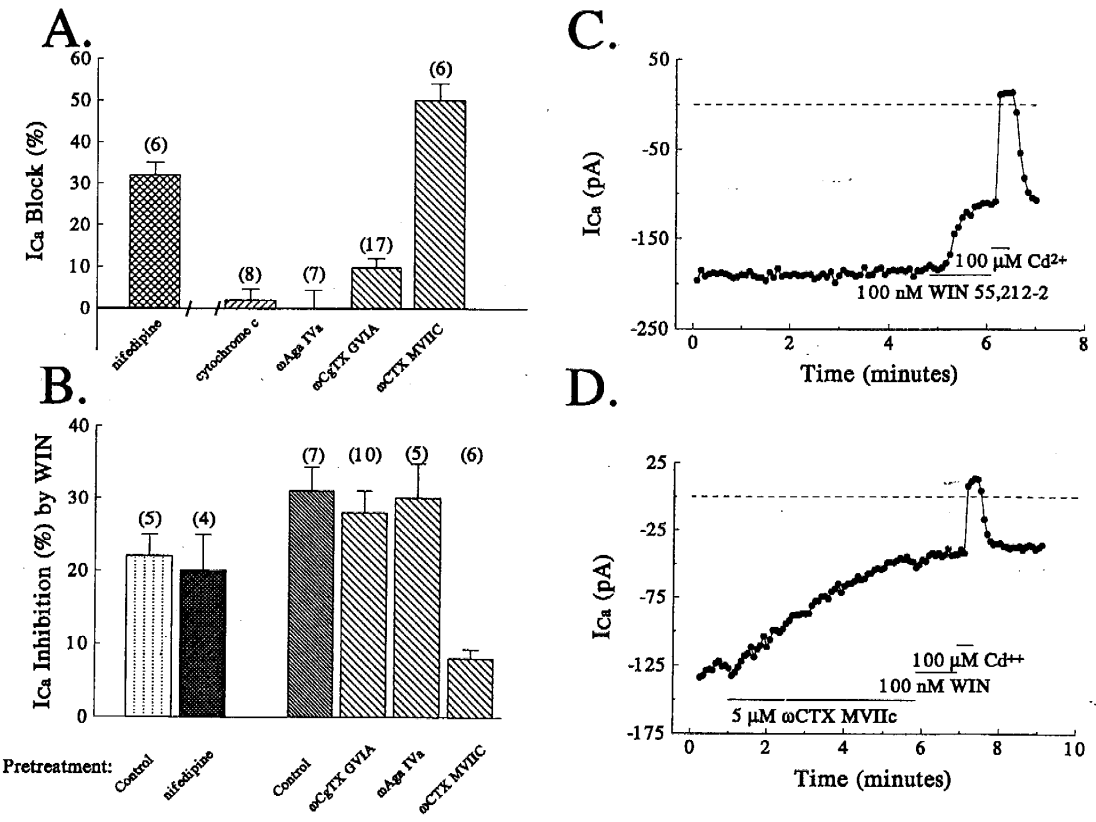

Figure 5. Calcium currents present in AtT20 cells expressing rat brain cannabinoid receptor and their modulation by calcium channel antagonists. *A*, Mean suppression of I_{Ca} by 2 μM nifedipine, 8 μM cytochrome c, 200 nM ωAga IVa, 1 μM ωCgTX GVIA, and 5 μM ωCTX MVIIC. Nifedipine block is expressed as a percentage of whole cell I_{Ca}, while block by the remaining agents are expressed as percentage of whole cell I_{Ca} after treatment of the cell with 2 μM nifedipine; 8 μM cytochrome c was used as the carrier for ωAga IVa, ωCgTX GVIA and ωCTX MVIIC. *B*, Inhibition of I_{Ca} by WIN 55,212-2 after treatment with calcium channel antagonists. *Left two bars,* preapplication of 2 μM nifedipine before treatment with 100 nM WIN 55,212-2 does not reduce inhibition by WIN 55,212-2. *Right four bars,* cells were continuously exposed to 2 μM nifedipine, then the indicated calcium channel antagonist, and finally 100 nM WIN 55,212-2. Inhibition by WIN 55,212-2 was normalized to I_{Ca} in nifedipine before application of the second calcium channel antagonist. Modulation of I_{Ca} by 100 nM WIN 55,212-2 in cells exposed to 8 μM cytochrome c (*C*) and 5 μM ωCTX MVIIC (*D*).

