Effect of (-)-cis-3-[2-Hydroxy-4-(1,1-dimethyl-heptyl)phenyl]-trans-4-(3-hydroxypropyl) cyclohexanol (CP55,940) on Intracellular Ca^{2+} Levels in Human Osteosarcoma Cells

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ABSTRACT

The study was undertaken to explore the effect of CP55,940 ((-)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol), a drug commonly used as a CB1/CB2 cannabinoid receptor agonist, on intracellular free Ca^{2+} levels ($[Ca^{2+}]_i$) in MG63 human osteoblast-like epithelial cells. $[Ca^{2+}]_i$ was measured in suspended cells by using the fluorescent dye fura-2 as an indicator. At concentrations between 2-20 μM, CP55,940 increased $[Ca^{2+}]_i$ in a concentration-dependent manner with an EC_{50} of 8 μM. The $[Ca^{2+}]_i$ signal comprised an initial rise, a slow decay, and a sustained phase. CP55940 (10 μM)-induced $[Ca^{2+}]_i$ signal was not altered by 5 μM of two cannabinoid receptor antagonists (AM-251, N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; AM-281, 1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-m3thyl-N-4-morpholinyl-1H-pyrazole-3-carboxamide). Extracellular Ca^{2+} removal decreased the maximum value of the Ca^{2+} signals by 50%. CP55,940 induced quench of fura-2 fluorescence by Mn^{2+} (50 μM), suggesting the presence of Ca^{2+} influx across the plasma membrane. CP55,940 (10 μM)-induced $[Ca^{2+}]_i$ increase in Ca^{2+}-free medium was inhibited by 84% by pretreatment with 1 μM thapsigargin, an endoplasmic reticulum Ca^{2+} pump inhibitor. Conversely, pretreatment with 10 μM CP55,940 in Ca^{2+}-free medium abolished thapsigargin-induced $[Ca^{2+}]_i$ increase. At 1 μM, nifedipine, verapamil, and diltiazem did not alter CP55, 940 (10 μM)-induced $[Ca^{2+}]_i$ increase. CP55,940 (20 μM)-induced Ca^{2+} release was not affected when phospholipase C was inhibited by 2 μM U73122 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino) hexyl)-1H-pyrrole-2,5-dione). CP55,940 (20 μM) did not induce acute cell death after incubation for 30 min as assayed by trypan blue exclusion. Collectively, CP55,940 induced significant $[Ca^{2+}]_i$ increases in osteoblasts by releasing store Ca^{2+} from thapsigargin-sensitive stores and by causing Ca^{2+} entry. The CP55,940's action appears to be independent of stimulation of CB1 cannabinoid receptors.

Key Words: CP55,940, $[Ca^{2+}]_i$, MG63 cells, osteoblast, thapsigargin

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Introduction

CP55,940 is a synthesized compound designed for selectively stimulating cannabinoid receptors (17), and has proven valuable for identifying receptor-mediated cannabinoid action in systems including several neuronal origins (6, 21, 24-26) and ileum (19). However, recently it has been shown that CP55, 940 induced an increase in cytoplasmic free Ca^{2+} levels ($[Ca^{2+}]_i$) in a canine renal tubular cell line in a manner dissociated from stimulation of CB1 receptors (11). An increase in $[Ca^{2+}]_i$ plays a pivotal role in initiating and regulating many pathophysiological cellular events (2, 3, 5). A $[Ca^{2+}]_i$ increase may occur via extracellular Ca2+ influx and/or store Ca^{2+} release from the endoplasmic reticulum and other compartments. Depletion of Ca^{2+} stores may cause Ca^{2+}-refilling by store-operated Ca^{2+} entry (22). Thus CP55,940 may affect cell functions via elevating $[Ca^{2+}]_i$ in a manner independent of stimulation of cannabinoid receptors.

This study examined whether the Ca^{2+}-elevating action of CP55,940 could be observed in osteoblasts. The fluorescent Ca^{2+}-sensitive dye fura-2 was used to measure $[Ca^{2+}]_i$ changes in suspended cells. MG63 human osteoblast-like cancer cells were used. MG63 cells have been used as a model of osteoblasts because they express many characteristics similar to that of normal osteoblasts. Several compounds have been shown to increase $[Ca^{2+}]_i$ in MG63 cells (7, 8, 15, 16). In this study, it has been found that CP55,940 induced marked $[Ca^{2+}]_i$ increases in MG63 cells. The concentration-response relationships of the Ca^{2+} signal in the presence and absence of extracellular Ca^{2+} were established, and the sources of the $[Ca^{2+}]_i$ response and possible underlying mechanisms were evaluated.

