Integrin clustering enables anandamide-induced Ca^{2+} signaling in endothelial cells via GPR55 by protection against CB_1-receptor-triggered repression

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Accepted 17 February 2008
Journal of Cell Science 121, 1704-1717 Published by The Company of Biologists 2008
doi:10.1242/jcs.020958

Summary

Although the endocannabinoid anandamide is frequently described to act predominantly in the cardiovascular system, the molecular mechanisms of its signaling remained unclear. In human endothelial cells, two receptors for anandamide were found, which were characterized as cannabinoid 1 receptor (CB_1R; CNR1) and G-protein-coupled receptor 55 (GPR55). Both receptors trigger distinct signaling pathways. It crucially depends on the activation status of integrins which signaling cascade becomes promoted upon anandamide stimulation. Under conditions of inactive integrins, anandamide initiates CB_1R-derived signaling, including G_i-protein-mediated activation of spleen tyrosine kinase (Syk), resulting in NFκB translocation. Furthermore, Syk inhibits phosphoinositide 3-kinase (PI3K) that represents a key protein in the transduction of GPR55-originated signaling. However, once integrins are clustered, CB_1R splits from integrins and, thus, Syk cannot further inhibit GPR55-triggered signaling resulting in intracellular Ca^{2+} mobilization from the endoplasmic reticulum (ER) via a PI3K-Bmx-phospholipase C (PLC) pathway and activation of nuclear factor of activated T-cells. Altogether, these data demonstrate that the physiological effects of anandamide on endothelial cells depend on the status of integrin clustering.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/121/10/1704/DC1

Key words: Anandamide, Bmx/Etk, Cannabinoid signaling, CB_1 receptor, GPR55, Ca^{2+} signaling, Integrins, Syk

Introduction

The endogenous agonists of cannabinoid receptors exhibit multiple biological functions in various tissues, such as neurons, the immune system and the cardiovasculature (Hillard, 2000; Randall et al., 2002). Among these endocannabinoids, arachidonoylethanolamide (anandamide) recognizes several receptors (Di Marzo et al., 2002) and acts predominantly in the cardiovascular system (Randall and Kendall, 1997; Randall and Kendall, 1998). Anandamide is produced by vascular endothelial cells (Deutsch et al., 1997; Sugiura et al., 1998) that express either the cannabinoid 1 receptor [CB_1R; CNR1 (Maccarrone et al., 2000)] or the CB_2 receptor [CB_2R; CNR2 (Zoratti et al., 2003)], depending on the source and species of the cells. Notably, most of our recent knowledge on the CB receptor type underlying anandamide-induced physiological effects is based on the use of two selective inhibitors of CB_1R and CB_2R: rimonabant (SR141716A; N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride) (Rinaldi-Carmona et al., 1994; Showalter et al., 1996) and SR144528 [N-[(1S)-endo-1,3,3-trimethyl bicyclo[2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazo le-3-carboxamide (Griffin et al., 2000; Rinaldi-Carmona et al., 1998; Showalter et al., 1996)], respectively. Endothelium-dependent relaxation was found to be sensitive to rimonabant (Chaytor et al., 1999; Mukhopadhyay et al., 2002; Wagner et al., 1999; White and Hiley, 1997) but not to gap junction inhibitors (Chaytor et al., 1999) and blockade of the vanilloid receptor 1 (Grainger and Boachie-Ansah, 2001; Mukhopadhyay et al., 2002), another putative receptor of anandamide on endothelial cells. However, in mice that lack CB_1R and CB_2R, anandamide-induced mesenteric vasodilation was still prevented by rimonabant (Offertaler et al., 2003), thus, leading to the concept of an 'atypical' endothelial anandamide receptor (e-aR) that is also sensitive to rimonabant. Hence, this receptor was found to be activated by the cannabinoid analogs abnormal cannabidiol (–)-4-(3-3,4-trans-p-menthadien-[1,8]-yl)-olivetol) and O1602 [5-methyl-4-[(1R,6R)-3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-1,3-benzenediol; an agonist of 'atypical' non-CB_1/CB_2 endothelial anandamide receptor (Offertaler et al., 2003), and to be inhibited by O1918 (Offertaler et al., 2003). The e-aR was shown to be coupled to $G_{i/o}$ protein and to trigger Ca^{2+}-activated formation of nitric oxide in endothelial cells (Mukhopadhyay et al., 2002).