did not inhibit I_{Ca} (0.9 ± 1.1%, $n = 4$, data not shown). Similarly, as was found for activation of K_{ir}, modulation of I_{Ca} by 100 nM WIN 55,212-2 is mediated via G protein(s) of the G_i or G_o class, as overnight incubation of A-2 cells with 500 ng/ml PTX abolished WIN 55,212-2-induced I_{Ca} inhibition (Fig. 4*B*, left). Application of 300 nM anandamide inhibited I_{Ca} as efficaciously as 100 nM WIN 55,212-2 (Fig. 4*B*, right). Inhibition of I_{Ca} by WIN 55,212-2 was potent, being half-maximal at 11 nM (Fig. 4*D*), and noncooperative, with a Hill coefficient of 1.1.

Cannabinoids inhibit a non-L, N, P, T calcium current

We next determined which subtype(s) of I_{Ca} cannabinoids inhibited in A-2 cells. Previous studies have found that AtT20 cells do not express a functional low-voltage-activated, or T-type, calcium current (Luini et al., 1986; Surprenant et al., 1992). Similarly, A-2 cells do not appear to express this current (Fig. 4*C*). Although the classification of high voltage-activated calcium currents in excitable cells is still developing, a classification useful for interpreting data from electrophysiological experiments is based on the sensitivity of different calcium currents to dihydropyridines and peptide toxins. For this study, we defined L-type calcium current as that I_{Ca} sensitive to the dihydropyridine, nifedipine; P type I_{Ca} was defined as that component of the current sensitive to low concentrations of the spider toxin, ωAga IVa; N-type I_{Ca} was defined as the current sensitive to the conus toxin, ωCgTX GVIA. In many neurons, a significant high voltage-activated I_{Ca} remains after treatment with a cocktail of the above blockers. This current is often sensitive to the conus toxin, ωCTX MVIIC, appears to play a role in neurotransmitter release, can be modulated by G protein coupled receptors, and has tentatively been labeled as Q-type I_{Ca} (Adams et al., 1993; Sather et al., 1993; Wheeler et al., 1994; Zhang et al., 1993). The residual high voltage-activated current after treatment with dihydropyridines and ωCTX MVIIC has been designated "R-type" by some investigators (Randall and Tsien, in press) although this current might actually consist of several components.

We first pharmacologically determined the high voltage-activated calcium current types present in AtT-20 cells. Nifedipine (2 μM) blocked about a third of the whole cell I_{Ca} elicited by a depolarization to 0 mV from a holding potential of −90 mV (Fig. 5*A*). In cells pretreated with 2 μM nifedipine, 200 nM ωAga IVa was ineffective in blocking I_{Ca} (Fig. 5*A*), while 1 μM ωCgTX GVIA blocked less than 10% of I_{Ca} (Fig. 5*A*). Therefore, AtT-20 cells don't express P-type I_{Ca} and express low levels of N-type I_{Ca}. However, 5 μM ωCTX MVIIC slowly blocked approximately 50% of the I_{Ca} in these cells (Fig. 5*A,D*). Inhibition during ωCTX MVIIC application did not appear to be a consequence of rundown as calcium currents were fairly stable un-

der the present recording conditions (Fig. 5*A,C*). Thus AtT-20 cells are distinctive among cell lines examined thus far in that they express a significant Q-type I_{Ca}.

