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Profiling Two Indole-2-Carboxamides for Allosteric Modulation of the CB1 Receptor

Kwang H. Ahn[1], **Mariam M. Mahmoud**[2], **Sushma Samala**[3], **Dai Lu**[3,*], and **Debra A. Kendall**[1,*]

[1]Department of Pharmaceutical Sciences, University of Connecticut, Storrs, Connecticut, United States

[2]Department of Molecular and Cell Biology, University of Connecticut, Storrs, Connecticut, United States

[3]Department of Pharmaceutical Sciences, Irma Lerma Rangel College of Pharmacy, Texas A&M Health Science Center, Kingsville, Texas, United States

Abstract

Allosteric modulation of G-protein coupled receptors (GPCRs) represents a novel approach for fine-tuning GPCR functions. The cannabinoid CB1 receptor, a GPCR associated with the CNS, has been implicated in the treatment of drug addiction, pain, and appetite disorders. We report here the synthesis and pharmacological characterization of two indole-2-carboxamides: 5-chloro-3-ethyl-1-methyl-N-(4-(piperidin-1-yl)phenethyl)-1*H*-indole-2-carboxamide (ICAM-a) and 5-chloro-3-pentyl-N-(4-(piperidin-1-yl)phenethyl)-1*H*-indole-2-carboxamide (ICAM-b). While both ICAM-a and ICAM-b enhanced CP55,940 binding, ICAM-b exhibited the strongest positive cooperativity thus far demonstrated for enhancing agonist binding to the CB1 receptor. Although it displayed negative modulatory effects on G-protein-coupling to CB1, ICAM-b induced β-arrestin-mediated downstream activation of ERK signaling. These results indicate that this compound represents a novel class of CB1 ligands that produce biased signaling via CB1.

Keywords

GPCR; CB1; allosteric modulator; ligand binding; G protein-coupling; biased signaling

INTRODUCTION

The CB1 cannabinoid receptor belongs to the class A rhodopsin-like G protein coupled receptor family. It binds Δ^9-tetrahydrocannabinol (Δ^9-THC), an active ingredient of *Cannabis sativa*. CB1 receptors are localized on pre-synaptic nerve terminals and are thought to play a direct role in the inhibition of neurotransmitter release (Mackie & Hille 1992). The abundance of CB1 in the central nervous system and its implication in many disease states underscores its importance to human pharmacology and as a target in drug discovery for the treatment of nausea, obesity, pain, neurodegenerative diseases and substance abuse disorders (Harkany *et al.* 2007, Porter & Felder 2001, Smith *et al.* 2010).

[*]Corresponding authors: Debra A. Kendall, Department of Pharmaceutical Sciences, 69 N. Eagleville Road, Storrs, CT 06269-3092. Phone: (860) 486-1891; Fax: (860) 486-5792; debra.kendall@uconn.edu. Dai Lu, Department of Pharmaceutical Sciences, Irma Lerma Rangel College of Pharmacy, 1010 West Avenue B, Kingsville, Texas 78363. Phone: (361) 221-0745; Fax: (361) 593-4303; dlu@tamhsc.edu.

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A few compounds have been identified as allosteric modulators of the CB1 receptor including ORG27569, ORG29647, ORG27759 (Price *et al.* 2005), and PSNCBAM-1 (Horswill *et al.* 2007). Despite acting as positive allosteric modulators of agonist affinity, ORG compounds act as inhibitors of agonist-induced G-protein coupling. We have further demonstrated that the ORG27569-induced conformational change of the CB1 receptor leads to its cellular internalization and downstream activation of ERK signaling. These observations provided the first evidence of signaling-pathway-selective allosteric modulation via CB1 (Ahn et al. 2012). Moreover, we discovered that ORG27569 not only acts as an allosteric modulator for orthosteric agonist binding and its efficacy, but also impacts CB1 activity in the absence of orthosteric ligands. Given the vital role of CB1 in the central nervous system and its potential therapeutic value (Gardner & Vorel 1998, Harkany et al. 2007, Porter & Felder 2001), the development of its allosteric modulators with distinct therapeutic properties resulting from receptor-selectivity and signaling-pathway-selectivity provides a promising avenue by which the current obstacles in cannabinoid-based drug discovery such as on- and off-target side effects can be overcome.

