Inhibitory Signaling by CB₁ Receptors in Smooth Muscle Mediated by GRK5/β-Arrestin Activation of ERK1/2 and Src Kinase

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ABSTRACT

We examined whether CB₁ receptors in smooth muscle conform to the signaling pattern observed with other G₁-coupled receptors that stimulate contraction via two Gβγ-dependent pathways (PLC-β3 and PI 3-kinase/integrin-linked kinase). Here we show that the anticipated Gβγ-dependent signaling was abrogated. Except for inhibition of adenyl cyclase via Gαi, signaling resulted from Gβγ-independent phosphorylation of CB₁ receptors by GRK5, recruitment of β-arrestin1/2, and activation of ERK1/2 and Src kinase. Neither uncoupling of CB₁ receptors from G₁ by PTx or G₁ minigene, nor expression of a Gβγ-scavenging peptide had any effect on ERK1/2 activity. The latter was abolished in muscle cells expressing β-arrestin1/2 siRNA. CB₁ receptor internalization and both ERK1/2 and Src kinase activities were abolished in cells expressing kinase-deficient GRK5(K215R). Activation of ERK1/2 and Src kinase endowed CB₁ receptors with the ability to inhibit concurrent contractile activity. We identified a consensus sequence (¹⁰²K⁹S⁹P⁹K⁹L⁹S¹⁰⁹) for phosphorylation of RGS4 by ERK1/2 and showed that expression of a RGS4 mutant lacking Ser¹⁰³/Ser¹⁰⁸ blocked the ability of anandamide to inhibit acetylcholine-mediated PI hydrolysis or enhance Gαq:RGS4 association and inactivation of Gαq. Activation of Src kinase by anandamide enhanced both M-RIP:RhoA and M-RIP:MYPT1 association and inhibited Rho kinase activity leading to increase of MLC phosphatase activity and inhibition of sustained muscle contraction. Thus, unlike other G₁-coupled receptors in smooth muscle, CB₁ receptors did not engage Gβγ but signaled via GRK5/β-arrestin activation of ERK1/2 and Src kinase: ERK1/2 accelerated inactivation of Gαq by RGS4, and Src kinase enhanced MLC phosphatase activity leading to inhibition of ACh-stimulated contraction.
INTRODUCTION

The properties and role of G protein-coupled cannabinoid receptors, CB1 and CB2, have been extensively studied in their primary locations, the central and peripheral nervous system and the immune system, respectively (3, 8, 22, 32). The location of CB1 receptors in the brain (cerebral cortex, hippocampus, basal ganglia, amygdala, and cerebellum) correlates with the observed psychotropic effects of cannabinoids. It is increasingly evident, however, that CB1 receptors and the endocannabinoid system that provides them with lipid ligands on demand are more widely distributed, though less abundantly than in neural tissue. Thus, CB1 receptors are expressed in both vascular and visceral smooth muscle (e.g., cerebral arterial smooth muscle (7), vas deferens (5), and myometrial smooth muscle (2)), where they signal variously via G\textsubscript{\alpha_i}-dependent inhibition of adenylyl cyclase and G\beta\gamma-dependent inhibition of voltage-gated L-type Ca\textsuperscript{2+} channels, and activation of various kinases (phosphatidylinositol 3-kinase (PI 3-kinase), extracellular signal-regulated kinase (ERK1/2), and Src kinase).

In the present study, we examined signaling by G\textsubscript{i}-coupled CB1 receptors in gastric smooth muscle cells, a model system used extensively to examine signaling by G protein-coupled receptors (16, 20). Earlier studies had shown that receptors coupled to G\textsubscript{i1} (e.g., opioid \mu, \delta, \kappa) (19), G\textsubscript{i2} (e.g., somatostatin sstr3) (17), or G\textsubscript{i3} (e.g., adenosine A\textsubscript{i}) (18) induce contraction by activating dual pathways initiated by G\beta\gamma: the first involves activation of PLC-\beta3, stimulation of IP\textsubscript{3}-dependent Ca\textsuperscript{2+} release, and phosphorylation of myosin light chain 20 (MLC\textsubscript{20}) by Ca\textsuperscript{2+}/calmodulin-dependent MLC kinase, resulting in a transient initial muscle contraction. The second pathway involves sequential activation of PI 3-kinase and integrin-linked kinase: the latter acts as a Ca\textsuperscript{2+}-
independent MLC kinase and inhibits MLC phosphatase by activating CPI-17 (PKC-
potentiated inhibitor 17-kDa protein), an endogenous inhibitor of the catalytic PP1cδ
subunit of MLC phosphatase (10). The dual effects of integrin-linked kinase result in
sustained MLC₂₀ phosphorylation and muscle contraction. We wondered whether CB₁
receptors would conform to the stimulatory pattern of signaling observed with other Gi-
coupled receptors or trigger pathways that attenuate smooth muscle contraction,
consistent with the inhibitory effect of CB₁ receptors in other locations. Here we show
that the expected activation of Gβγ-dependent pathways that lead to stimulation of
contraction was abrogated. Except for inhibition of adenylyl cyclase via Gαₐ, signaling
resulted from Gβγ-independent phosphorylation of CB₁ receptors by GRK5, binding of
β-arrestin to the phosphorylated receptors, and activation of ERK1/2 and Src kinase (26).
Both ERK1/2 and Src kinase inhibited concurrent contraction stimulated by acetylcholine
(ACh) by targeting specific steps in the pathways that mediate contraction: ERK1/2 via
phosphorylation of RGS4 (regulator of G protein signaling) and rapid inactivation of
Gαq.GTP, and Src kinase via enhanced association of RhoA and MYPT1 (myosin
phosphatase targeting subunit) with M-RIP (myosin phosphatase RhoA-interacting
protein) leading to increase in MLC phosphatase activity.

