Cannabinoids and Dopamine Receptors' Action on Calcium Current in Rat Neurons

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ABSTRACT: ***Objective:*** To study the effects of cannabinoid, glutamate, and dopamine agonists and antagonists on the calcium current in rat sympathetic neurons. ***Methods:*** Calcium current was recorded using the whole-cell variant of the patch-clamp technique. After expression in neuronal membranes of the cannabinoid CB1, glutamate mGluR2, or dopamine D1 receptor (by microinjection of the relevant receptor's cDNA into the neuron's nucleus) agonists' and antagonists' effects were observed. ***Results:*** Applications of agonists of the expressed receptor (0.1-10 μM) decreased the calcium current. The calcium current was increased after application of cannabinoid antagonists (AM251 and AM630); these compounds thus act as inverse agonists in this preparation. Glutamate and dopamine antagonists had no effects on the calcium current by themselves. Combined application of cannabinoids and dopamine, but not glutamate, agonists produced a decrement in the calcium current that was bigger than either of the effects seen when one agonist was applied alone. ***Conclusions:*** These results suggest that cannabinoid with dopamine receptors have an interactive inhibitory effect on the calcium current in this preparation, indicating that within the nervous system, receptor interactions may be important in the regulation of ion-channel functions.

RÉSUMÉ: Effet des cannabinoïdes et des récepteurs dopaminergiques sur les courants calciques dans des neurones de rat. ***Objectif:*** Étudier les effets d'agonistes et d'antagonistes de cannabinoïdes, du glutamate et de la dopamine sur le courant calcique dans des neurones sympathiques de rat. ***Méthodes:*** Le courant calcique a été enregistré au moyen de la technique patch-clamp sur cellules intactes. Des effets agonistes et antagonistes ont été observés après expression dans les membranes neuronales du récepteur cannabinoïde de type CB1, du récepteur métabotropique du glutamate mGluR2 ou du récepteurs D1 de la dopamine par micro injection de l'ADNc correspondant au récepteur dans le noyau du neurone. ***Résultats:*** L'application d'agonistes du récepteur exprimé (0,1 à 10 mmol) diminuait le courant calcique. Le courant calcique était augmenté suite à l'application d'antagonistes cannabinoïdes tels l'AM251 et l'AM630. Ces substances agissent donc comme des agonistes inverses dans cette préparation. Les antagonistes du glutamate et de la dopamine n'avaient pas d'effet sur le courant calcique par eux-mêmes. Une application combinée d'agonistes de cannabinoïdes et de dopamine diminuait davantage le courant calcique que l'application de chacun d'eux seul, ce qui n'était pas le cas des agonistes du glutamate. ***Conclusions:*** Ces résultats suggèrent un effet inhibiteur interactif des récepteurs cannabinoïdes et des récepteurs dopaminergiques sur le courant calcique dans cette préparation. Ceci indique que, dans le système nerveux, l'interaction de récepteurs pourrait être importante dans la régulation des fonctions des canaux ioniques.

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The brain CB1 cannabinoid receptor is a member of the G-protein-coupled receptor superfamily.[1] Among the wide variety of effects induced by activation of CB1 cannabinoid receptors are: inhibition of glutamatergic and $GABA_A$ synaptic transmission,[2-4] inhibition of dopamine release in rat brain,[5] and inhibition of serotonin release in mouse brain.[6] Thus, the CB1 cannabinoid receptor modulates neuronal excitability and neurotransmitter release, and thereby regulates Ca^{2+}- and K^+-currents.[7] Activation of CB1 receptors has been shown to inhibit N- and P-/Q-type Ca^{2+} channels in cultured hippocampal neurons and in heterologous expression systems.[8-9] On the other hand, CB1 receptors activate inwardly rectifying K+ channels,[10] and it was recently shown that cannabinoids decrease the K^+ M-current in hippocampal CA1 neurons.[11] Additionally, there is evidence for associations and signaling interactions between cannabinoids and both glutamate and dopamine receptors.[12-16] However, to our knowledge such interactions have not been investigated at the ion-channel level. We now report that combined activation of D1 and CB1 receptors in rat sympathetic neurons results in a greater

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Ca^{2+} current inhibition than that observed on activation of either receptor alone. These data seem to indicate a cooperative action on the Ca^{2+} current, and indicate that within the nervous system, receptor interactions may be important in the regulation of the function of ion channels.

Materials and Methods

Neuron Preparation

Sympathetic neurons were isolated from the superior cervical ganglia (SCG) of adult male Wistar rats of five-six months of age (350-375g). All procedures were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals in Research, and were approved by the Committee on Animal Use for Research and Education at the University of Colima. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data. The ganglia were dissected in cold Hank's balanced salt solution, then incubated in a flask containing modified Earle's balanced salt solution with 0.9 mg/ml collagenase type D, 0.3 mg/ml trypsin (both from Boehringer Mannheim), and 0.1 mg/ml DNase type I (Sigma Company, St. Louis, MO, USA.). This incubation, at 35°C for 1 hour, was conducted in a shaking water-bath. Cells were then dissociated by vigorous shaking of the flask by hand for 10 sec. Cells were washed with, and plated in, Minimum Essential Medium (Gibco, Invitrogen, USA) containing 10% fetal bovine serum (Gibco, Invitrogen, USA), 1% penicillin/streptomycin, and 1% glutamine (both from Sigma) on poly-L-lysine-coated 35 mm culture dishes. Cells were then maintained at 37°C in a humidified atmosphere containing 5% CO_2, and allowed to attach for four-five hours before microinjection.