Indole-2-carboxamides have been noted to have great potential as CB1 allosteric modulators (Piscitelli *et al.* 2012, Ross 2006). To better understand the impact of this class of compounds on the allosteric modulation of the CB1 receptor, we synthesized two indole-2-carboxamides: ICAM-a and ICAM-b (Figure 1), which have significant structural changes in comparison with ORG27569, and report here our characterization for their allostery on the CB1 receptor. We found that ICAM-b was the more potent modulator of CB1 with a very high cooperativity factor and ligand affinity shifts over 14-fold for CP55,940. Although ICAM-b inhibited agonist-induced and basal GTPγS binding, it still induced an activation of downstream ERK1/2 in the absence and presence of the orthosteric agonist CP55,940. Using a siRNA knockdown strategy, we demonstrated that ICAM-b-induced signaling is β-arrestin 1 dependent. These results elucidate β-arrestin-mediated CB1 biased signaling by an allosteric modulator.

MATERIALS AND METHODS

Synthesis of the ICAM compounds

The target compounds (ICAM-a and b) were prepared from commercially available ethyl 5-chloro-1*H*-indole-2-carboxylate **1** (Sigma-aldrich, St. Louis, MO) and 2-(4-(piperidin-1-yl)phenyl)ethanamine (Oakwood Products, Inc, West Columbia, SC) according to the synthetic routes described in Figure 1. The final compounds ICAM-a and ICAM-b exhibited poor solubility in common organic solvents except tetrahydrofuran (THF).

CB1 Expression and Membrane Preparation

HEK293 cells were transfected with the human CB1 cDNA cloned into pcDNA3.1 using the calcium phosphate precipitation method (Chen & Okayama 1987). 24 h post-transfection, membranes of transfected cells were prepared as described previously.(Ahn *et al.* 2009)

Radioligand Binding Assay

Assays used to determine the cooperativity between allosteric and orthosteric ligands have been detailed previously (Ahn et al. 2012). The total assay volume and the amount of membrane samples were adjusted to avoid ligand depletion by keeping the bound ligand less than 10% of the total.

GTPγS Binding Assay

GTPγS binding assays were performed as described previously (Ahn et al. 2012). The basal GTPγS binding was measured in the absence of ligand. Nonspecific binding was determined with 10 μM unlabeled GTPγS (Sigma).

Ligand and GTPγS Binding Data Analysis

All ligand binding assays and GTPγS binding assays were carried out in duplicate. Data are presented as the mean ± S.E. value or the mean with the corresponding 95% confidence limits from at least three independent experiments. The K_d, B_{max}, K_B and α cooperativity values were calculated by nonlinear regression as mentioned previously (Ahn et al. 2012).

Immunoblotting Studies

siRNA transfection was carried out as described (Ahn *et al.* 2003). Chemically synthesized, duplex siRNAs targeting β-arrestin 1 and β-arrestin 2, and control siRNA were purchased from Qiagen. The siRNA sequences targeting β-arrestin 1 and β-arrestin 2 are 5'-CTCGACGTTCTGCAAGGTCTA-3' and 5'-CTCGAACAAGATGACCAGGTA-3', respectively. A non-silencing RNA duplex as indicated by the manufacturer was used as control. HEK293 cells that were 40–50% confluent in a 6-well plate were transfected with 2.6 μg siRNA. Cells expressing CB1 receptors were exposed to the different compounds for the times indicated and immunoblotting was performed as described previously (Ahn et al. 2012) using phospho- and total ERK1/2 antibodies (1:3000 phospho-p44/42 and p44/42 antibodies; Cell Signaling Technology, Danvers, MA). Immunoblots were quantified with the ImageJ program (http://rsb.info.nih.gov/ij/). Data are expressed as mean ± S.E. Statistical significances were assessed by one-way analysis of variance followed by the Bonferroni's post hoc test.