MATERIALS AND METHODS

Reagents. N-arachidonylethanolamine (anandamide; AEA) was obtained from Sigma, St.
Louis, MO; AM251, N⁶-Cyclopentyladenosine and [D-Pen²,D-Pen⁵]enkephalin (DPDPE)
was obtained from Tocris, Ellisville, MO; [¹²⁵I]cAMP, [γ⁻³²P]ATP, [³⁵S]GTPγS,
CP55940 and [³H]myo-inositol were from PerkinElmer Life Sciences, Boston, MA;
Collagenase CLS type II and soybean trypsin inhibitor were from Worthington, Freehold, NJ; Western blotting, Dowex AG-1 X 8 resin (100-200 mesh in formate form), chromatography material and protein assay kit, 10% Tris-HCl Ready Gels were from Bio-Rad Laboratories, Hercules, CA; antibodies to CB1 receptor (SC-10066), Gaq, Gas1, Gas2, Gas3, Gai12, Gai13, Gas, RGS4, MYPT1, M-RIP, RhoA, Src kinase, Rho kinase, ERK1/2 were from Santa Cruz biotechnology, Santa Cruz, CA and antibody to CB2 receptor (AB5640P) from Chemicon, Billerica, MA; myelin basic protein (MBP) was from Upstate Biotechnology; Y27632, PP2, PD98049, pertussis toxin, forskolin, cAMP were from Calbiochem, La Jolla, CA; RNAqueous™ kit was obtained from Ambion, Austin, TX; Effectene Transfection Reagent, QIAEX®II Gel extraction Kit and QIAprep®Spin Miniprep Kit were from QIAGEN Sciences, Maryland; PCR reagents were from Applied Biosystems, Roche; SuperScript™ II Reverse Transcriptase and TOPO TA Cloning® Kit Dual Promoter were from Invitrogen, CA; EcoR I was from New England Bio Labs; Dulbecco’s modified Eagle’s medium (DMEM) was from Fisher Scientific. All other chemicals were obtained from Sigma, St. Louis, MO.

Experimental Animals. New Zealand white rabbits (weight: 4-5 lbs) were purchased from RSI Biotechnology, Clemmons, NC. The rabbits were housed in the animal facility administered by the Division of Animal Resources, Virginia Commonwealth University, and maintained in a temperature-controlled environment with free access to food and water. The rabbits were killed by sodium pentobarbital overdose (100 mg/kg); this and all other procedures were performed in accordance with the recommendations of the Institutional Animal Care and Use Committee of Virginia Commonwealth University.
Preparation of dispersed and cultured smooth muscle cells. Smooth muscle cells were isolated from strips of circular muscle layer of rabbit stomach by sequential enzymatic digestion, filtration, and centrifugation as described in detail previously (17-19). Freshly dispersed muscle cells were suspended in a medium consisting of 120 mM NaCl, 4 mM KCl, 2.6 mM KH$_2$PO$_4$, 2 mM CaCl$_2$, 0.6 mM MgCl$_2$, 25 mM HEPES, 14 mM Glucose, and 2.1% Eagle’s essential amino acid mixture (pH 7.4). In some experiments, smooth muscle cells were cultured in Dulbecco’s modified Eagles’s medium (DMEM) containing 10% fetal bovine serum until they attained confluence; the cells were re-dispersed and passaged once for use in various studies (20).

Detection of cannabinoid receptor expression by PCR. Total RNA was extracted from cultured gastric smooth muscle cells by treatment with RNaqueous reagent and contaminant genomic DNA removed by treatment with TURBO DNase as described previously (23). RNA (5 μg) was reversibly transcribed and amplified by PCR under standard conditions. Specific primers for rabbit CB$_1$ and CB$_2$ receptors were designed based on identical sequences in human, rat, and mouse cDNA.

CB$_1$
F: 5’ACATGGCATCCAAATTAGG3’
R: 5’CAGTTTGAACAGAAACAC3’

CB$_2$
F: 5’AGCTGACTTCCTGGCCAG3’
R: 5’AGTCTTGGCCAACCTCAC3’

PCR products were purified and sequenced.

Detection of cannabinoid receptors by Western blot. The expression of CB$_1$ or CB$_2$ receptors was determined by Western blot as described previously (23) using homogenates prepared from freshly dispersed gastric smooth muscle cells. Proteins were resolved by 15% SDS-PAGE and electrophoretically transferred to nitrocellulose...
membranes. The membranes were incubated for 12 h at 4°C with specific antibodies for
CB1 or CB2 receptors and then for 1 h with secondary antibody. The bands were
identified by enhanced chemiluminescence.

*Binding of CB1 receptor agonist [3H]CP55,940 to muscle cells.* Cultured gastric smooth
muscle cells were re-suspended in HEPES medium containing 1% BSA, 10 μM
amastatin, 1 μM phosphoramidon, and 0.7 mM bacitracin. Triplicate aliquots (0.3 ml) of
cell suspension (10^6 cells/ml) were incubated for 60 min at 4°C with 50 pM
[^3H]CP55,940 alone or with 1 μM AEA. Bound and free radioligand were separated by
rapid filtration through 5 μm polycarbonate nucleopore filters followed by washing with
HEPES medium. Nonspecific binding (26 ± 5%) was determined as the amount of
radioactivity associated with the muscle cells in the presence of 1 μM AEA.

*Identification of G proteins coupled to CB1 receptors.* G proteins activated by AEA were
identified by an adaptation of the method of Okamoto et al. as described previously (21,
34). Homogenates of dispersed muscle cells were centrifuged at 30,000g for 30 min at 4°C,
and the membranes solubilized at 4°C in 20 mM HEPES (pH 7.4) buffer. Solubilized
membranes were incubated for 20 min at 37°C with 100 nM[^35S]GTPγS in 10 mM
HEPES in the presence or absence of 1 μM AEA. The reaction was stopped and the
membranes were re-incubated for 2 h on ice in wells precoated with specific antibodies to
Gαs, Gαq, Gαi1, Gαi2, Gαi3. Radioactivity from each well was counted by liquid
scintillation.