Materials and Methods

Cell Culture

MG63 cells obtained from American Type Culture Collection were cultured in modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum at 37°C in 5% CO_2-containing humidified air.

Solutions

Ca^{2+}-containing medium (pH 7.4) contained 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM Hepes, and 5 mM glucose. In Ca^{2+}-free medium, Ca^{2+} was substituted with 1 mM EGTA. The experimental solution contained less than 0.1% of solvent (dimethyl sulfoxide or ethanol) which did not affect $[Ca^{2+}]_i$ (n=3).

$[Ca^{2+}]_i$ Measurements

Trypsinized cells (10^6/ml) were loaded with fura-2/acetoxymethyl (2 μM) for 30 min at 25°C in Ca^{2+}-containing medium. Cells were washed and resuspended in Ca^{2+}-containing medium before use. Fura-2 fluorescence measurements were performed in a water-jacketed cuvette (25°C) with continuous stirring; the cuvette contained 1 ml of medium and 0.5 million cells. Fluorescence was monitored with a Shimadzu RF1503PC spectrofluorophotometer (Schimadzu Corp., Kyoto, Japan) by recording excitation signals at 340 nm and 380 nm and emission signal at 510 nm at 1-s intervals. Maximum and minimum fluorescence values were obtained by adding 10 μM digitonin (plus 5 mM $CaCl_2$) and 10 mM EGTA sequentially at the end of each experiment. $[Ca^{2+}]_i$ was calculated as described previously (7-11, 15, 16, 18) assuming a K_d of 155 nM (13).

Mn^{2+} quench of fura-2 fluorescence was performed in Ca^{2+}-containing medium containing 50 μM $MnCl_2$, by recording the excitation signals at 340, 360, and 380 nm and emission signal at 510 nm at 1-s intervals.

Viability Assay

Fifty μl of cell suspension was mixed with 50 μl of trypan blue isotonic solution (0.2%; w/v) and cell viability was determined on a hemocytometer under a microscope. The cell density in the assay solution was 0.5 million/ml.

Chemicals

The reagents for cell culture were from Gibco. Fura-2/acetoxymethyl (1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methylphenoxy)-ethane-N,N,N,N-tetraacetic acid pentaacetoxymethyl ester) was from Molecular Probes. CP55,940 ((-)-cis-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl) cyclohexanol) and AM-281 (1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-m3thyl-N-4-morpholinyl-1H-pyrazole-3-carboxamide) were from Tocris Cookson Ltd. (Bristol, UK). U73122 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione), U73343 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-2, 5-pyrrolidine-dione), and AM-251 (N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide) were from Biomol (Plymouth Meeting, PA, USA).

All other reagents were from Sigma-Aldrich Co. (St. Louis, MO, USA). Statistics All values were reported as meansSEM of 3-5 replicates. Statistical