RESULTS AND DISCUSSION

To elucidate the structural features of CB1 allosterism including the mode of binding of the allosteric modulator to the receptor, we synthesized two novel structural analogues of ORG27569 (i.e. ICAM-a and ICAM-b) as depicted in Figure 1. The two indole-2-carboxamides were obtained by coupling of 5-chloro-3-alkyl-indole-2-carbolic acids with 2-(4-(piperidin-1-yl)phenyl)ethanamine. Since it was noted that indole-2-carboxamides with lower alkyl group substitutions at C3 of the indole ring impact orthosteric ligand binding (Piscitelli et al. 2012), the C3 alkyl chain in ICAM-b was instead elongated from an ethyl to a *n*-pentyl group in comparison with ORG27569. In ICAM-a, only the nitrogen of the indole ring was methylated in comparison with ORG27569.

To evaluate the allosteric effects of the ICAM compounds on orthosteric ligand binding, we measured changes in specific binding of the CB1 agonist [^{3}H]CP55,940 and inverse agonist [^{3}H]SR141716A to the CB1 wild-type receptor in the presence of each ICAM compound at various concentrations. Although both compounds increased the level of specific [^{3}H]CP55,940 binding, ICAM-b showed a more drastic effect (Figure 2A) than ICAM-a. In contrast, when the inverse agonist [^{3}H]SR141716A was used as a tracer, ICAM-b exhibited a substantial decrease in [^{3}H]SR141716A binding while ICAM-a produced little effect in the concentration range tested (Figure 2B). Due to compound solubility limits and possible non-CB1-mediated secondary effects, concentrations over 32 μM ICAM-b were not tested in this study. These data suggest that ICAM-b exhibits a higher allosteric potency on orthosteric ligand binding than ICAM-a. Figure 2C shows the equilibrium dissociation constant (K_B) and the cooperactivity factor (α) for ICAM-a and ICAM-b determined from the [^{3}H]CP55,940 binding curves (Figure 2A). While ICAM-a showed only a weak enhancement of CP55,940 binding (K_B = 5778 nM and α value of 11.9), ICAM-b exhibited

a remarkably high cooperativity for CP55,940, evident by the K_B value of 469.9 nM and α value of 17.6. These data suggest that while the methylation of the indole-1-position of ORG27569 enhanced the α value, the structural change also substantially reduced K_B. Most strikingly, ICAM-b exhibited significantly higher cooperativity for CP55,940 than ORG27569 (Ahn et al. 2012). To our knowledge, such an impressively high α cooperativity factor has never been reported for CB1 and possibly for all GPCRs. Consistent with that result, ICAM-b exhibited a significant decrease in inverse agonist SR141716A binding (K_B = 831.6 nM and α value of 0.11) (Figure 2C).

Since ICAM-b exhibited exquisite allosteric effects on CP55,940 binding, saturation binding experiments were performed for [^{3}H]CP55,940 and [^{3}H]SR141716A in the presence and absence of ICAM-b to measure its impact on affinity shifts for both CB1 ligands. Consistent with the cooperativity factor determined above, the CB1 receptor exhibited a 14-fold higher affinity for CP55,940 in the presence of 3.2 μM ICAM-b (K_d = 0.16 nM) relative to that in the absence of ICAM-b (K_d = 2.27 nM) (Figure 2D). In contrast, the presence of ICAM-b shifted the K_d values for [^{3}H]SR141716A in the opposite direction as expected for an inverse agonist from 2.17 nM in the absence of ICAM-b to 20.03 nM in the presence of 3.2 μM ICAM-b (Figure 2D). Both binding affinity shifts for CP55,940 and SR141716A by the ICAM-b are more pronounced than any other CB1 allosteric modulators reported to date. Negligible B_{max} changes were observed in the presence of ICAM-b for both CP55,940 and SR141716A.