Molecular biology and microinjection

The cDNAs for the human CB1 cannabinoid receptor (hCB1), the metabotropic glutamate receptor 2 (mGluR2), and the dopamine receptor 1 (D1) were subcloned into the mammalian expression vector pCI (Invitrogen), hCB1 between the *Xba*I and *BamH*I restriction sites, mGluR2 between the *Sal*I and *Not*I restriction sites, and D1 between the *Xba*I and *EcoR*I restriction sites. Preparation of plasmid DNAs was accomplished using a plasmid prep kit (Qiagen).

The nuclei of single SCG neurons were microinjected with pCI containing hCB1, mGluR2, and/or D1 cDNA (either separately or combined, as the experiment required). Plasmids were diluted with Tris/EDTA pH 8 to a final injection concentration of 100 ng/µl. The pEGFP-N1 plasmid (10 ng/µl) containing the cDNA encoding enhanced green fluorescent protein (Clontech, Palo Alto, CA, USA) was used as a co-injection marker. The plasmid solution was centrifuged (16,000Xg) in non-heparinized hematocrit tubes for 20 minutes to remove particles. Injection pipettes were made from fiber-filled capillary glass (1B100F-4; World Precision Instruments, Sarasota, FL, USA) pulled on a P-97 Flaming-Brown micropipette puller (Sutter Instrument Co., Novato, CA, USA). Nuclear microinjection was performed with the aid of an Eppendorf FemtoJet and 5171 micromanipulator (Eppendorf, Madison, WI, USA) using an injection pressure of 90-150 hPa and an injection time of 0.4-0.6 seconds. The actual amount of DNA injected is unknown, but we estimated it to be of the order of 0.1 ng per cell. Successful injections were subsequently confirmed by observing the cells for fluorescence on an inverted microscope (Olympus CK40) equipped with an epifluorescence unit (Figure 1).

Electrophysiological recording of Ca^{2+} current

Ca^{2+} currents from rat SCG neurons were recorded at room temperature (22-24°C) some 16-20 hours after microinjection using the whole-cell variant of the patch-clamp technique[17] with the aid of an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA, USA). Patch electrode pipettes were made by pulling borosilicate glass capillaries (Corning 7052; Garner Glass Co., Claremont, CA, USA) on a P-97 Flaming-Brown micropipette puller (Sutter Instruments). The patch electrodes were coated with Sylgard 184 (Dow Corning Corp., Midland, MI, USA) and fire-polished on a microforge (Narishige, Tokyo, Japan). The cell membrane capacitance and

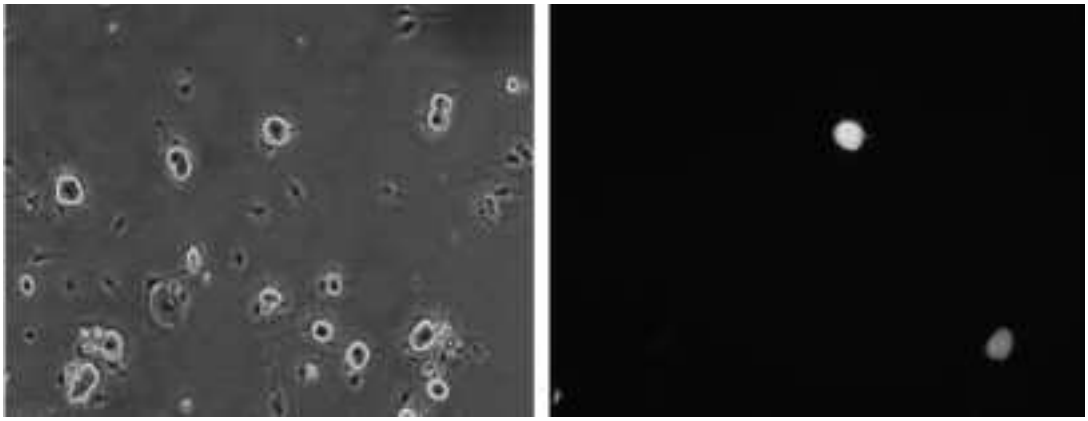

Figure 1: *Microinjected superior cervical ganglion neurons. A) Phase contrast photomicrograph. B) Photomicrograph of the same field under fluorescence optics showing neurons successfully injected. Neurons are approximately 25-30 µm in diameter.*

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series resistance were electronically compensated to >80%. Whole-cell currents were low-pass filtered at 5 kHz (-3dB) using the 4-pole Bessel filter of the clamp amplifier. In addition, we applied suppression of capacitive transients and leak-subtraction protocols.

To isolate Ca^{2+} currents for whole-cell recording, cells were bathed in an external solution containing (in mM): 140 tetraethylammonium methanesulfonate, 10 HEPES, 15 glucose, 10 $CaCl_2$, 0.0001 tetrodotoxin, pH 7.4 (adjusted with methanesulfonic acid). The intracellular solution consisted of (in mM): 120 N-methyl-D-glucamine, 20 tetraethylammonium chloride, 10 HEPES, 11 EGTA, 1 $CaCl_2$, 4 MgATP, 0.1 Na_2GTP, 14 phosphocreatine, pH 7.2 (adjusted with methanesulfonic acid).