We next determined the component of I_{Ca} cannabinoids inhibited. WIN 55,212-2 does not appear to inhibit L-type I_{Ca}, as block of these channels by 2 μM nifedipine does not decrease the fraction (normalized to prenifedipine levels) of I_{Ca} inhibited by 100 nM WIN 55,212-2 (Fig. 5*B*). As an expected consequence of their modest effects on whole cell I_{Ca}, prior application of 200 nM ωAga IVa or 1 μM ωCgTX GVIA did not significantly alter I_{Ca} inhibition by 100 nM WIN 55,212-2 (Fig. 5*B*). In contrast, prior treatment by 5 μM ωCTX MVIIC reduced subsequent inhibition by 100 nM WIN 55,212-2 from 31 ± 3.3% (n = 7) to 8 ± 1.2% (n = 6) (Fig. 5*B,D*). Thus the majority of the WIN 55,212-2-inhibited I_{Ca} is carried via Q-type calcium channels sensitive to ωCTX MVIIC but insensitive to nifedipine, ωAga IVa, or ωCgTX GVIA.

Immunocytochemical identification of class A, B, C, D, and E calcium channels in A-2 cells

Immunocytochemistry was carried out to further verify the presence of L-, N-, and Q-type calcium channels in the A-2 cell line, using anti-peptide antibodies specific to class A (CNA1), class B (CNB2), class C (CNC1), class D (CND1), or class E (CNE2) neuronal calcium channels. The biochemical properties and localization of CNA1 and CNB2 antibodies in rat brain have been reported previously (Westenbroek et al., 1992, 1995; Hell et al., 1993). Using the CNA1 antibody we observed a relatively dense pattern of staining in the cell body and along the extensions of the A-2 cells (Fig. 6*A*). Occasionally there was staining in the foot processes of the cells, however, staining in this region was variable. The pattern of CNB2 antibody localization (Fig. 6*B*) was similar to that observed for the CNA1 antibody. There was specific CNB2 staining in the cell body, along the extensions and in the foot process. The CNB2 staining in the foot process was much more common than staining of this region with the CNA1 antibody. The pattern of staining for CNA1 and CNB2 antibodies appears to be punctate, suggestive of clustering of calcium channels. In addition, the staining appears to be mainly in the membrane as suggested by a z-series through several cells at 1 μm steps. The immunoreactivity observed with the CNC1 and the CND1 antibodies was localized predominatly to the cell body and in the proximal portion of the extensions (Fig. 6*C,D*). Occasionally, there was relatively faint staining of CND1 in the foot process. The staining with the CNC1 also appeared to be punctate. In N18 neuroblastoma cells the high-voltage-activated I_{Ca} is blocked by a combination of 2 μM nifedipine and 1 μM ωCgTX GVIA, implying that class B, C, and/or D, but not A, calcium channels are functionally expressed (Mackie et al., 1993). Consistent with this electrophysiological observation, N18 cells were intensely labeled with antibodies against class B, C, and D channels, but not with antibodies against class A channels (data not shown).

Antipeptide antibodies to class E calcium channels (Yokoyama et al., 1995) were also used to investigate the presence of other voltage-dependent calcium channels in A-2 cells. Using CNE2 antibodies we observed a relatively dense pattern of staining in the cell body of A-2 cells (Fig. 6*E*). There was also weak CNE2 staining along the extensions with relatively little staining observed in the foot process. We observed no staining with the CNE2 antibody in N18 cells. The current electrophysiological studies suggest that R-type currents are present in A-2 cells and earlier work demonstrates a prominent T-type current in N-18 cells (Mackie et al., 1993; Mackie, unpublished observations). Thus, our immunocytochemical results are consistent with the hypotheses that class E calcium channels mediate "R-type" currents in A-2 cells and they do not mediate T-type currents in N18 cells. Control sections in which the primary antibody was omitted (Fig. 6*F*) indicate that the staining observed using the CNA1, CNB2, CNC1, CND1, or CNE2 antibodies is specific.