Given the strength of ICAM-b on CB1 agonist binding it was critical to evaluate the impact of the allosteric modulator on CP55,940-induced [^{35}S]GTPγS binding to CB1 in the presence of various concentrations of ICAM-b. Interestingly, we observed that ICAM-b inhibited CP55,940-induced [^{35}S]GTPγS binding in a concentration-dependent manner evident by a progressive decrease in the E_{max} values with an increasing concentration of ICAM-b for the CB1 receptor (Figure 3A). The addition of 0.32 and 1 μM ICAM-b resulted in a reduction in E_{max} values (E_{max} = 78.7 and 59.3 fmol/mg), respectively, compared with that in the absence of ICAM-b (E_{max} = 98.9 fmol/mg). Treatment with ICAM-b at 3.2 μM showed complete inhibition of CP55,940-induced [^{35}S]GTPγS binding. Moreover, the treatment with 3.2 μM ICAM-b further inhibited [^{35}S]GTPγS binding to a point (42.1 fmol/mg) that is below the basal level (in the absence of agonist) of [^{35}S]GTPγS binding (50.1 fmol/mg) observed for the CB1 wild-type receptor and that is comparable to the level observed in a mock-transfected sample (Figure 3B). These data suggest that ICAM-b binds the CB1 receptor and results in differential effects on agonist affinity and G protein signaling efficacy.

The opposite effects of ICAM-b on orthosteric ligand binding affinity and G-protein-coupling brought up questions as to whether the compound impacts downstream signaling. We therefore investigated the effect of ICAM-b on ERK1/2 phosphorylation, since its analog ORG27569 has been shown to activate G-protein-independent ERK1/2 in the absence and presence of agonist (Ahn et al. 2012). Additionally, we used an RNA interference technique to silence the expression of endogenous β-arrestin 1 or β-arrestin 2 to determine whether β-arrestins contribute to ERK1/2 activation induced by this compound. ERK1/2 phosphorylation reached maximal levels at 5 min of treatment with 0.5 μM CP55,940, 3.2 μM ICAM-b, or both and then rapidly declined by 10 min. Levels of total ERK1/2 (lower panel) are shown for comparison. It is noteworthy that although ICAM-b alone did not significantly alter the time course of ERK1/2 phosphorylation, it produced a larger increase in transient ERK1/2 phosphorylation compared with the stimulation by CP55,940 alone in HEK293 cells expressing CB1 (Figure 4A). Most strikingly, Figure 4B shows that the reduced expression of β-arrestin 1 nearly abolished ICAM-b-induced ERK1/2 phosphorylation, whereas co-transfection with β-arrestin 2 siRNA did not alter patterns of

ERK1/2 phosphorylation compared to those shown by control siRNA transfection (Figure 4C). In mock-transfected cells as control, suppression of β-arrestin expression showed no effect on ICAM-b-induced ERK1/2 phosphorylation (Figure 4D) suggesting that ICAM-b does not induce non-CB1-mediated ERK1/2 phosphorylation in HEK293 cells. Taken together, these results indicate that β-arrestin 1, but not β-arrestin 2, is key to ICAM-b-induced ERK1/2 phosphorylation (Figure 4E).

Collectively, our current investigation of the indole-2-carboxamides provides evidence that the indole-2-carboxamide is a viable template for developing allosteric ligands for the CB1 receptor. To obtain enhanced orthosteric ligand binding activity, a lengthened C3 alkyl chain on the indole-2-carboxamide yielded a highly enhanced α value and similar K_B relative to ORG27569. Elongation of the alkyl group at this position enhanced the orthosteric ligand binding, possibly through increased hydrophobic interactions with their binding site. Unlike the conformation induced by orthosteric agonist alone, this modulator-induced conformation excludes G-protein from association with the CB1 receptor even while bound to the orthosteric agonist, CP55,940. More importantly, ICAM-b induced β-arrestin 1-mediated pathway-biased signaling, which provides new opportunities for regulating the function of the CB1 receptor to selectively activate a specific signaling pathway. Such functional selectivity (biased agonism) is hypothesized to result from ligand-dependent stabilization of receptor conformations that favor coupling to specific downstream effectors. This selective modulation increases the specificity of the GPCR for pharmaceutical applications. Thus, allosteric modulators offer significant promise for the development of new classes of GPCR-targeted drugs.