Voltage-clamp protocols were generated by a Compaq S500 computer (Pentium III) using the program pClamp 8.0 (Axon Instruments, Foster City, CA, USA). Data were digitized using a DIGIDATA 1200 interface (Axon Instruments, Foster City, CA, USA) and stored on the computer's hard disc. Ca^{2+} currents were elicited by voltage steps from a holding potential of -80 mV and digitized at 180 µsec/point. A pulse-protocol consisting of one 50 ms step to +5 mV was used to elicit Ca^{2+} currents. Figures were generated using Clampfit (Axon Instruments, Foster City, CA, USA) with final preparation in Sigmaplot (SPSS Inc., Chicago, IL, USA).

Drug solutions were applied to isolated cells by bath perfusion. All compounds were diluted to their final concentrations in the external solution (from concentrated stock solution) just before use. Stock solutions (each, 10 mM), were made up as follows: WIN 55,212-2 mesylate, AM251 and AM630 (Tocris Cookson Inc., Ballwin, MO, USA) in dimethylsulfoxide; anandamide (Tocris Cookson Inc., Ballwin, MO, USA) and methanandamide (Research Biochemicals International, Natick, MA, USA) in ethanol; L-CCG-I, DCG-IV, (RS)-APICA, and EGLU in NaOH; dihydrexidine, SKF-38393, and SCH-23390 in water. The final concentrations of dimethylsulfoxide, ethanol, and NaOH were <0.01%; none had any effect on the Ca^{2+}-current. Stock solutions were stored at –20ºC.

Statistical significance was determined by means of a Student's t-test or ANOVA test as indicated, differences being considered significant at $P<0.05$. Results are presented as means ± S.E.M.

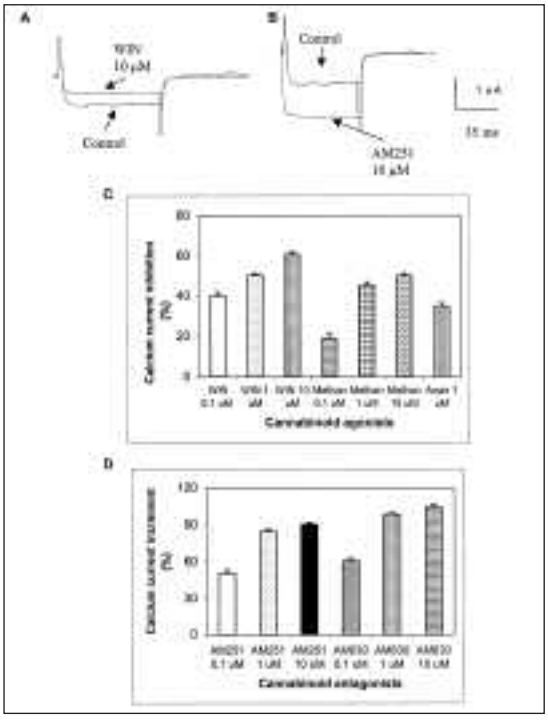

Figure 2: *Effects of cannabinoid agonists and antagonists on Ca^{2+} current in sympathetic neurons. A) Superimposed traces of Ca^{2+} currents recorded from an SCG neuron in the absence (Control) and presence of WIN 55,212-2 (10 µM WIN). Currents were induced by depolarizing voltage pulses from –80 mV to a test potential of +5 mV. B) Superimposed traces of Ca^{2+} currents from the same neuron in the absence (Control) and presence of AM251 (10 µM AM251). C) Bar graph showing the Ca^{2+} current inhibitions induced by the concentrations used of WIN 55,212-2, methanandamide, and anandamide (cannabinoid agonists). D) Bar graph showing the increments in the Ca^{2+} current induced by the concentrations used of AM251 and AM630 (cannabinoid antagonists). All data are from SCG neurons microinjected with human cannabinoid receptor CB1 cDNA.*

Results

Effects of cannabinoid agonists and antagonists

In the present study, the cannabinoid agonist WIN 55,212-2 significantly reduced the calcium current in neurons microinjected with hCB1 cDNA by 60.7±1.2 ($p<0.0001$), 50.2±0.9 ($p<0.0001$), and 40.1±1.7% ($p<0.001$) at concentrations of 10, 1, and 0.1 µM, respectively (n=3 for each concentration) (Figure 2A and 2C). However, it had no effect in uninjected neurons. Anandamide (1 µM) and methanandamide (1 µM), endogenous cannabinoids, inhibited the calcium current too (Figure 2C). AM251, a cannabinoid antagonist, as well as blocking the effect of the WIN 55,212-2 (the corresponding values being 0.3±0.5 (10 µM WIN 55,212-2), 1.3±0.5 (1 µM), and 1.2±0.2% (0.1 µM)), increased the calcium current by 90.4±1.6 ($p<0.0001$), 84.3±1.7 ($p<0.0001$), and 50.4±2.9% ($p<0.001$), at concentrations of 10, 1, and 0.1 µM, respectively, when applied by itself (n=3 for each concentration) (Figure 2B and 2D). Anandamide (0.1-10 mM), methanandamide (0.1-10 µM), and AM251 (0.1-10 µM) had no effects on uninjected neurons (n=3 for each drug). AM630, another cannabinoid antagonist, as well as blocking the effect of WIN 55,212-2 (the corresponding values being 1.1±0.2 (10 mM WIN 55,212-2), 1.0±0.7 (1 µM), and 1.4±0.3% (0.1 µM)), increased the calcium current just as AM251 did, when applied alone (n=3 for each concentration; Figure 2D). These results indicate that the cannabinoid antagonists AM251 and AM630 act as inverse agonists in this preparation, suggesting that some expressed cannabinoid receptors are in a constitutively active state, as previously described in this and other preparations.[18-22] The above effects were all reversible.