Collectively our studies confirm the presence of class A (Q-type), class B (N-type), class C and D (L-type), and class E (R type?) calcium channels in the A-2 cells. Since this is not quantitative immunocytochemistry, the differences in the relative amount of staining among the antibodies can not be interpreted as absolute differences in the amount of channel present. The staining depends on such variables as the titer of the antibodies and the access of the antibody to the antigenic site.

Discussion

Here we have found that cannabinoids activate a K_{ir} current and inhibit a Q-type I_{Ca} in AtT20 cells transfected with the rat brain cannabinoid receptor. Thus, it is likely that cannabinoids will do the same in neurons expressing the cannabinoid receptors, these ion channels, and N-type calcium channels. Our favored interpretation of the results of these and earlier studies is that activation of the cannabinoid receptor decreases the excitability of those neurons expressing it. Specifically, inhibition of N- and Q-type calcium currents will decrease the likelihood of neurotransmitter release and successful synaptic transmission as well as suppressing other calcium-dependent processes. Activation of K_{ir} currents will decrease excitability by holding the membrane potential negative to the activation threshold for sodium and calcium channels making pre- and postsynaptic depolarization, action potential generation, and impulse propagation less likely.

AtT20 cells transfected with the rat brain cannabinoid receptor (A-2 cells) expressed functional cannabinoid receptors as evidenced by the development of specific WIN 55,212-2 binding sites and the ability of WIN 55,212-2 to inhibit forskolin-stimulated adenylyl cyclase. The inward current activated by cannabinoids in A-2 cells has the characteristics of a K_{ir} current—it was sensitive to 1 mM Ba^{2+} and its current–voltage relationship showed inward rectification (Fig. 2*B*). Activation of K_{ir} current by cannabinoids in A-2 cells appears similar in most regards to activation of similar currents in other cells and neurons as it is mediated by a PTX-sensitive G protein (North, 1989).

The endogenous cannabinoid, anandamide, has a lower intrinsic efficacy than the potent synthetic cannabinoids, WIN 55,212-2 or CP 55,940 (Mackie et al., 1993). In N18 cells, anandamide acts as a partial agonist in inhibiting N-type I_{Ca}. In this study partial agonism by anandamide was not found for either activation of K_{ir} current or inhibition of Q-type I_{Ca}. This might be a consequence of A-2 cells having a higher density of CB-1 receptors than N18 cells (<100 fmol/mg, data not shown), or because different G proteins transduce the response (Hoyer and Boddeke, 1993). In either case, because of the high density of cannabinoid receptor expression in many brain regions, anandamide likely acts as a full agonist in activation of K_{ir} current or inhibition of Q-type I_{Ca} in those regions.

Cannabinoids inhibit N-type calcium channels in N18 and NG108-15 cells (Mackie and Hille, 1992; Mackie et al., 1993), two cell lines descended from a murine peripheral neuroblastoma. N-type I_{Ca} mediates a portion of neurotransmitter release at some mammalian CNS synapses (Takahashi and Momiyama,