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The abbreviations used are

GPCR	G-protein coupled receptor
CB1	cannabinoid receptor 1
ORG27569	5-chloro-3-ethyl-1H-indole-2-carboxylic acid [2-(4-piperidin-1-yl-phenyl)-ethyl]-amide
CP55,940	(1*R*,3*R*,4*R*)-3-[2-hydroxy-4-(1,1-dimethylheptyl)-phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol
SR141716A	*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide
HEK293	human embryonic kidney cells
GTPγS	guanosine 5'-3-O-(thio)triphosphate
ERK	extracellular signal-regulated kinase

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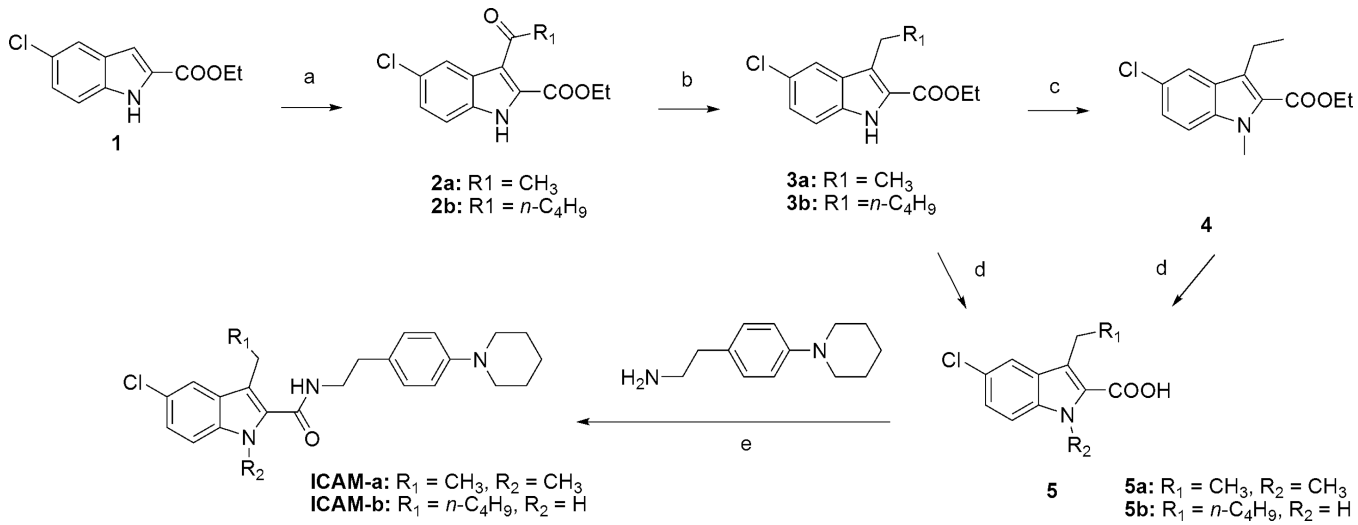
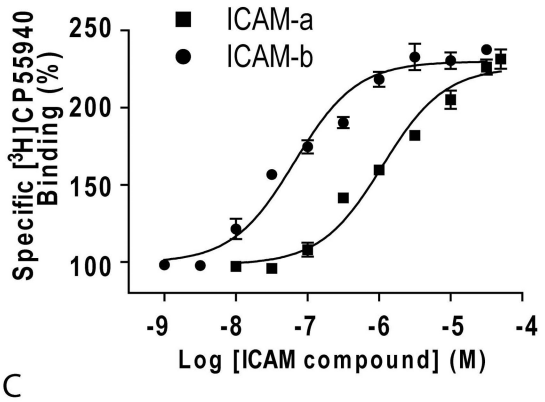


Figure 1. Synthesis and structure of ICAM compounds

Reagents and conditions: a) R_1COOH, $(CF_3CO)_2O$, H_3PO_4, CH_3CN, rt, 12h; b) Et_3SiH, CF_3COOH, 0°C-rt, 24h; c) LiOH, THF/H_2O, 60°C 36h; d) CH_3I, K_2CO_3, DMF, rt 12h; e) HATU, EDCI, *N*-Methyl-morpholine, DMF, rt, 12h.