Effects of glutamatergic compounds

In the present study, we tested the effects of two agonists of these receptors [L-CCG-I and DCG-IV, type 2 metabotropic

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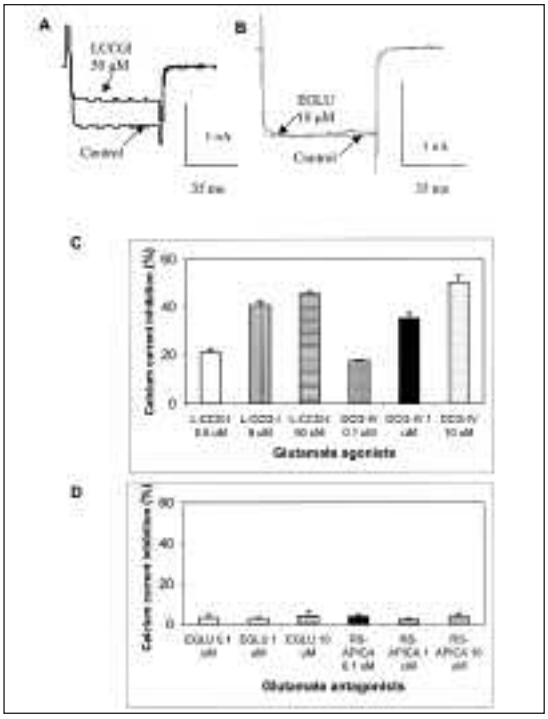

Figure 3: *Effects of glutamate agonists and antagonists on Ca^{2+} current in sympathetic neurons. A) Superimposed traces of Ca^{2+} currents recorded from another SCG neuron (i. e., a different neuron from that in Fig. 1) in the absence (Control) and presence of L-CCG-I (50 µM). Currents were induced by depolarizing voltage pulses from –80 mV to a test potential of +5 mV. B) Superimposed traces of Ca2+ currents recorded from the same neuron as in A) in the absence (Control) and presence of EGLU (10 µM EGLU). C) Bar graph showing the Ca^{2+} current inhibition induced by all concentrations used of L-CCG-I and DCG-IV (glutamate agonists). D) Bar graph showing the effects of EGLU and (RS)-APICA (glutamate antagonists). All data are from neurons microinjected with mGluR2 receptor cDNA.*

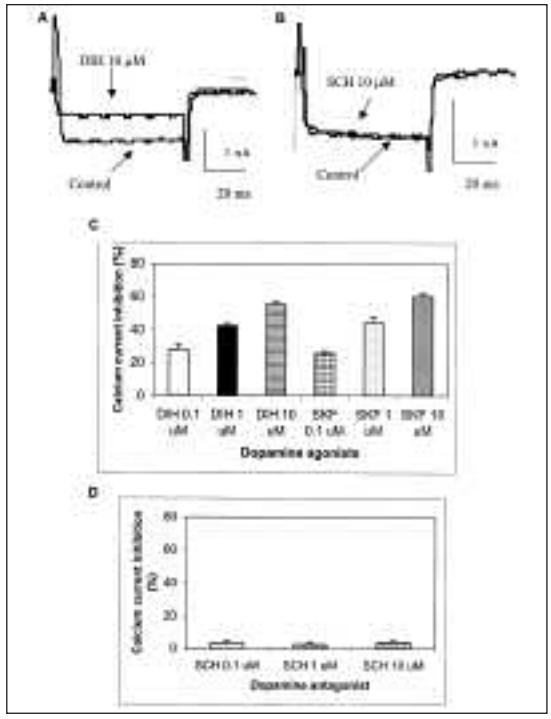

Figure 4: *Effects of dopamine agonists and antagonists on Ca^{2+} current in sympathetic neurons. A) Superimposed traces of Ca^{2+} currents recorded from an SCG neuron in the absence (Control) and presence of dihydrexidine (10 µM DIH). The neuron was depolarized from a holding potential of –80 mV to a test potential of +5 mV. B) Superimposed traces of Ca^{2+} currents recorded from an SCG neuron in the absence (Control) and presence of SCH-23390 (10 µM SCH). Currents were induced by depolarizing voltage pulses from –80 mV to a test potential of +5 mV. C) Summary of the effects of two dopamine agonists, DIH and SKF. D) Bar graph showing the effects of a dopamine antagonist, SCH (all concentrations used) on the Ca^{2+} current. All data are from SCG neurons microinjected with D1 receptor cDNA.*