*Transfection of GRK2, GRK5, and RGS4 mutants and β-arrestin1 and -2 siRNA into
cultured smooth muscle cells.* Wild type GRK2, GRK5, RGS4, kinase-deficient
GRK2(K220R) and GRK5(K215R), phosphorylation-deficient RGS4(S103A/S108A),
and β-arrestin1 and -2 siRNA were subcloned into the multiple cloning site (EcoR I) of the eukaryotic expression vector pcDNA3. Recombinant plasmid cDNAs were transiently transfected into smooth muscle cultures for 48 h. The cells were co-transfected with 2 µg pcDNA3 vector and 1 µg of pGreen Lantern-1 DNA to monitor transfection efficiency (23).

Cannabinoid receptor phosphorylation. Phosphorylation of CB1R was determined from the amount of 32P incorporated after immunoprecipitation with specific antibody for CB1R. Cultured muscle cells were transfected with control vector or vector containing kinase-deficient GRK5(K215R) in wells containing 2x10⁶ cells and labeled with 0.5 µCi/ml of [32P] orthophosphate for 3 h. The cells were then treated with or without 1 µM AEA for 15 min. The reaction was terminated with an equal volume of lysis buffer (final concentrations: 1% Triton X-100, 0.5% SDS, 0.75% deoxycholate, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 100 µg/ml aprotinin, 10 mM Na₂P₂O₇, 50 mM NaF, 0.2 mM Na₃VO₄) and placed on ice for 30 min. Cell lysates were separated by centrifugation at 13,000 rpm for 15 min at 4°C. The lysates were incubated with CB1R antibody for 2 h at room temperature, and then overnight at 4°C after addition of 25 µl of Protein A/G plus agarose. The immunoprecipitates were washed with lysis buffer and extracted by boiling with Laemmlli buffer for 15 min, and separated by SDS gel electrophoresis. 32P-labeled CB1R was visualized by autoradiography (15).

Protein:protein association (M-RIP:RhoA, M-RIP:MYPT1, and Gαq:RGS4). Sequential immunoprecipitation and immunoblot with selective antibodies was used to determine the association of M-RIP with RhoA and MYPT1. Dispersed or cultured muscle cells were treated with 1 µM AEA, 0.1 µM ACh, or a combination of AEA and ACh for 5 min. In
some experiments, the cells were treated in the same fashion in the presence of the Src kinase inhibitor, PP2 (1 μM), or the CB1 receptor antagonist, AM251 (1 μM). For Gαq:RGS4 association, the cells were treated with 1 μM AEA and 0.1 μM ACh or a combination of AEA and ACh for 30 s.

**Assay of phosphoinositide hydrolysis.** Phosphoinositide (PI) hydrolysis was measured by anion exchange chromatography and expressed as total inositol phosphate formation as described previously (34). Freshly dispersed muscle cells (10⁶ cells/ml) were labeled with myo-[³H]inositol (0.5 μCi/ml) for 3 h, then centrifuged at 350 g for 10 min and resuspended in 10 ml fresh HEPES medium. Cell aliquots (2x10⁶ cells/ml) were treated with ACh for 60 s in the presence or absence of 1 μM AEA. In other studies, muscle cells were cultured in wells (2x10⁶ cells/well) and labeled with myo-[³H]inositol for 24 h, and then treated with AEA and ACh as described above for dispersed muscle cells. [³H]inositol phosphate radioactivity was determined by liquid scintillation.

**Measurement of ERK1/2 and Rho-kinase activities.** ERK1/2 and Rho-kinase activities were determined in cell extracts by immunokinase assay as described previously (9, 30). Dispersed smooth muscle cells (10⁶ cells/ml) or cultured muscle cells (2x10⁶ cells/well) were treated with AEA for 5 min and with ACh for 10 min and then centrifuged for 5 min. Cell pellets were solubilized and equal amounts of protein extracts were incubated with ERK1/2 or Rho kinase-2 antibody plus protein A/G agarose overnight at 4℃. The immunoprecipitates were washed and incubated for 5 min on ice with 5 μg of myelin basic protein. The assay was initiated by the addition of 10 μCi of [³²P]ATP (3,000Ci/mmol) and 20 μM ATP. ³²P-labeled myelin basic protein was absorbed onto
phosphocellulose disks and the amount of radioactivity was measured by liquid scintillation.

**Measurement of Src Kinase activity.** Src kinase activity was measured by Western blot using a phospho-Src (Tyr^{416}) antibody. Dispersed muscle cells (10^6 cells/ml) or cultured muscle cells (2x10^6 cells/well) were treated with AEA for 5 min in the presence or absence of PP2 (1 μM), AM251 (1 μM), or PTx (400 ng/ml) and solubilized on ice for 2 h in 20 mM Tris/HCl medium. The proteins were resolved by SDS-PAGE and phosphorylation of Src kinase was analyzed by Western blot using phospho-Src (Tyr^{416}) antibody.

**Measurement of cAMP in smooth muscle cells.** Cyclic AMP production was measured by radioimmunoassay using ^{125}I-cAMP. Cells (3×10^6 cells) were treated with AEA for 60 s in the presence of 100 μM Isobutylmethylxanthine 1-Methyl-3-Isobutylxanthine or 3,7-dihydro-1-methyl-3-(2-methylpropyl)-1H-purine-2,6-dione (IBMX), and the reaction was terminated with 10% trichloroacetic acid. The samples were acetylated with triethylamine/acetic anhydride (2:1) for 30 min and cAMP was measured in duplicate using 100 μl aliquots. The results were expressed as picomoles/mg protein.