A



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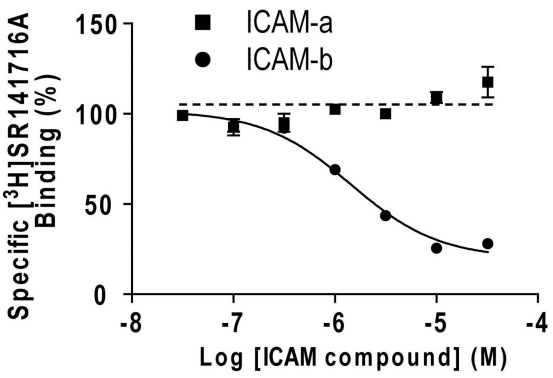

C

	[3H]CP55940				[3H]SR141716A			
	ICAM-a		ICAM-b		ICAM-a		ICAM-b	
	K_B (nM)	Cooperativity factor (α)	K_B (nM)	Cooperativity factor (α)	K_B (nM)	Cooperativity factor (α)	K_B (nM)	Cooperativity factor (α)
CB1 WT	5778 (3108-10740)	11.9	469.9 (126.2-1750)	17.6	NC[a]	NC[a]	831.6 (567.3 – 1219)	0.11

[a] NC, no significant change in [3H]SR141716A binding was observed up to 32 μM ICAM-a.

D

	[3H] CP55940					[3H] SR141716A				
	Vehicle alone		ICAM-b		Fold change	Vehicle alone		ICAM-b		Fold change
	K_d (nM)	B_{max} (fmol/mg)	K_d (nM)	B_{max} (fmol/mg)	(Vehicle: KM05/51)	K_d (nM)	B_{max} (fmol/mg)	K_d (nM)	B_{max} (fmol/mg)	(Vehicle: KM05/51)
CB1 WT	2.33±0.43	3072±256	0.16±0.02	5186±147	15:1	2.17±0.42	5616±288	20.03±2.25	5977±324	1:9

Figure 2. Effect of ICAM-a and ICAM-b on [^{3}H]CP55,940 and [^{3}H]SR141716A binding

Binding assays were performed using (A) [^{3}H]CP55,940 and (B) [^{3}H]SR141716A on membrane preparations from HEK293 cells expressing CB1 in the presence of the ICAM-a (■) or ICAM-b (●) as described in Materials and Methods. Each data point represents the mean ± S.E. of three independent experiments performed in duplicate. (C) Binding parameters of the ICAM-a and ICAM-b compounds to CB1 receptors were determined using [^{3}H]CP55,940 and [^{3}H]SR141716A. Note that since ICAM-a produced no effect (indicated by dashed line) on [^{3}H]SR141716A binding, the K_B and α values for binding of [^{3}H]SR141716A were not determined. (D) Saturation binding parameters for [^{3}H]CP55,940 and [^{3}H]SR141716A were determined in the presence and absence of 3.2 μM ICAM-b. Data are the mean ± S.E. of three independent experiments performed in duplicate.