glutamate receptor (mGluR2) agonists] on the calcium current (Figure 3A and 3C). At 50 µM, L-CCG-I decreased the calcium current by 45.5±0.8% ($p<0.0001$, n=3), at 5 µM by 40.7±1.5% ($p<0.0001$, n=3), and at 0.5 µM by 20.9±1.8% ($p<0.005$, n=3) in microinjected neurons, but it had no effect in uninjected neurons. At 10 µM, DCG-IV decreased the calcium current by 50.4±2.9% ($p<0.001$, n=3), at 1 µM by 35.2±2.2% ($p<0.001$, n=3), and at 0.1 µM by 17.5±0.5% ($p<0.005$, n=3) in microinjected neurons, but it too had no effect in uninjected neurons. EGLU and (RS)-APICA, mGluR2 antagonists, blocked the effects of DCG-IV (the corresponding values being for EGLU 1.6±0.2 (10 µM), 2.1±0.4 (1 µM), and 0.6±0.3% (0.1 µM) and for (RS)-APICA 1.3±0.1 (10 µM), 1.1±0.5 (1 µM), and 1.6±0.7% (0.1 µM)). However, by themselves they had no effect on microinjected neurons at the concentrations used (Figure 3B and, 3D). They too had no effect on uninjected neurons. These results indicate that L-CCG-I and DCG-IV are adequate pharmacologic tools for investigations of the modulation of calcium channels via mGluR2 receptors.

Effects of dopaminergic compounds on Ca^{2+} currents in sympathetic neurons

We tested the effects of dihydrexidine, SKF-38393, and SCH-23390 on Ca^{2+} currents in sympathetic neurons microinjected with D1 receptor cDNA (Figure 4). Dihydrexidine, a dopamine D1 receptor agonist, reduced the calcium current by 55.5±1.3% ($p<0.001$), 42.4±1.2% ($p<0.0001$), and 28.1±2.9% ($p<0.01$) at concentrations of 10, 1, and 0.1 µM, respectively (n=3 for each concentration) (Figure 4A and 4C). SKF-38393, another dopamine D1 receptor agonist, inhibited the calcium current just as dihydrexidine did (n=3 for each concentration, Figure 4C). By itself, the dopamine D1 receptor antagonist SCH-23390 had no effect on the calcium current (Figure 4B and 4C), but it blocked the above agonists´ effects at concentrations within the range 0.1-10 µM (n=3 for each concentration, the corresponding values being 1.4±0.8 (10 µM), 1.6±0.2 (1 µM), and 0.9±0.5% (0.1 µM). The above agents had no effects in uninjected neurons.

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Table 1: Comparison of the effects (percentage reduction in Ca2+ current) of a drug combination with the effects of the same concentrations of individual drugs (cannabinoids and glutamate agonists)

WIN	L-CCG-I	WIN + L-CCG-I	p*
40.1±1.7% (0.1 μM)	20.9±1.8% (0.5 μM)	20.1±1.8% (0.1 + 0.5 μM)	0.21
50.2±0.9% (1 μM)	40.7±1.5% (5 μM)	40.3±2.7% (1 + 5 μM)	0.47
60.7±1.2% (10 μM)	45.5±0.8% (50 μM)	50.2±2.9% (10 + 50 μM)	0.24

*p, ANOVA test

Table 2: Comparison of the effects (percentage reduction in Ca^{2+} current) of a drug combination with the effects of the same concentrations of individual drugs (cannabinoids and glutamate agonists)

METHANANDAMIDE	DCG-IV	METHANANDAMIDE+ DCG-IV	p*
19.0±2.0% (0.1 μM)	17.5±0.5% (0.1 μM)	19.5±2.7% (0.1 + 0.1 μM)	0.65
45.4±1.6% (1 μM)	35.2±2.2% (1 μM)	38.5±2.1% (1 + 1 μM)	0.19
50.2±1.0% (10 μM)	50.4±2.9% (10 μM)	50.1±1.1% (10 + 10 μM)	0.93

*p, ANOVA test

Combined effects of cannabinoids and glutamatergic compounds

To explore the possible interactions between cannabinoid and glutamate receptors, we tested the effects of combined applications of agonists for these receptors. Application of the mGluR2 agonist L-CCG-I (50 μM) plus the CB1 agonist WIN 55,212-2 (10 μM) decreased the calcium current by 50.2±2.9% (p=0.003, n=3) (Figure 5A and 5C), while with 5 μM of L-CCG-I plus 1 μM of WIN 55,212-2 it was decreased by 40.3±2.7% (p<0.005, n=3), and with 0.5 μM of L-CCG-I plus 0.1 μM of WIN 55,212-2 by 20.1±1.8% (p<0.005, n=3). Only those neurons microinjected were affected, not uninjected ones. Application of 0.5 μM L-CCG-I plus 0.1 μM of WIN 55,212-2 tended to have a weaker inhibitory effect than WIN alone; however, a comparison of the three groups revealed no significant difference among them (Table 1, top row). Likewise, combined application of the cannabinoid agonist methanandamide and the glutamate mGluR2 agonist DCG-IV produced no additional effect (Table 2 and Figure 5B and 5C). Again, these effects were seen only in microinjected neurons, not in uninjected ones.