**Measurement of [Ca^{2+}]_i in cultured smooth muscle cells.** Dispersed muscle cells were plated on cover-slips for 12 h in DMEM. The cells were washed and loaded with 10 μM fura 2-AM in HEPES medium. [Ca^{2+}]_i was measured by fluorescence in single smooth muscle cells loaded with the fluorescent Ca^{2+} dye fura 2-AM as previously described (11). The cells were alternately excited at 380 and 340 nm and imaged at 15 s intervals. The emitted light was imaged at 510 nm. Background and autofluorescence were corrected from images of unloaded control cells.
Measurement of contraction in dispersed smooth muscle cells. Muscle cell contraction was measured in freshly dispersed muscle cells by scanning microscopy as described previously (11, 34). Cell aliquots containing \(\sim 10^4\) muscle cells/ml were treated with 1 \(\mu M\) AEA and 0.1\(\mu M\) ACh for different time periods (30 s to 10 min) and the reaction was terminated with 1% acrolein. The lengths of treated muscle cells were compared with the lengths of untreated cells, and contraction was expressed as the percent decrease in cell length from control.

Statistical Analysis. The results were expressed as means ± SE of n experiments and analyzed for statistical significance using Student’s t test for paired and unpaired values. Differences among groups were tested using ANOVA and checked for significance using Fisher’s protected least significant difference test. P<0.05 was considered significant.

RESULTS

Selective expression of CB\(_1\) receptors in smooth muscle cells. CB\(_1\) receptors were detected by RT-PCR and Western blot in cultured rabbit gastric smooth muscle cells and in brain homogenates, whereas CB\(_2\) receptors were detected in spleen homogenates but not in cultured smooth muscle cells (Figs. 1A). The partial amino acid sequence of rabbit CB\(_1\) receptors was closely similar to the corresponding amino acid sequences of human (96%), rat (96%) and mouse (97%). Binding of \(^{[3]}H\)CP55,940 to dispersed smooth muscle cells was inhibited by AEA in a concentration-dependent fashion (IC\(_{50}\) 5 nM), consistent with expression of CB\(_1\) receptors in these cells (Fig. 1B).

Preferential activation of G\(\alpha_{i2}\) by CB\(_1\) receptors. Anandamide (AEA) increased \(^{[35]}S\)GTP\(\gamma\)S binding to G\(\alpha_{i1}\), G\(\alpha_{i2}\), and G\(\alpha_{i3}\) by 66±13%, 182±20%, and 54±11%,
respectively, but had no effect on \[^{35}\text{S}]\text{GTP}\gamma\text{S} binding to \(\text{G}\alpha_{q/11}\) or \(\text{G}\alpha_{s}\) (Fig. 1C). The results implied that CB1 receptors are preferentially coupled to \(\text{G}\alpha_{i2}\).

Consistent with the coupling of CB1 receptors to \(\text{G}\alpha_{i1-3}\), AEA inhibited forskolin-stimulated cAMP formation in dispersed muscle cells in a concentration-dependent fashion (IC\(_{50}\) 13 nM) (Fig. 2A). The inhibition was blocked by the CB1 receptor antagonist, AM251, and by preincubation of the muscle cells for 60 min with 400 ng/ml of pertussis toxin (PTx). By itself, AEA had no effect on basal cAMP formation, and AM251, which can act as a reverse agonist in some cells (6, 27), had no effect on basal or forskolin-stimulated cAMP formation in smooth muscle cells (Fig. 2B). AEA inhibited forskolin-stimulated cAMP formation in cultured muscle cells also; the inhibition was blocked in cells expressing \(\text{G}\alpha_{i}\) minigene (Fig. 2C). Our previous studies had shown that \(\text{G}\alpha_{i}\) minigenes selectively block responses mediated by \(\text{G}\alpha_{i}\) (34).

Absence of \(\text{G}\beta\gamma\)-dependent stimulation of phosphoinositide hydrolysis and muscle contraction by CB1 receptor agonist. Receptors coupled to \(\text{G}\alpha_{i1-3}\) stimulate phosphoinositide hydrolysis and smooth muscle contraction via \(\text{G}\beta\gamma\)-dependent activation of PLC-\(\beta3\) (17-19). Activation of CB1 receptors with AEA, however, did not stimulate PI hydrolysis or induce muscle contraction (Figs. 3A and 3B). In contrast, as shown previously (18, 19) and confirmed in this study, activation of \(\text{G}\alpha_{i3}\)-coupled adenosine \(\text{A}_{1}\) receptors with cyclopentyl adenosine (CPA) and \(\text{G}\alpha_{i2}\)-coupled \(\delta\) opioid receptors with \([\text{D-Pen}^2,\text{D-Pen}^5]\text{enkephalin (DPDPE)}\) stimulated PI hydrolysis and induced muscle contraction (Figs. 3A and 3B). The results implied that CB1 receptors did not bind or activate \(\text{G}\beta\gamma\).
CB₁ receptor phosphorylation by GRK5 mediates receptor internalization. Treatment of cultured smooth muscle cells with 1 μM AEA for 15 min induced CB₁ receptor phosphorylation; receptor phosphorylation was abolished in cells transfected with kinase-deficient GRK5 (GRK5(K215R)) (Fig. 4A). CB₁ receptor internalization, determined from the decrease in binding of [³H]CP55,940 to cultured smooth muscle cells after treatment with 1 μM AEA, was about 10% within 5 min, 50% within 15 min, and 80% within 30 min (Fig. 4B). Expression of kinase-deficient GRK5(K215R) blocked CB₁ receptor internalization whereas expression of kinase-deficient GRK2(K220R) had no effect, suggesting that CB₁ receptor internalization was dependent on receptor phosphorylation by GRK5 and not by GRK2/3 (9, 11) (Fig. 4B).