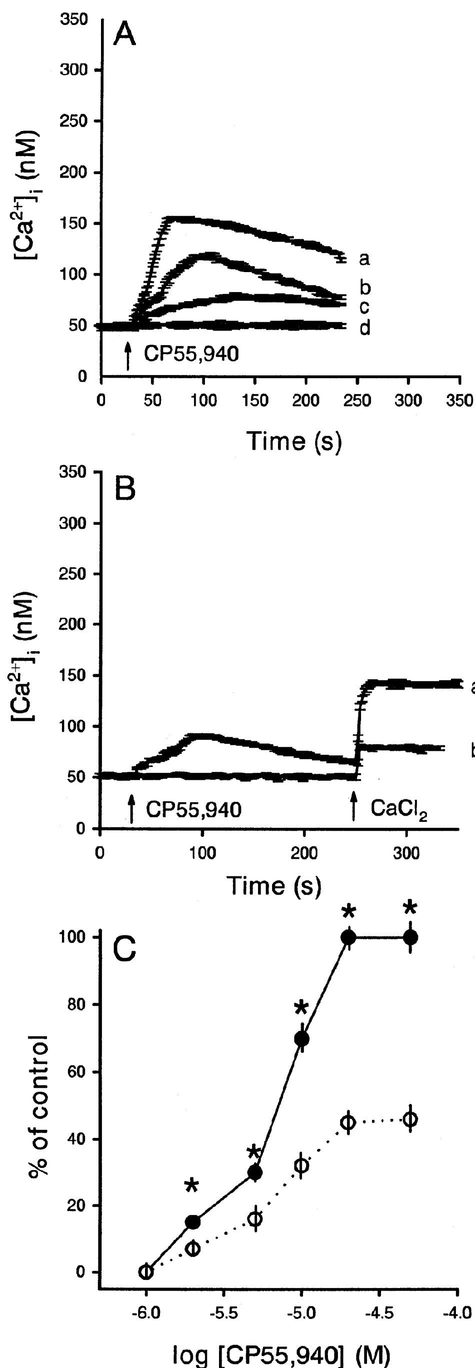

Fig. 1. Effect of CP55,940 on $[Ca^{2+}]_i$ in MG63 cells. (A) $[Ca^{2+}]_i$ increases induced by CP55,940 in Ca^{2+}-containing medium. The concentration of CP55,940 was 20 µM in trace a, 10 µM in trace b, 5 µM in trace c, and 1 µM in trace d. Baseline (no drug was added) was identical to trace d. (B) Effect of removing extracellular Ca^{2+} on CP55,940-induced $[Ca^{2+}]_i$ increase and the effect of reintroduction of extracellular Ca^{2+}. Trace a: CP55,940 (20 µM) was added at 30 s followed by 3 mM $CaCl_2$ added at 250 s. Trace b: Ca^{2+} was added without CP55,940 pretreatment. The experiments were performed in Ca^{2+}-free medium. (C) Concentration-response plots of CP55,940-induced Ca^{2+} signals in Ca^{2+}-containing medium (filled circles) and Ca^{2+}-free medium (open circles). Y axis is the percentage of control which is defined as 20 µM CP55940-induced net (baseline subtracted) maximum $[Ca^{2+}]_i$ value. Data were means±SEM of 3-5 replicates. *P<0.05.

comparisons were determined by using Student's t test, and significance was accepted when P<0.05.

Results

In Ca^{2+}-containing medium, the basal $[Ca^{2+}]_i$ was 50±3 nM (n=5). At concentrations between 2-20 µM, CP55,940 increased $[Ca^{2+}]_i$ in a concentration-dependent manner. Figure 1A shows the responses induced by 20 µM (trace a), 10 µM (trace b) and 5 µM (trace c) of CP55,940. At a concentration of 1 µM, CP55,940 had no effect (trace d). The Ca^{2+} signal saturated at 20 µM of CP55,940 because 50 µM of the drug did not induce a greater response. Over a time period of 250 s the $[Ca^{2+}]_i$ increase consisted of an initial rise and an elevated phase. At a concentration of 20 µM (trace a), CP55,940 induced an immediate, gradual increase in $[Ca^{2+}]_i$ which reached a net (baseline subtracted) maximum value of 102±3 nM (n=5). This Ca^{2+} signal was followed by a gradual decay which reached a net $[Ca^{2+}]_i$ of 75±2 nM at the time point of 250 s. The rise of the Ca^{2+} signal was slower in response to lower concentrations of CP55, 940.

Removing extracellular Ca^{2+} significantly reduced the Ca^{2+} signals induced by CP55,940. Figure 1B (trace a) shows 20 µM CP55940-induced response (time points of 30-250 s). The response had a net maximum value of 51±3 nM (n=5) and a net $[Ca^{2+}]_i$ of 15±2 nM at the time point of 250 s. The concentration-response plots obtained both in Ca^{2+}-containing medium (solid circles) and Ca^{2+}-free medium (open circles) are shown in Figure 1C. The data indicate an EC_{50} value of about 8 µM.

The role of store-operated Ca^{2+} entry in CP55, 940-induced Ca^{2+} influx was explored. Store-operated Ca^{2+} entry was measured by adding 3 mM Ca^{2+} to cells pretreated with CP55,940 in Ca^{2+}-free medium. Figure 1B (trace a; time points of 250-350 s) shows that after depleting Ca^{2+} stores for 220 s with 20 µM CP55,940, addition of 3 mM Ca^{2+} at the time point of 250 s induced an immediate increase in $[Ca^{2+}]_i$ with a net maximum value of 70±2 nM (n=5) which was 2.90.1-fold of control response (P<0.05) (adding Ca^{2+} without CP55,940 treatment; 24±2 nM; trace b).