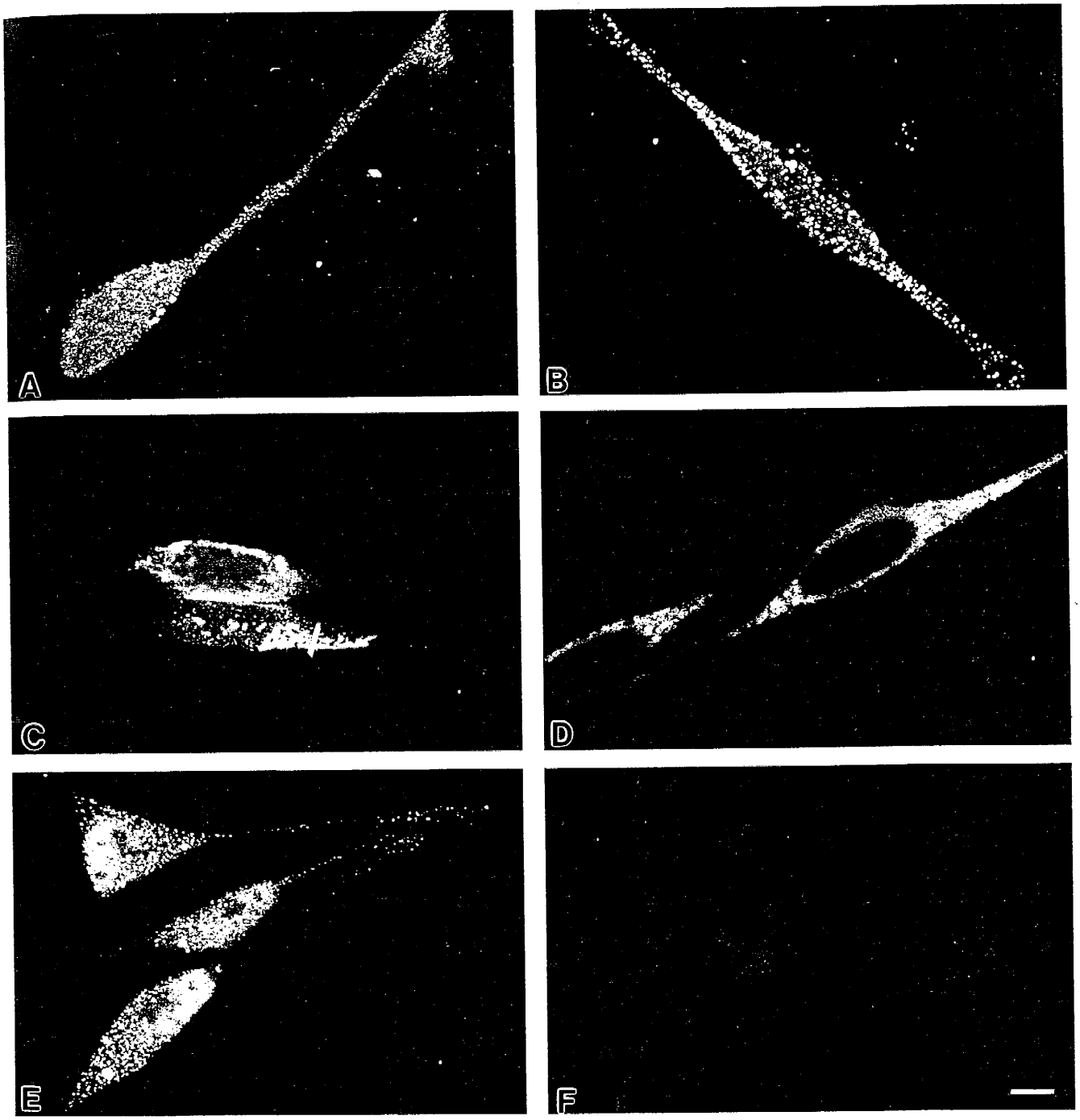

Figure 6. Localization of class A–E calcium channels in A-2 cells. A-2 cells were stained using the immunofluoresence technique as described in Materials and Methods. *A*, A-2 cells stained with CNA1 antibody to rat brain class A calcium channels. *B*, A-2 cells stained with the CNB2 antibody against the rat brain class B N-type calcium channels. *C*, Cells labeled with CNC1 demonstrating the presence of class C L-type calcium channels. *D*, A-2 cell line labeled with class D L-type calcium channels. *E*, A-2 cells stained with CNE2 antibody against the rat brain class E calcium channels. *F*, A-2 cells with primary antibody omitted. Scale bar, 10 μm.