Considering the large amount of recent reports using rimonabant and the variety of vascular effects of anandamide, which include inhibition of endothelin 1 expression (Ronco et al., 2006), relaxation of mesenteric arteries (Hoi and Hiley, 2006), or the inhibition of CB_1R-mediated tumor growth and metastatic spreading (Portella et al., 2003), endocannabinoid-triggered signaling in endothelial cells needs to be explored in more detail.

Despite the intriguing reports on the existence of e-aR in human endothelial cells, most reports on endothelial cells did not

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differentiate between the signal transduction triggered by the two receptor types present. Thus, the underlying signal transduction cascades beyond each individual receptor type are still unclear. Moreover, as these receptors are presumably activated by anandamide at the same time, interplay between the two signaling cascades activated by each of these receptors may occur and needs further investigation.

Consequently, in this study we intended to explore the signaling cascades downstream of CB_1R and e-aR in human endothelial cells. Furthermore, the interrelation and integration of the two signaling pathways on the initiation of cytosolic Ca^{2+} signaling as a highly specific readout of activation of e-aR were investigated.

Results

The effect of anandamide on endothelial Ca^{2+} signaling inversely depended on external Ca^{2+} and was due to atypical endothelial anandamide receptors

In the presence of 2 mM external Ca^{2+}, addition of 10 μM anandamide yielded very small cytosolic Ca^{2+} elevation in only 10% of the cells, which was far less than that obtained with 100 μM histamine under identical conditions (Fig. 1A). Similarly, the hydrolytically more stable analog methanandamide failed to initiate Ca^{2+} elevation in the presence of external Ca^{2+}, thus excluding Ca^{2+}-dependent hydrolysis as being responsible for the lack of Ca^{2+} signaling in response to anandamide in the presence of external Ca^{2+}.

However, in the nominal absence of extracellular Ca^{2+}, both agonists increased cytosolic Ca^{2+} levels in endothelial cells (Fig. 1B) and, similar to histamine, over 95% of the cells responded to the endocannabinoid while there was no effect upon stimulation with 10 μM arachidonic acid (supplementary material Fig. S1). Moreover, consequent to a strong endoplasmic reticulum (ER) depletion by either anandamide or histamine (Fig. 1C), capacitative Ca^{2+} entry was detected upon re-addition of extracellular Ca^{2+} (Fig. 1B). The EC_{50} for anandamide to initiate Ca^{2+} signaling in nominally Ca^{2+}-free solution was 7.3 (4.5-11.7) μM (n=10), which was comparable with that of the metabolically stable analog methanandamide [EC_{50}=10.6 (9.9-11.3) μM, n=10] (Fig. 1D), thus indicating that anandamide-induced Ca^{2+} signaling is directly evoked by the compound itself and not by a metabolite.

To define the receptor being involved in anandamide-triggered endothelial Ca^{2+} signaling, the expression of CB_1R but not CB_2R was confirmed by RT-PCR in the human endothelial cells used in this study (Fig. 2A). Involvement of CB_2R was further excluded by the lack of the CB_2R antagonist SR144528 [1 μM (Rinaldi-Carmona et al., 1998)] (Table 1) to inhibit anandamide-induced Ca^{2+} signaling in the nominal absence of extracellular Ca^{2+} (supplementary material Fig. S2A). Similarly, inhibition of TRPV1 (VR1) receptors by SB366791 [4′-chloro-3-methoxycinnamanilide (Fowler et al., 2003)] (10 μM) failed to affect anandamide-induced Ca^{2+} signaling under the same conditions (supplementary material Fig. S2B). By contrast, rimonabant (1 μM), an inhibitor of CB_1R and e-aR (Jarai et al., 1999; Wagner et al., 1999), inhibited anandamide-induced Ca^{2+} signaling in nominally Ca^{2+}-free solution (Fig. 2B).