ERK1/2 and Src kinase activation by CB₁ receptors depends on receptor phosphorylation and activation of β-arrestin1/2. Treatment of cultured smooth muscle cells with AEA stimulated ERK1/2 activity in a time dependent fashion; ERK1/2 activity was blocked by AM251 (Fig. 5A). The increase in ERK1/2 activity was significant within 1 min and attained a sustained maximum within 10 min (Fig. 5A). ERK1/2 activity was abolished by the ERK1/2 inhibitor PD98059, but was not affected by the PKC inhibitor, bisindolylmaleimide, PTx, and in cells expressing a Gᵢ minigene implying that it was not dependent on activation of Gαᵢ (Fig. 5B). ERK1/2 activity was not affected also in cells expressing the C-terminal sequence of GRK2 (GRK2CT(495-689)), which competes with cytosolic GRK2 for binding to Gβγ (Fig. 5B), or in cells expressing kinase-deficient GRK2(K220R) (Fig. 5C), implying that ERK1/2 activity was not dependent on recruitment of GRK2 to the receptor by Gβγ or receptor phosphorylation by GRK2. ERK1/2 activity was blocked, however, in cells expressing kinase-deficient
GRK5(K215R) (Fig. 5C) and in cells expressing siRNA for β-arrestin1 and 2 (Fig. 5D), implying that ERK1/2 activation resulted from AEA-induced phosphorylation of CB₁ receptors by GRK5 and binding of β-arrestin1/2 to phosphorylated CB₁ receptors.

In similar fashion, treatment of cultured muscle cells with AEA for 5 min stimulated Src kinase activity. Activation of Src kinase was blocked by PP2 and AM251 (Fig. 6A), and in cells expressing GRK5(K215R) (Fig. 6B), but not in cells expressing GRK2CT(495-689). Thus, Src kinase activation also resulted from AEA-induced phosphorylation of CB₁ receptors via Gβγ-independent GRK5 and the binding of β-arrestin to phosphorylated CB₁ receptors.

*Activation of ERK1/2 and Src kinase modulates ACh-stimulated contraction via phosphorylation of RGS4 by ERK1/2 and M-RIP and MYPT1 by Src kinase.* Activation of ERK1/2 and Src kinase raised the possibility that one or both kinases could modulate concurrent smooth muscle contraction. As shown previously (20) and confirmed in the present study, ACh acting via m3 receptors (i.e., ACh in the presence of the m2 receptor antagonist, methoctramine) stimulates PI hydrolysis and induces a biphasic contraction consisting of an initial 30 s peak followed by a sustained contraction. AEA inhibited both initial and sustained ACh-stimulated contraction (Fig. 7A). AEA inhibited ACh-stimulated PI hydrolysis, [Ca²⁺], and initial contraction in dispersed smooth muscle cells by 65±6% (p<0.01), 66±13% (p<0.01), and 54±2% (p<0.001), respectively: the inhibition of PI hydrolysis and initial contraction was selectively blocked by PD98059, but not by bisindolylmaleimide or PP2 (Figs. 7B and 7C), implying that it was mediated by ERK1/2.

We next examined the mechanism by which ERK1/2 inhibited PI hydrolysis and muscle contraction. ACh-stimulated PI hydrolysis in smooth muscle is mediated by Gαq-
dependent activation of PLC-β1 and regulated by RGS4, which binds to and enhances the intrinsic GTPase activity of Gαq,GTP (12). We identified a proline-rich consensus site (102KS\textsuperscript{P}SKL\textsuperscript{P}SP\textsuperscript{109}) in RGS4 for phosphorylation by ERK1/2 and hypothesized that phosphorylation of Ser\textsuperscript{103} and/or Ser\textsuperscript{108} by ERK1/2 could further enhance the GTPase activity of Gαq,GTP leading to rapid inactivation of Gαq and inhibition of Gαq-dependent PI hydrolysis. In control cultured muscle cells, AEA inhibited ACh-stimulated PI hydrolysis and increased Gαq:RGS4 association, whereas in cultured muscle cells expressing a mutant RGS4(S103A/S108A) that lacks ERK1/2 phosphorylation sites, AEA did not inhibit PI hydrolysis or increase Gαq:RGS4 association (Fig. 7D). The results suggest that inhibition of PI hydrolysis and initial muscle contraction by anandamide-stimulated ERK1/2 is mediated via stimulatory phosphorylation of RGS4, resulting in rapid inactivation of Gαq,GTP.

Unlike initial contraction, sustained contraction is mediated by Rho kinase-dependent phosphorylation of MYPT1 and inhibition of MLC phosphatase activity (16, 20). Measurements of sustained contraction and Rho kinase activity were, therefore, made 5 min after treatment with ACh and AEA. AEA inhibited ACh-stimulated Rho kinase activity and sustained contraction in dispersed muscle cells by 48±8% and 48±5%, respectively; p<0.01 (Figs. 8A and 8B). Inhibition of sustained contraction and Rho kinase activity was blocked by AM251 and PP2, but not by PD98059 (Figs. 8A and 8B). As previously shown (16, 20, 30), the decrease in Rho kinase activity should lead to decrease in MYPT1 phosphorylation by Rho kinase and thus enhance MLC phosphatase activity.
We hypothesized that Src kinase enhances the association of M-RIP (MYPT1/RhoA interacting protein) \(^{(13, 24)}\) with both RhoA and MYPT1 leading to increase in MLC phosphatase activity \(^{(16, 20)}\). Treatment of cultured muscle cells with AEA for 10 min enhanced association of M-RIP with RhoA (Fig. 9A) and MYPT1 (Fig. 9B). The increase in M-RIP:RhoA and M-RIP:MYPT1 association was blocked by AM251 and PP2 (Fig. 9A and 9B), and in smooth muscle cells expressing kinase-deficient GRK5(K215R) (Figs. 10A and 10B). Thus, activation of Src kinase upon phosphorylation of CB\(_1\) receptors by GRK5 enhanced the association of M-RIP with both RhoA and MYPT1. The increase in M-RIP:RhoA association inhibited Rho kinase activity and thus should inhibit Rho kinase-dependent MYPT1 phosphorylation leading to increase in MLC phosphatase activity. The increase in M-RIP:MYPT1 association should enhance further the activity of the MLC phosphatase holoenzyme by fostering its binding to the actomyosin complex \(^{(13)}\). Both mechanisms result in inhibition of sustained muscle contraction.