Combined effects of cannabinoids and dopaminergic compounds

To explore the possible interactions between cannabinoid and dopamine receptors, we tested combined applications of agonists for these receptors. Application of the D1 agonist dihydrexidine (10 μM) plus the CB1 agonist WIN 55,212-2 (10 μM) decreased the calcium current by 100.6±0.9% (p<0.0001, n=3) (Figure 6A and 6C), 1 μM of each drug decreased it by 80.5±1.3% (p<0.0005, n=3), and 0.1 μM of each drug by 55.6±1.4% (p<0.0001, n=3). Only those neurons microinjected were affected, not uninjected ones. Such combined applications had an additional effect (Table 3) over and above each of those seen when either drug was applied separately (e.g., compare data in Figures 2C and 4C with that in Figure 6C). Likewise, combined application of the cannabinoid agonist methanandamide and the D1 dopamine agonist SKF-38393 (Table 4) produced an additional effect (compare data in Figures 2C and 4C with that in Figure 6C), again only in microinjected neurons, not in uninjected ones.

Discussion

The main purpose of the present study was to characterize the combined effects of cannabinoids and glutamate- or dopamine-receptor agonists on the Ca^{2+}-current in sympathetic neurons microinjected with the cDNAs for those receptors. We found that cannabinoids, and glutamate and dopamine agonists each inhibited the Ca^{2+} current when applied separately. In this respect, our results confirm similar effects described in neurons expressing these receptors or in heterologous expression systems[8-10], for cannabinoid receptors. In the case of dopamine receptors, Bigornia et al[23] observed that dopamine D2 receptors reduced calcium channel currents. Moreover, in retinal horizontal cells dopamine has been reported reduce T calcium currents,[24] while in adrenal glomerulosa cells the T-type Ca^{2+}

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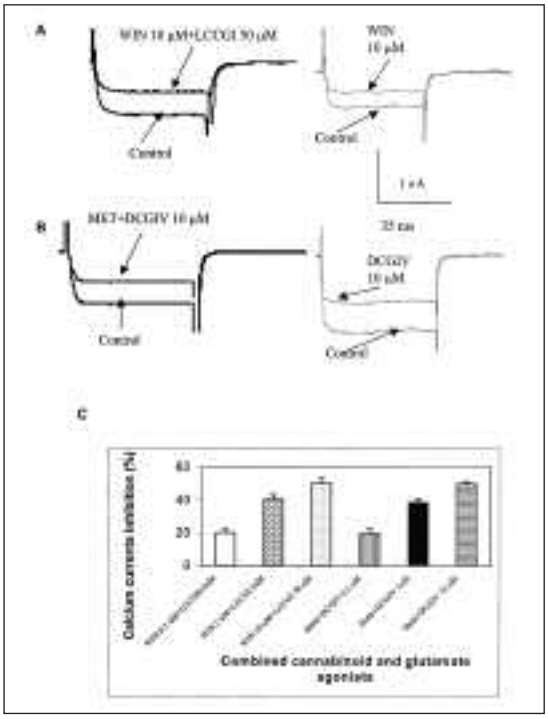

Figure 5: *Combined effects of cannabinoid and glutamate agonists on Ca^{2+} current in sympathetic neurons. A) Left part, superimposed traces of Ca^{2+} currents recorded from an SCG neuron in the absence (Control) and presence of WIN 55,212-2 (10 μM WIN) plus L-CCG-I (50 μM LCCGI). Right part, superimposed traces of Ca^{2+} currents in the presence of a single agonist (WIN 55,212-2 (10 μM WIN)). The neurons were depolarized from a holding potential of –80 mV to a test potential of +5 mV. B) Left part, superimposed traces of Ca^{2+} currents recorded from an SCG neuron in the absence (Control) and presence of methanandamide (10 μM METHAN) plus DCG-IV (10 μM DCGIV). Right part, superimposed traces of Ca^{2+} currents in the presence of a single agonist (DCG-IV (10 μM DCGIV)).Currents were induced by depolarizing voltage pulses from –80 mV to a test potential of +5 mV. C) Summary of the effects on Ca^{2+} currents induced by combined application of all the concentrations used of cannabinoid and glutamate agonists. All data are from SCG neurons microinjected with cDNAs for human cannabinoid CB1 and mGluR2 receptors.*