1993; Turner et al., 1993; Wheeler et al., 1994). It is likely that cannabinoid agonists decrease neurotransmitter release in presynaptic terminals expressing cannabinoid receptors and N-type I_{Ca}. Recent studies have implicated Q-, O-, or possibly other types of I_{Ca} (defined as being insensitive to ωCgTX GVIA and dihydropyridines, sensitive to only high concentrations of ωAga IVa, and sensitive to ωCTX MVIIC; Adams et al., 1993; Zhang et al., 1993) and P-type I_{Ca} (sensitive to low concentrations of ωAga IVa) as major facilitators of neurotransmission at mammalian CNS synapses (Wheeler et al., 1994). It has been proposed based on differential toxin sensitivities that the Q-type current described in cerebellar granule cells (Zhang et al., 1993) corresponds to the Ba^{2+} current described in *Xenopus* oocytes expressing a rabbit α_{1A} (together with rabbit α_2/δ and β subunits) calcium channel (Sather et al., 1993). The G protein modulation of Q-type I_{Ca} found in the current study appears to have at least

two properties in common with modulation of N-type I_{Ca}. In particular, it is voltage dependent and PTX-sensitive (Mintz and Bean, 1993; Swartz, 1993; Wheeler et al., 1994; current study).

The pharmacology of the I_{Ca} inhibited by cannabinoids in CB-1-transfected AtT-20 cells is similar to that described for Q-type I_{Ca} (Zhang et al., 1993; Randall and Tsien, in press). As activation of m_4 muscarinic and somatostatin receptors also inhibit most of the dihydropyridine-insenstive I_{Ca} in these cells in a voltage-dependent fashion (data not shown), it is likely that these receptors can also inhibit Q-type I_{Ca} in neurons. It is possible that Q-type current may actually be composed of several components, but the present operational definition of Q-type I_{Ca} as a I_{Ca} insensitive to ωCgTX GVIA and dihydropyridines, sensitive to high concentrations of ωAga IVa, and sensitive to ωCTX MVIIC is useful. There are two differences between Q-type I_{Ca} previously defined (Zhang et al., 1993; Randall and Tsien, in press) and our present results. The first is that 200 nM ωAga IVa was ineffective at blocking I_{Ca} in our experiments, whereas this concentration of ωAga IVa did block a portion of the I_{Ba} in oocytes expressing rabbit α_{1A} (Sather et al., 1993). This may be a consequence of a charge carrier, species, or expression difference. The second is that 5 μM ωCTX MVIIC blocked only about 50% of the dihydropyridine-insensitive current in A-2 cells. The immunocytochemical detection of class E calcium channels in these cells suggests that at least a portion of the ωCTX MVIIC-insensitive or "R-type" I_{Ca} is carried via these channels. It is also possible that ωCTX MVIIC block had not come to completion after 5 min with our recording conditions. It has been noted that ωCTX MVIIC block is slow in onset compared to other peptide blockers (Sather et al., 1993; Wheeler et al., 1994). Supporting the incomplete block hypothesis is the observation that block was greater (58%, $n = 2$) and WIN 55,212-2 I_{Ca}-inhibition less (4%, $n = 2$) after 10 min of toxin exposure. This latter observation also suggests that cannabinoids modulate "R-type" I_{Ca}, weakly, if at all. Another explanation for incomplete block is that ωCTX MVIIC block is dependent on external divalent concentration, being prevented by 40 mM Ba^{2+} (Sather et al., 1993), and 5 mM Ca^{2+} was used in the current study. In summary, as CB-1 receptors are highly concentrated at presynaptic endings in the CNS, including at some endings that have few N-type calcium channels (Herkenham et al., 1991; Matsuda et al., 1993), cannabinoid-mediated inhibition of Q-type I_{Ca} is a potential mechanism to decrease neurotransmission and other calcium dependent processes at these synapses. Whether this is the basis for some or all of the psychoactive effects of the cannabinoids awaits further experimentation.

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