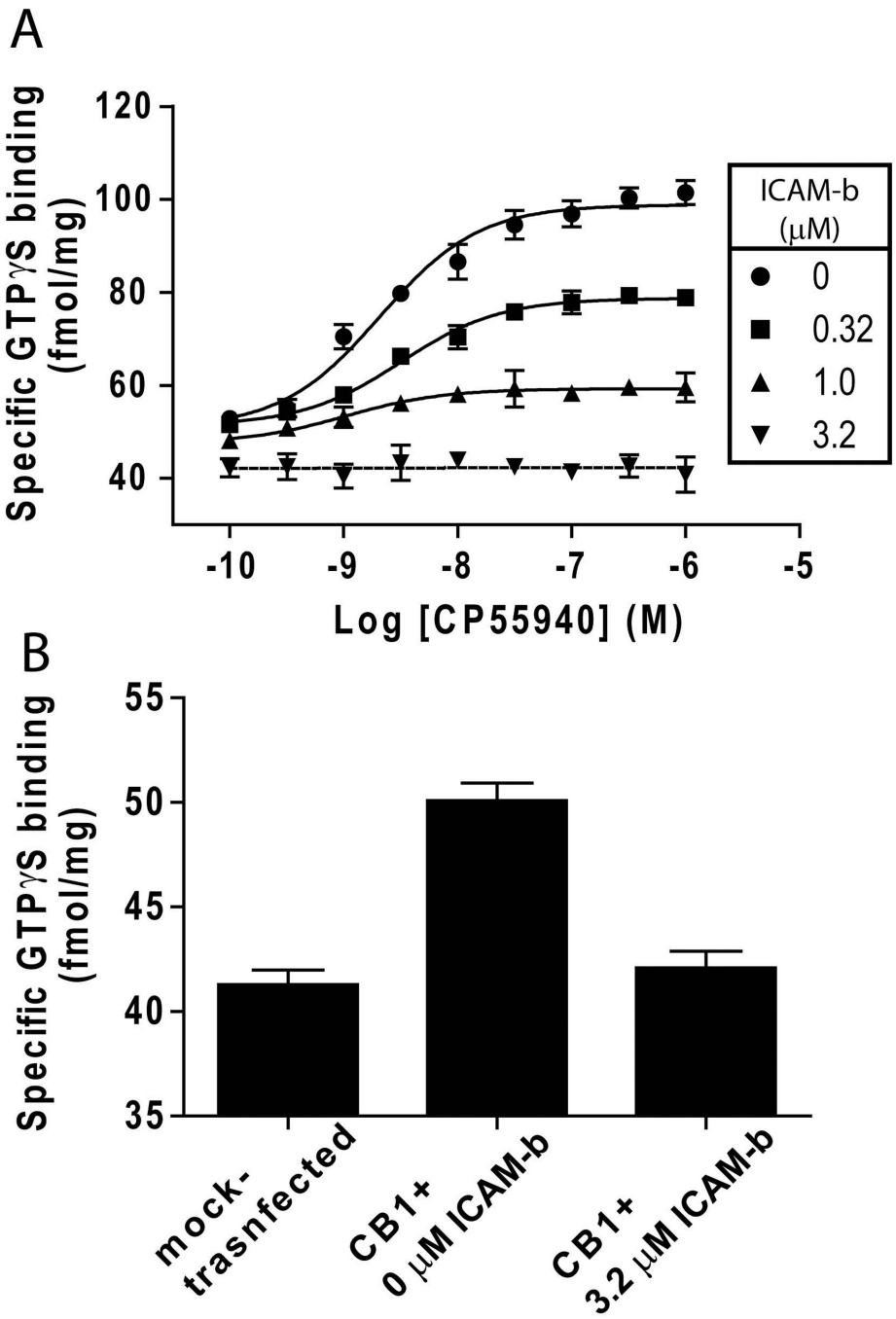

Figure 3. Effect of ICAM-b on the stimulation of [^{35}S]GTPγS binding to HEK293 cell membranes expressing the CB1 wild-type receptor

(A) Dose-response curves for CP55,940-induced [^{35}S]GTPγS binding in membrane preparations of HEK293 cells expressing the wild-type receptor in the absence (●) or presence of 0.32 μM (■), 1 μM (▲), and 3.2 μM (▼) ICAM-b. Dashed line indicates no stimulation of [^{35}S]GTPγS binding. (B) Inhibition of basal [^{35}S]GTPγS binding by ICAM-b. The level of non CB1-mediated GTPγS binding was obtained from [^{35}S]GTPγS binding to the mock-transfected membrane sample. Data are presented as specific binding of GTPγS to the membranes. Nonspecific binding was determined in the presence of 10 μM unlabeled

GTPγS. Each data point represents the mean ± S.E. of at least three independent experiments performed in duplicate.