These experiments were performed to confirm that CP55,940 really induced Ca^{2+} influx and the smaller CP55,940-induced response seen in Ca^{2+}-free medium was not due to EGTA-induced store Ca^{2+} depletion. Mn^{2+} enters cells through similar pathways as Ca^{2+} but quenches fura-2 fluorescence at all excitation wavelengths (20). Thus, quench of fura-2 fluorescence excited at the Ca^{2+}-insensitive excitation wavelength of 360 nm by Mn^{2+} indicates Ca^{2+} influx. Figure 2 shows that 10 µM CP55,940 induced an immediate and gradual decrease in the 360 nm

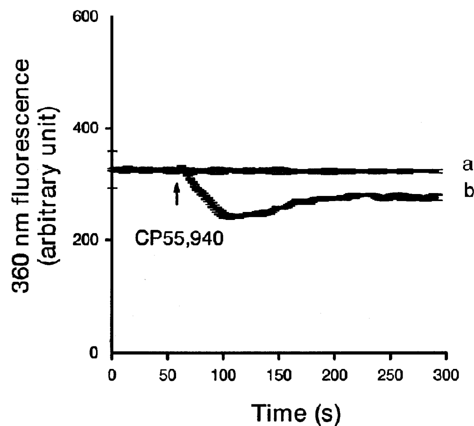

Fig. 2. Effect of CP55,940 on Ca^{2+} influx by measuring Mn^{2+} quench of fura-2 fluorescence. Experiments were performed in Ca^{2+}-containing medium. $MnCl_2$ (50 μM) was added to cells before fluorescence measurements. Trace a: control (in the absence of CP55,940). Trace b: CP55,940 (10 μM) was added at time indicated by the arrow. Data were mean±SEM of three replicates.

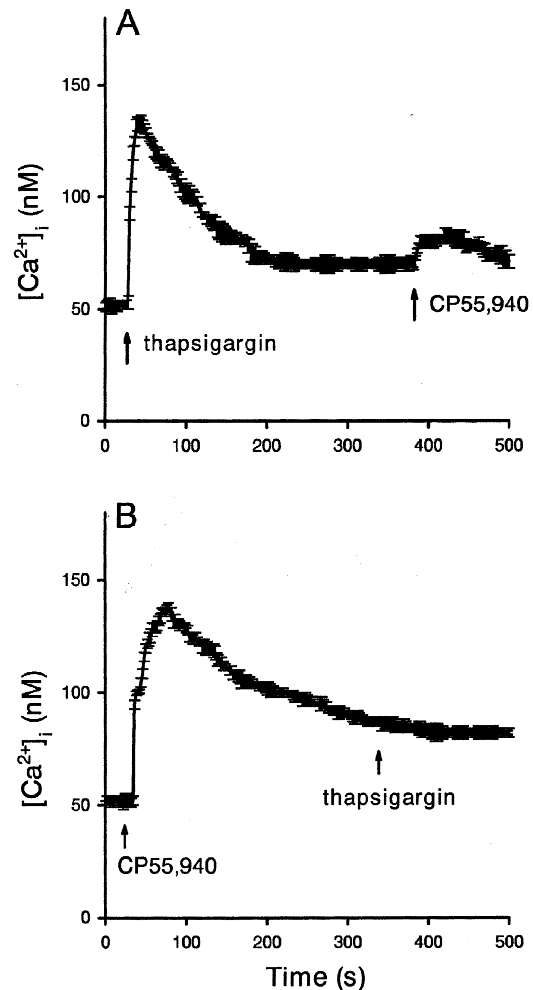

Fig. 3. Intracellular Ca^{2+} stores of CP55,940-induced $[Ca^{2+}]_i$ increase. The experiments were performed in Ca^{2+}-free medium. (A) 1 μM thapsigargin was added at 30 s followed by 20 μM CP55,940 added at 390 s. (B) 20 μM CP55,940 was added at 30 s followed by 1 ,M thapsigargin added at 350 s. Data were means±SEM of 3-5 replicates.

excitation signal leading to a net value of 101±2 (n=6) arbitrary units within 45±2 s (trace b), which was significantly lower than control (trace a, no CP55,940 was added) (P<0.05). Subsequently, the CP55,940-induced Mn^{2+} influx gradually attenuated and returned toward baseline. The CP55,940-induced Mn^{2+} quench of fura-2 fluorescence was abolished by La^{3+} (50 μM), but was unchanged by 1 μM of nifedipine, nimodipine, nicardipine, verapamil and diltiazem (n=4; not shown).

In Ca^{2+}-containing medium, pretreatment with 5 μM AM-251 or AM-281 to inhibit cannabinoid receptors did not alter 10 μM CP55,940-induced $[Ca^{2+}]_i$ increase (n=4; P>0.05; not shown).