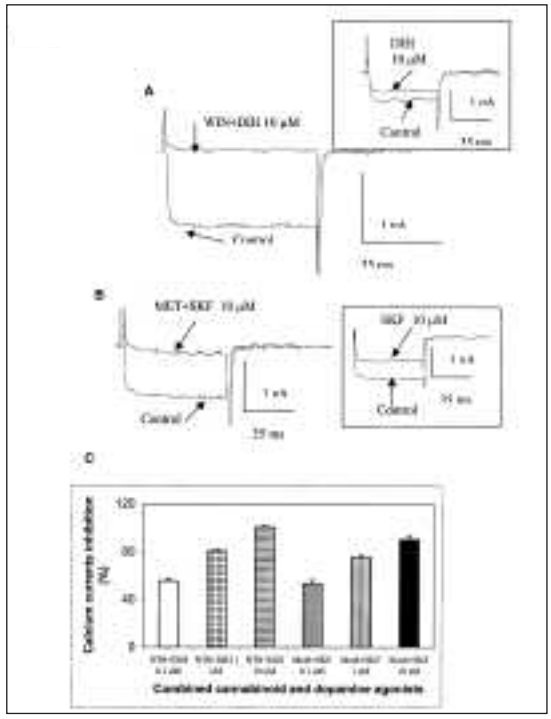

Figure 6: *Combined effects of cannabinoid and dopamine agonists on Ca^{2+} current in sympathetic neurons. A) Superimposed traces of Ca^{2+} currents recorded from an SCG neuron in the absence (Control) and presence of WIN 55,212-2 (10 μM WIN) plus dihydrexidine (10 μM DIH). Inset, superimposed traces of Ca^{2+} currents in the presence of a single agonist (DIH (10 μM DIH)). The neurons were depolarized from a holding potential of –80 mV to a test potential of +5 mV. B) Superimposed traces of Ca^{2+} currents recorded from an SCG neuron in the absence (Control) and presence of methanandamide (10 μM METHAN) plus SKF-38393 (10 μM SKF). Inset, superimposed traces of Ca^{2+} currents in the presence of a single agonist (SKF (10 μM SKF)). Currents were induced by depolarizing voltage pulses from –80 mV to a test potential of +5 mV. C) Summary of the effects on the Ca^{2+} currents induced by combined application of all the concentrations used of cannabinoid and dopamine agonists. All data are from SCG neurons microinjected with cDNAs for human cannabinoid CB1 and D1 receptors.*

current has been found to be blocked by activation of the D2 receptor.[25] In addition, D1 receptor activation inhibits Ca^{2+} currents (N- and P-types) (in striatal neurons[26]), and it has been demonstrated that in rat adrenal glomerulosa cells, D1 inhibition is mediated by the Gs-coupling G protein (through its βγ-subunit) and an increase in the cAMP concentration.[27] On the other hand, glutamate has been found to inhibit the N-type Ca^{2+} current in sympathetic neurons microinjected with the cDNA for the mGluR2 receptor,[28] while group II mGlu receptor agonists inhibit voltage-gated Ca^{2+} channels in the rat cerebellum,[29] and activation of the group II mGlu receptor inhibits voltage-gated Ca^{2+} channels in myenteric neurons.[30] Thus, the cellular mechanism by which CB1, mGluR2, and D1 receptors modulate the Ca^{2+} channels in sympathetic neurons microinjected with the cDNAs for these receptors would appear to be by activation of the pertussis toxin-sensitive G protein pathway involving the Gi family (such as G_i or G_o) and the G_s-coupling G protein, through its βγ-subunit.[8-10,27,30] Inhibition of these Ca^{2+} channels would be expected to decrease the likelihood both of neurotransmitter release and of successful synaptic transmission, and also to suppress other calcium-dependent processes in these cells. An important functional insight would be obtained if we were able to establish the type of Ca^{2+} channels affected by this synergism. This might be achieved using a specific voltage-command protocol and/or a pharmacological solution for each voltage-dependent calcium channel. We suggest that the N-type may be the channel involved in our preparation because: 1) the voltage command (amplitude and duration) used in our experiment is specific for activating N-type Ca^{2+} channels, 2) 80-85% of the whole-cell peak current in these cells is carried through N-type Ca^{2+} channels, and 3) previous reports have indicated that CB1 and D1 receptors inhibit N-or T-type Ca^{2+} channels, and SCG neurons do not have T-type Ca^{2+} channels.[8-10,23-27,31-33]

The behavior of over-expressed receptors not normally

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Table 3: Comparison of the effects (percentage reduction in Ca^{2+} current) of a drug combination with the effects of the same concentrations of individual drugs (cannabinoids and dopamine agonists)

WIN	DIHYDREXIDINE	WIN + DIHYDREXIDINE	p*
40.1±1.7% (0.1 μM)	28.1±2.9% (0.1 μM)	55.6±1.4% (0.1 + 0.1 μM)	<0.005
50.2±1.0% (1 μM)	42.4±1.2% (1 μM)	80.5±1.3% (1 + 1 μM)	<0.001
60.7±1.2% (10 μM)	55.5±1.3% (10 μM)	100.6±0.9% (10 + 10 μM)	<0.0001

*p, ANOVA test

Table 4: Comparison of the effects (percentage reduction in Ca^{2+} current) of a drug combination with the effects of the same concentrations of individual drugs (cannabinoids and dopamine agonists)

METHANANDAMIDE	SKF	METHANANDAMIDE+ SKF	p*
29.0±2.0% (0.1 μM)	25.6±1.4% (0.1 μM)	53.4±2.7% (0.1 + 0.1 μM)	<0.005
45.4±1.6% (1 μM)	44.3±3.4% (1 μM)	75.3±1.3% (1 + 1 μM)	<0.005
53.0±1.5% (10 μM)	60.2±1.9% (10 μM)	90.1±2.4% (10 + 10 μM)	<0.005

*p, ANOVA test

present in the cell-type being examined and the use of a heterologous expression system instead of physiological neuronal/glial environments could be said to be limitations of our observations. However, although the density of receptors expressed in SCG neurons in our study is unknown, Landsman et al[20] reported transfected cells expressing 2.6 pmol/mg protein hCB1 receptors, while *in vivo* the CB1 cannabinoid receptors density is 6.3 pmol/mg protein in the rat substantia nigra and 4.1 pmol/mg in the hippocampal dentate gyrus molecular layer.[34] Thus, there are brain areas with physiologically high expression levels of these receptors. Further, there are brain areas that physiologically co-express dopamine and cannabinoid receptors (striatal neurons, globus pallidus, substantia nigra[14,35,36]) and areas that physiologically co-express glutamate and cannabinoid receptors (cerebral cortex and thermosensitive regions of the hypothalamus[15,37,38]). It remains possible that the expression of receptors in central neurons might differ from those in our experimental conditions to such an extent that cooperativity between receptors occurs either not occur at all or at least not to the same extent.