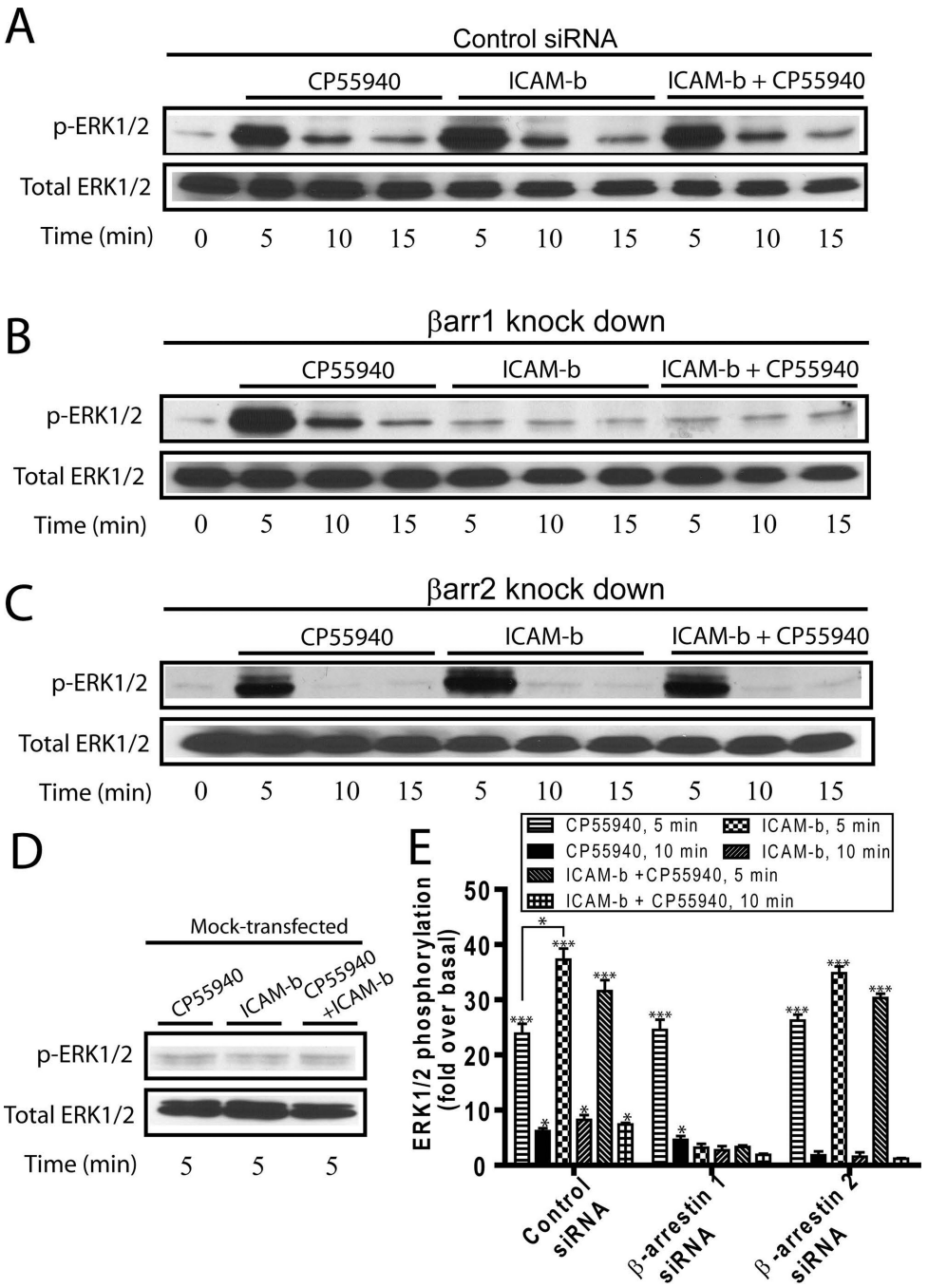

Figure 4. Effect of siRNA-mediated suppression of β-arrestin levels on ICAM-b-stimulated ERK1/2 phosphorylation

HEK293 cells co-expressing CB1 and either (A) control, (B) β-arrestin 1, or (C) β-arrestin 2 siRNAs were exposed to CP55,940 (0.5 μM) or ORG27569 (10 μM) for 0, 5, 10 or 15 min as indicated. (D) Mock-transfected HEK293 cells were used as control. The immunoblotting study was performed as described in Materials and Methods. Representative blots of phosphorylated and total ERK1/2 of at least three separate experiments are shown for each condition. Note that the two bands correspond to the predominant isoforms, p42 (ERK2) and p44 (ERK1) for ERK signaling. (E) Graphs provide the quantified ERK1/2 phosphorylation levels for 5 min and 10 min deduced from the mean ± S.E. of at least three experiments.

Data are expressed as the fold increase above the basal level of phosphorylation. The statistical significance of the differences between each data point and the basal level under corresponding conditions was assessed using one-way analysis of variance and Bonferroni's post –hoc test; *, $p < 0.05$; ***, $p < 0.001$. ERK1/2 phosphorylation levels with CP55,940 alone and ICAM-b alone under control siRNA-transfected conditions are compared and their statistical significance is indicated by a bracket and an asterisk.