We have previously reported that the endoplasmic reticulum plays a dominant role in store Ca^{2+} release in MG63 cells (7, 8, 15, 16). Figure 3A shows that in Ca^{2+}-free medium, addition of 1 μM thapsigargin (an inhibitor of the endoplasmic reticulum Ca^{2+} pump) (27) induced a marked $[Ca^{2+}]_i$ transient with a net maximum value of 84±3 nM (n=4), suggesting a large portion of the endoplasmic reticulum Ca^{2+} store had been depleted. Subsequently added CP55,940 (20 μM) induced a small $[Ca^{2+}]_i$ increase with a net maximum value of 15±2 nM (n=6) which was smaller than control CP55,940 response (Figure 3B; 912 nM; n=5) by 84±2% (P<0.05). Also shown in Figure 3B was that after CP55,940 pretreatment for 5 min, addition of 1 μM thapsigargin failed to induce a $[Ca^{2+}]_i$ increase (n=5; P>0.05).

Figure 4A shows that in Ca^{2+}-free medium, 10 μM ATP, a phospholipase C-dependent Ca^{2+} mobilizer, induced a transient $[Ca^{2+}]_i$ increase with a net peak value of 75±2 nM (n=4). This suggests the presence of a phospholipase C-dependent Ca^{2+} mobilizing machinery in MG63 cells. Figure 3B shows that incubation with 2 μM U73122 (a phospholipase C inhibitor) (28) to inhibit phospholipase C did not alter basal $[Ca^{2+}]_i$ but abolished 10 μM ATP-induced $[Ca^{2+}]_i$ increases (n=5). U73343 (10 μM), an inactive U73122 analogue (Thompson et al. 1991), did not alter basal $[Ca^{2+}]_i$ or ATP-induced $[Ca^{2+}]_i$ increase (n=4; not shown). This indicates that U73122 effectively inhibited phospholipase C. After U73122 pretreatment for 250 s, addition of 20 μM CP55,940 induced a $[Ca^{2+}]_i$ increase with a magnitude comparable to the control shown in Figure 3B (n=5; P>0.05).

Because a prolonged increase in $[Ca^{2+}]_i$ may result in cell death (2, 3, 5), trypan blue exclusion

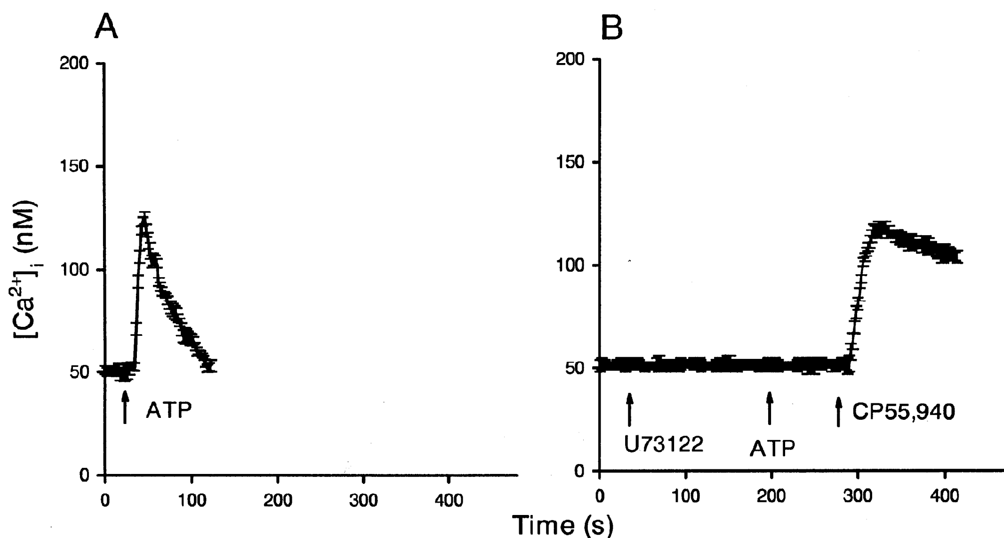

Fig. 4. Effect of inhibition of phospholipase C activity on CP55,940-induced $[Ca^{2+}]_i$ increase. The experiments were performed in Ca^{2+}-free medium. (A) 10 µM ATP was added at 20 s. (B) The phospholipase C inhibitor U73122 (2 µM) was added at 30 s followed by 10 µM ATP and 20 µM CP55, 940 added at 200 s and 280 s, respectively. Data were means±SEM of 3-5 replicates.

assays were performed to explore whether CP55,940 induce acute cell death. It was found that incubation with 20 µM CP55,940 for 30 min did not alter cell viability (n=3; P>0.05; not shown). The control viability was 98±1 % (n=3).