It has previously been reported that SR141716A, a cannabinoid antagonist, acts as an inverse agonist on hCB1 receptors.[19-22] The authors suggested that cannabinoid receptors are in a constitutively active state. When we tested two other cannabinoid antagonists, AM251 and AM630, each of which has also been demonstrated to act as an inverse agonist,[39,40] we found evidence that they act as inverse agonists in SCG neurons expressing hCB1 cannabinoid receptors (*viz*. when applied by themselves, they altered ionic currents). These effects were the opposite of those induced by the cannabinoid agonists. For an cannabinoid antagonist to have an effect, some receptors must be in an active state. The active state of the receptor could arise through two different mechanisms: 1) activation by an endogenous agonist, or 2) adoption of a spontaneously active state. In the former case, the effect of a cannabinoid antagonist would be that of a classical antagonist, whereas in the latter case the cannabinoid antagonist would be acting as an inverse agonist, as in the present study. To account for the phenomenon of inverse agonism, a two-state receptor model has been proposed. In this model, receptors exist in an equilibrium between inactive (R) and active (R*) states. Agonists stabilize the R* state, inverse agonists stabilize the R state, and antagonists have equal preferences for both states.[41-43] Thus, for an antagonist to be an inverse agonist some receptors must be in the active R* state.[19-22]

In the present study, we found that the responses elicited by co-application of cannabinoid and glutamate agonists were no greater than either of the responses obtained by individual application of one of the agonists. These data indicate that these cannabinoid and glutamate agonists activate a common set of Ca^{2+} channels in SCG neurons microinjected with CB1 and mGluR2 cDNAs, probably because both receptors use the same $G_{i/o}$ proteins.[8-10,28] This absence of an interaction between cannabinoid and glutamate receptors could be important in determining the strength of neurotransmission at synapses at which anandamide and glutamate are co-transmitters. In this

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regard, there are *in vivo and in vitro* findings suggesting an increase in cortical glutamatergic transmission via CB1 receptors,[37] and there is recent evidence to suggest (a) that interactions between NMDA and CB1 receptors lead to synergistic hypothermia,[15] and (b) that there are direct and indirect interactions between the CB1 receptor and the group II metabotropic glutamate receptor in the rat prefrontal cortex.[38] The nature of any functional interaction between cannabinoid and glutamate receptors may also depend on the specific expression of these receptors, as earlier suggested for the subunit composition of nicotinic cholinergic and P2X purine receptors.[44]

The cooperativity we observed in respect of Ca^{2+} current inhibition when cannabinoid and dopamine agonists were applied together indicates interactions at some level in their signaling pathways. Most likely, individual Ca^{2+} channels require the binding of multiple βγ subunits, and individual Ca^{2+} channels share a pool of G-proteins that can be activated by multiple receptors. Another possibility is that the two receptors use different G proteins (cannabinoid receptors $G_{i/o}$, dopamine receptors G_s),[8-10,45] even though it has been reported that cannabinoid receptors can couple to both G proteins.[14] Whatever its molecular basis, this cooperativity may be significant physiologically, the basal extracellular levels of these transmitters within some nervous system regions perhaps being high enough to produce Ca^{2+} current inhibition in neurons in those regions. Such effects would be enhanced by cooperativity among the actions of multiple transmitters. This raises the possibility of interactions between hormones and neurotransmitters coupled to G-protein receptors in the control of neurotransmitter secretion by neurons. In this regard, there is interesting experimental evidence of interactions between cannabinoid and dopaminergic systems: (a) a predominant role for dopamine D1 receptors in the regulation of the cataleptic response to cannabinoids has been found;[46] (b) a concurrent stimulation of cannabinoid CB1 and dopamine D2 receptors has been observed to augment cAMP accumulation in striatal neurons;[14] (c) D2 receptors may have a significant modulatory role in determining the G protein coupling specificity of CB1 receptors;[47] and (d) interactions have been described between cannabinoids and the dopaminergic system with effects on rotational behavior in rats.[35,36,48] Additionally, there are results suggesting that functional interactions between endocannabinoid and dopaminergic systems may contribute to striatal signaling.[49] This cooperativity indicates that there is some type of interaction, most likely via release of endogenous ligands, on application of such drug combinations.[50] Further study will be required to confirm or deny this. The micromolar concentrations of ligands that are needed to show cooperative activity raise questions about the specificity of these drugs. However, those are the concentrations used in all previous reports of these various receptors, and at these micromolar concentrations glutamate agonists induced no detectable effects. On the other hand, non additive effects have been observed with high concentrations of cannabinoid and dopamine agonists.[51] Additionally, a recent report that utilized concurrent activation of dopamine and cannabinoid receptors employed concentrations in the micromolar range.[45]

To the best of our knowledge, this is the first reported example of an interaction between cannabinoid and dopamine receptors on ionic currents, specifically a Ca^{2+} current, indicating that receptor interactions within the nervous system may be important events in the regulation of the functions of ionic channels.

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