**DISCUSSION**

The present study shows that CB\(_1\) receptors are selectively expressed in smooth muscle cells and signal via G\(\alpha_i\) causing inhibition of adenylyl cyclase activity, but do not signal via G\(\beta\gamma\) as do other G\(_i\)-coupled receptors in these cells \(^{(17-19)}\). CB\(_1\) receptors signal also via \(\beta\)-arrestin1/2 in a G protein-independent fashion to activate ERK1/2 and Src kinase. Uncoupling of CB\(_1\) receptors from G\(_i\) by PTx or by expression of G\(_i\) minigene abolished inhibition of cAMP but had no effect on ERK1/2 activity, whereas
expression of kinase-deficient GRK5(K215R) or silencing of β-arrestin1/2 with siRNA abolished ERK1/2.

Anandamide-induced CB₁ receptor phosphorylation was blocked in smooth muscle cells expressing kinase-deficient GRK5 (GRK5(K215R)), implying that CB₁ receptors were phosphorylated by GRK5. Unlike GRK2/3, which are located in the cytosol and are recruited to G protein-coupled receptors by activated Gβγ (9, 11), GRK5 and GRK6 are constitutively tethered to the plasma membrane where they directly phosphorylate agonist-bound receptors (4). Activation of ERK1/2, which was significant within 1 min and maximal within 10 min, not affected by expression of kinase-deficient GRK2(K220R) or a Gβγ-scavenging peptide, implying that it was not dependent on activation of Gβγ or recruitment of GRK2. ERK1/2 activity was blocked, however, by expression of kinase-deficient GRK5(K215R) or β-arrestin1/2 siRNA, implying that ERK1/2 activation resulted from agonist-induced phosphorylation of CB₁ receptors by GRK5 and binding of β-arrestin1/2 to phosphorylated CB₁ receptors.

GRK5-dependent activation of ERK1/2 and Src kinase endowed CB₁ receptors with the ability to inhibit concurrent contractile activity. We had previously shown that the initial transient contraction induced by ACh is mediated by Gαq-dependent activation of PLC-β1 and terminated by RGS4 (9, 11). In this study, we identified a consensus sequence (102KS₅PSK₅LS₅P₁₀₉) for phosphorylation of RGS4 by ERK1/2 and showed that expression of a phosphorylation-deficient RGS4 mutant lacking Ser₁₀³ and Ser₁₀₈ blocked the ability of AEA to inhibit of ACh-stimulated PI hydrolysis or enhance Gαq;RGS4 association. Huang et al. (12) had previously reported a similar mechanism involving
phosphorylation of RGS4 at Ser$_{52}^{52}$ by cyclic AMP- and cGMP-dependent protein kinases that resulted in inhibition of ACh-stimulated initial contraction and PI hydrolysis (12).

Unlike the initial transient contraction, sustained contraction is mediated in part by Rho kinase-dependent phosphorylation of the myosin phosphatase-targeting subunit, MYPT1 (16, 29, 31). Phosphorylation of MYPT1 at Thr$_{696}^{696}$ by Rho kinase inhibits the activity of the catalytic subunit of MLC phosphatase and fosters sustained MLC$_{20}$ phosphorylation and contraction. Stimulation of Src kinase activity by anandamide enhanced both M-RIP:RhoA and M-RIP:MYPT1 association. The increase in M-RIP:RhoA association inhibited Rho kinase activity and therefore, should attenuate MYPT1 phosphorylation by Rho kinase leading to increase in MLC phosphatase activity, whereas the increase in M-RIP:MYPT1 association should enhance further the ability of MYPT1 to activate MLC phosphatase holoenzyme by fostering its binding to the actomyosin complex. The dual mechanism initiated by Src kinase upon phosphorylation of CB$_1$ receptors increased MLC phosphatase activity and inhibited sustained contraction.

The results of this study may be usefully compared with those of Shenoy et al. (25) obtained in cell lines expressing a β2 adrenergic receptor mutant (β2ARTYY) incapable of G protein activation. Over-expression of GRK5 or -6 in these cells enhanced recruitment of β-arrestin to the receptor and its ability to induce prompt, sustained activation of ERK1/2. GRK2 expression was effective only when targeted to the membrane by co-expression of a prenylation signal (CAAX). In our study, CB$_1$ receptors constitutively expressed in a native smooth muscle cell, signaled concurrently via G$_{ai}$ and β-arrestin, the latter upon receptor phosphorylation by GRK5. Uncoupling CB$_1$ receptors from G proteins by PTx or expression of G$_i$ minigene had no effect on β-
arrestin-dependent activation of ERK1/2 and Src kinase. Unlike other G\(_i\)-coupled receptors in smooth muscle, CB\(_1\) receptors did not engage G\(\beta\gamma\) to initiate signaling that would have resulted in activation of dual pathways involving PLC-\(\beta\)3 and PI 3-
kinase/integrin linked kinase (17-19).

The present study does not resolve the fate or role of G\(\beta\gamma\) in CB\(_1\) receptor signaling. It is possible that interaction of G\(\beta\gamma\) with both CB\(_1\) receptor and G\(\alpha\)\(_i\) allows for productive guanine nucleotide exchange on G\(\alpha\)\(_i\) without sustaining downstream signaling via G\(\beta\gamma\). Alternatively, the G\(\beta\gamma\) dimer could be atypical, consisting, for example, of G\(\beta\)\(_5\) bound to the G\(\gamma\)-like domain of R7 subfamily of RGS proteins (e.g., RGS6,-7,-9,-11) (1, 28). This subfamily is defined by the presence of three domains: an N-terminal DEP (Dishevelled, Egl, Pleckstrin) domain, linked via a DEP helical extension to a central G\(\gamma\)-like (GGL) domain tightly bound to a C-terminal catalytic domain with selectivity for G\(\alpha\)\(_i\). Although these atypical G protein dimers are preferentially expressed in neural tissues (28), a member of this subfamily, G\(\beta\)\(_5\)-RGS6, has also been found in smooth muscle (14). A G\(\beta\)\(_5\)-RGS6 heterodimer could bind to G\(\alpha\)\(_i\) and permit CB\(_1\) receptor-mediated nucleotide exchange without being able to substitute for, or signal in place, of a canonical G\(\beta\gamma\). This intriguing notion, however, remains speculative.