Discussion

Many researchers have used CP55,940 as a selective agonist of CB1/CB2 cannabinoid receptor agonist, and its possible other effects have been ignored until a recent report showing that CP55,940 can induce a significant $[Ca^{2+}]_i$ increase in a canine kidney tubular cell line (10, 11). Given the fact that a $[Ca^{2+}]_i$ increase in any cell type can alter diverse cellular functions, it is important to explore whether CP55,940 also increases $[Ca^{2+}]_i$ in other cell types. The present study has examined the effect of CP55, 940 on $[Ca^{2+}]_i$ in a human osteoblast-like cell line and has evaluated the underlying mechanisms. It was found that CP55,940 induced a concentration-dependent increase in $[Ca^{2+}]_i$ in MG63 osteosarcoma cells starting at 2 µM with an EC_{50} of 8 µM. The Ca^{2+} signal consisted of an initial increase and a sustained phase during the measurement of 4 min, suggesting that CP55,940 caused a prolonged $[Ca^{2+}]_i$ increase. Extracellular Ca^{2+} removal inhibited CP55,940-induced $[Ca^{2+}]_i$ increase by half, indicating comparable contribution of Ca^{2+} entry and Ca^{2+} release to CP55, 940-inudced $[Ca^{2+}]_i$ increase.

CP55,940 is thought to exert its cellular effect mainly by stimulating CB1 cannabinoid receptors (12, 14). However, our findings suggest that CB1 cannabinoid receptors do not participate in CP55, 940-induced Ca^{2+} signal because the CP55,940-induced Ca^{2+} signal was not affected by a rather high concentration (5 µM) of AM-251 and AM-281 (two CB1 cannabinoid receptor antagonists). AM-251 and AM-281 have been shown to block CB1 receptors at 1 µM and 0.5 µM, respectively (1, 23).

The intracellular Ca^{2+} store for CP55,940-induced $[Ca^{2+}]_i$ increases is mainly the thapsigargin-sensitive endoplasmic reticulum pool because depleting the endoplasmic reticulum Ca^{2+} pool with thapsigargin inhibited 84% of 20 µM CP55,940-induced Ca^{2+} release, and consistently, pretreatment with CP55,940 abolished thapsigargin-induced Ca^{2+} release. Consistently, the endoplasmic reticulum Ca^{2+} store has been shown to be the primary Ca^{2+} store in MG63 cells in previous studies (7, 8, 15, 16). Many Ca^{2+} mobilizers can release Ca^{2+} from the endoplasmic reticulum by phospholipase C-dependent formation of inositol 1,4,5-trisphosphate (2, 3). CP55,940 does not appear to cause phospholipase C-dependent Ca^{2+} release because when the activity of phospholipase C was effectively inhibited by U73122, CP55,940 still caused a normal Ca^{2+} release. How CP55,940 releases Ca^{2+} awaits further investigation.

CP55,940-induced Ca^{2+} influx may involve store-operated Ca^{2+} entry, a Ca^{2+}-refilling process initiated by depletion of Ca^{2+} store (22), because reintroduction of extracellular Ca^{2+} to cells that were depleted of Ca^{2+} by CP55,940 induced a $[Ca^{2+}]_i$ increase. However, CP55,940 may produce similar effects by opening some plasmalemmal Ca^{2+} channels independently of store Ca^{2+} depletion. These two

possibilities were difficult to distinguish due to the lack of selective blockers for store-operated Ca^{2+} entry (Birnbaumer et al. 2000). One characteristic of the CP55,940-induced Ca^{2+} influx is its insensitivity to voltage-gated Ca^{2+} channel blockers nifedipine, verapamil and diltiazem.

Together, this study shows that CP55,940 at a concentration above 2 μM induced significant $[Ca^{2+}]_i$ increases in osteoblasts without causing acute cell death. CP55,940 acts by releasing store Ca^{2+} from the endoplasmic reticulum in a phospholipase C-independent manner, and by inducing Ca^{2+} entry. Furthermore, CP55,940 may increase $[Ca^{2+}]_i$ via pathways dissociated from stimulation of CB1 cannabinoid receptors.

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