**ACKNOWLEDGMENTS**

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**FIGURE LEGENDS**

**Fig. 1.** (A) Selective expression of cannabinoid CB₁ receptor in smooth muscle cells. Total RNA isolated from cultured gastric smooth muscle cells in first passage and from brain homogenates was reverse transcribed. PCR product of the expected size (472 bp) was obtained for CB₁ receptor, but not for CB₂ receptor. CB₂ receptor was detected in spleen homogenates. Western blot analysis confirmed expression of the corresponding receptor protein. (B) Inhibition of binding of CB₁ receptor agonist [³H]CP55,940 to smooth muscle cells by anandamide (AEA). Results are expressed as percent of control specific binding (IC₅₀ 5 nM). Measurements were done in triplicate and the values are means ± SE of 5 experiments. (C) Preferential activation of Gαᵢ₂ by AEA in smooth muscle. AEA increased the binding of [³⁵S]GTPγS to Gαᵢ₁, Gαᵢ₂, and Gαᵢ₃, but not to Gαᵣ or Gαₛ. Values are means ± SE of 4 experiments; **p<0.01

**Fig. 2.** (A) Concentration-dependent inhibition of forskolin-stimulated cAMP formation by AEA in dispersed smooth muscle cells, and (B) selective blockade of inhibition by AM251 and PTx. (C) Inhibition of forskolin-stimulated cAMP by AEA in cultured smooth muscle cells selectively blocked in cells expressing Gᵢ minigene but not in cells expressing control, random order Gᵢ minigene. Results are means ± SE of 3 experiments.

**Fig. 3.** Differential effect of Gᵢ₂-coupled CB₁, Gᵢ₂-coupled opioid δ, and Gᵢ₃-coupled adenosine A₁ receptor agonists on (A) PI hydrolysis and (B) muscle contraction. Anandamide (1 µM) had no effect on PI hydrolysis or muscle contraction, whereas DPDPE (1 µM) and CPA (1 µM) stimulated PI hydrolysis and muscle contraction.
Muscle contraction was expressed as percent decrease in cell length from control. PI hydrolysis was expressed as cpm/mg protein. Results are means ± SE of 3 experiments.

Fig. 4. GRK5-mediated phosphorylation and internalization of CB1 receptors. (A) Cultured smooth muscle cells transfected with control vector or vector expressing kinase-deficient GRK5 (GRK5(K215R)) were treated for 15 min with 1 μM anandamide. (B) Time course of decrease in [3H]CP55,940 binding to cultured smooth muscle cells after treatment with 1 μM anandamide in cells transfected with control vector, vector expressing kinase-deficient GRK5 (GRK5(K215R)), or vector expressing kinase-deficient GRK2 (GRK2(K220R)). Receptor phosphorylation and internalization were blocked in cells expressing kinase-deficient GRK5. Results are means ± SE of 4 experiments.

Fig. 5. Activation of ERK1/2 by CB1 receptors is dependent on receptor phosphorylation by GRK5 and recruitment of β-arrestin1/2. (A) Time course of ERK1/2 activation by anandamide (1 μM) in cultured smooth muscle cells in the presence or absence of the CB1 receptor antagonist AM251 (1 μM). ERK1/2 activity was determined by immunokinase assay and expressed as cpm/mg protein. The increase in ERK1/2 activity was significant within 1 min (p<0.01) and near maximal within 10 min. Values are means ± SE of 4 experiments. (B) G protein-independent activation of ERK1/2. Treatment of cultured smooth muscle cells with PD98059 abolished AEA-stimulated ERK1/2 activity, whereas treatment with PTx (400 ng/ml for 1 h) and expression of Goαi minigene or GRK2CT(495-689), a Gβγ-scavenging peptide, PKC inhibitor (Bisindolylmaleimide, 1
μM for 10 min) had no effect. ERK1/2 activity was not affected by PKC or p38 MAP kinase inhibitors (data not shown). Values are means ± SE of 4 experiments.  

(C) Selective inhibition of anandamide-stimulated ERK1/2 activity in smooth muscle cells expressing kinase-deficient GRK5(K215R). Cultured smooth muscle cells expressing vector alone (control), kinase-deficient GRK2 (GRK2(K220R)), or kinase-deficient GRK5 (GRK5(K215R)) were treated with 1 μM AEA for various times up to 20 min. Values are means ± SE of 4 experiments.  

(D) Inhibition of anandamide-stimulated ERK1/2 activity in cells expressing siRNA for β-arrestin1 and -2. Values are means ± SE of 3 experiments.

Fig. 6. Activation of Src kinase by CB₁ receptors is dependent on receptor phosphorylation by GRK5 and independent of Gβγ. (A) Cultured smooth muscle cells were treated for 5 min with AEA in the presence and absence of 1 μM AM251 or 1 μM PP2 (Src kinase inhibitor). Src kinase activity was determined using phospho-specific Src(Tyr⁴¹⁶) antibody. Values are means ± SE of 4 experiments. (B) Selective inhibition of anandamide-stimulated Src kinase activity in cultured muscle cells expressing kinase-deficient GRK5(K215R). Cultured smooth muscle cells expressing vector (control), kinase-deficient GRK5 (GRK5(K215R)), and GRK2CT(495-689), a Gβγ scavenging peptide, were treated for 5 min with AEA. Values are means ± SE of 4 experiments.

Fig. 7. Inhibition of acetylcholine-stimulated initial contraction by anandamide is mediated by ERK1/2. (A) Time course of inhibition of ACh-stimulated initial and sustained muscle contraction by AEA. Dispersed smooth muscle cells were treated with
0.1 μM ACh in the presence of 0.1 μM methoctramine (m2 receptor antagonist) with or without 1 μM AEA for various time periods. Cell contraction was measured by scanning micrometry and expressed as percent decrease in cell length from control (109±4 μm). Values are means ± SE of 4 experiments. (B) Dispersed smooth muscle cells were treated with 1 μM AEA for 10 min followed by 0.1 μM ACh and 0.1 μM methoctramine for 30 s. The experiments were repeated in the presence of 10 μM PD98059, 1 μM PP2, or 1 μM bisindolylmaleimide. PD98059 reversed AEA-induced inhibition of peak 30-s muscle contraction. Values are means ± SE of 4-5 experiments. (C) Dispersed smooth muscle cells were labeled with myo-[3H]inositol for 3 h, and then treated with 1 μM AEA for 10 min followed by 0.1 μM ACh and 0.1 μM methoctramine for 30 s. The experiments were repeated in the presence of 10 μM PD98059, 1 μM PP2, or 1 μM bisindolylmaleimide. PD98059 reversed AEA-induced inhibition of PI hydrolysis. Values are means ± SE of 3 experiments. (D) Blockade of AEA-induced inhibition of PI hydrolysis in cells expressing phosphorylation-deficient RGS4 mutant. (Lower panel) Cultured smooth muscle cells expressing wild-type RGS4 (vector alone) or phosphorylation-deficient RGS4(S103A/108A) were labeled with myo-[3H]inositol for 24 h, and then treated with AEA for 10 min followed by 0.1 μM ACh and 0.1 μM methoctramine for 30 s. Values are means ± SE of 4 experiments. (Upper panel) The bands show that the increase in Gαq:RGS4 association induced by ACh was augmented upon addition of AEA in control cells but not in cells expressing RGS4(S103A/108A).

Fig. 8. Inhibition of acetylcholine-stimulated sustained contraction and Rho kinase activity by anandamide is mediated by Src kinase. (A and B) Dispersed smooth muscle
cells were treated with 1 μM AEA for 10 min followed by 0.1 μM ACh and 0.1 μM methoctramine for 5 min (the time point corresponds to the start of sustained contraction, see Fig. 7A). The experiments were repeated in the presence of 10 μM PD98059, 1 μM PP2, or 1 μM AM251. Smooth muscle contraction was measured by scanning micrometry and expressed as percent decrease in cell length from control (106±4 μm). Values are means ± SE of 4-5 experiments. Rho kinase activity was determined by immunokinase assay and expressed as cpm/mg protein. Values are means ± SE of 3 experiments. AEA-induced inhibition of sustained muscle contraction and Rho kinase activity was reversed by the Src kinase inhibitor, PP2, and the CB1 receptor antagonist, AM251. **p<0.01.

Fig. 9. Stimulation of M-RIP:RhoA and M-RIP:MYPT1 association by AEA is mediated by Src kinase. Dispersed smooth muscle cells were treated separately with (i) 0.1 μM ACh plus 0.1 μM methoctramine, (ii) 1 μM AEA, and (iii) 1 μM AEA plus 0.1 μM ACh plus 0.1 μM methoctramine for 5 min. The experiments were repeated in the presence of 1 μM PP2 or 1 μM AM251. Immunoprecipitates derived from 500 μg of protein using (A) RhoA antibody or (B) MYPT1 antibody were separated on SDS-PAGE and immunoblotted with M-RIP antibody. AEA alone or with ACh increased M-RIP:MYPT1 association. The increase in M-RIP:RhoA and M-RIP:MYPT1 association induced by a combination of AEA and ACh was abolished by PP2 and AM251. Values are means ± SE of 4 experiments.
Fig. 10. Inhibition of M-RIP:RhoA and M-RIP:MYPT1 association in cells expressing kinase-deficient GRK5. Cultured smooth muscle expressing vector or GRK5(K215R) were treated with 1 μM AEA, 0.1 μM ACh, and 0.1 μM methoctramine for 5 min. Immunoprecipitates derived from 500 μg of protein using (A) RhoA antibody or (B) MYPT1 antibody were separated on SDS-PAGE and immunoblotted with M-RIP antibody. The increase in M-RIP:RhoA and M-RIP:MYPT1 association was abolished in cells expressing kinase-deficient GRK5(K215R). Values are means ± SE of 4 experiments.

Fig. 11. Scheme depicting the inhibition of smooth muscle contraction via CB₁ receptors. CB₁ receptor phosphorylation by GRK5 leads to recruitment of β-arrestin1/2 and activation of ERK1/2 and Src kinase. ERK1/2 phosphorylates RGS4 at Ser¹⁰³/¹⁰⁸ and increases its association with and activation of Gaq-GTPase, resulting in inhibition of the Gaq/PLC-β/IP₃/Ca²⁺/MLCK pathway that mediates initial contraction. Activation of Src kinase stimulates: (1) M-RIP:MYPT1 association enhancing the ability of MYPT1 to activate MLC phosphatase, and (2) M-RIP:RhoA association, causing inhibition of Rho kinase and decreasing its ability to phosphorylate and inhibit MYPT1. Thus, Src kinase acting via M-RIP enhanced MLCP activity leading to inhibition of the Ga₁₃/RhoA/Rho kinase/MYPT1/MLCP pathway that mediates sustained contraction.
Figure 6

A) Src Kinase activity (% density)

B) Src Kinase activity (% density)
Figure 7

A) Contraction (% decrease in cell length) over time for ACh and ACh + AEA.

B) Initial Contraction (% decrease in cell length) with statistical significance marked.

C) PI Hydrolysis (pmol/minute per mg protein) with various treatments.

D) Western blot showing protein expression for Control Vector and RGS4 (S103A/S108A).
Figure 8

A) 
Sustained Contraction (% decrease in cell length)

B) 
Rho kinase Activity (cpm/ml)

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**P-values:** **P < 0.01**
Figure 10

A) 

![Graph showing M-RIP-RhoA Association (% density) for Control Vector and GRK5 (K215R) groups with Basal and AEA conditions.](image)

B) 

![Graph showing M-RIP-MIF Association (% density) for Control Vector and GRK5 (K215R) groups with Basal and AEA conditions.](image)