To further differentiate whether CB_1R or e-aR is involved in anandamide-induced Ca^{2+} signaling, the effects of specific receptor agonists and antagonists were tested. Inhibition of CB_1R by 10 μM of the potent CB_1R antagonist AM251 [N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide] (Table 1) (Pertwee, 2005) unmasked anandamide

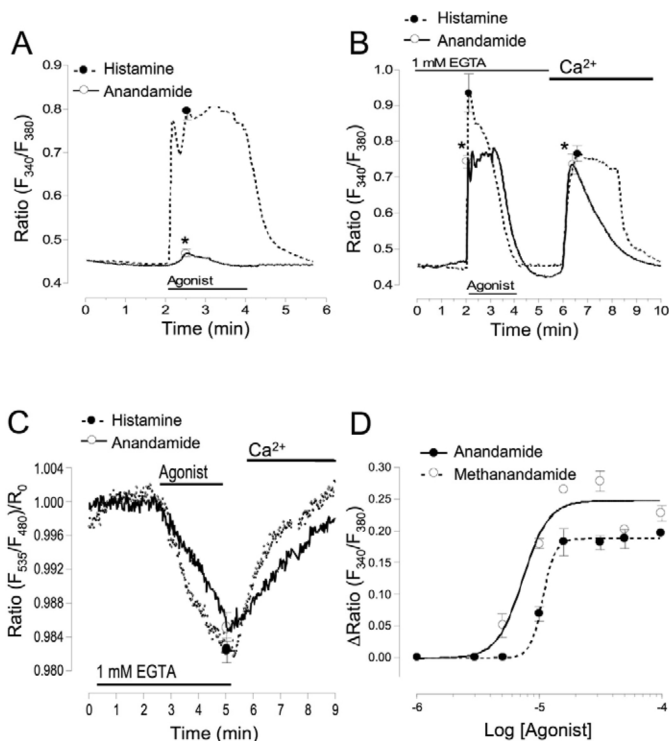

Fig. 1. Endocannabinoids trigger Ca^{2+} signaling and ER depletion in human endothelial cells. (A) Comparison of the effects of 100 μM histamine (n=18) and 10 μM anandamide (n=31) on cytosolic free-Ca^{2+} concentration in the presence of 2 mM extracellular Ca^{2+}. (B) Cytoplasmic free-Ca^{2+} concentration upon stimulation with 100 μM histamine (n=19) and 10 μM anandamide (n=21) in the absence of extracellular Ca^{2+}, followed by re-addition of 2 mM extracellular Ca^{2+} to reveal store-operated Ca^{2+} entry due to previous ER depletion. (C) Comparison of the effects of 100 μM histamine (n=4) and 10 μM anandamide (n=3) on free ER Ca^{2+} concentration in the absence of extracellular Ca^{2+}, followed by re-addition of 2 mM extracellular Ca^{2+} to visualize ER Ca^{2+} refilling efficiency. (D) Concentration-response curves for anandamide (n=10) and metanandamide (n=10) on peak responses of cytosolic Ca^{2+} elevation in the nominal absence of extracellular Ca^{2+}. Cytosolic (A,B,D) and ER (C) Ca^{2+} concentrations were recorded using fura-2 and YC4er, respectively. *P<0.01 versus histamine.

(10 μM)-induced Ca^{2+} signaling even in the presence of extracellular Ca^{2+} (Fig. 2C). In addition, the synthetic CB_1R agonist HU-210 [10 μM (Felder et al., 1995)], which has been shown to couple with intracellular Ca^{2+} signaling in HEK 293 cells, cultured hippocampal neurons (Lauckner et al., 2005) and rat insulinoma beta cells (De Petrocellis et al., 2007), failed to initiate endothelial Ca^{2+} signaling in the presence and nominal absence of external Ca^{2+} (supplementary material Fig. S2C). By contract, 10 μM of O1602, an agonist of the e-aR (Table 1) (Bukoski et al., 2002; Jarai et al., 1999) evoked a very strong cytosolic Ca^{2+} elevation even in the presence of extracellular Ca^{2+} (Fig. 2D), which was not affected by the TRPV1 receptor inhibitor SB366791 (10 μM) (supplementary material Fig. S2D). In line with these findings, 10 μM of O1918 [1,3-dimethoxy-5-methyl-2-[(1R,6R)-3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-benzene] (Table 1), a cannabidiol analog that acts as a selective antagonist of ‘atypical’ cannabidiol at the non-CB_1/CB_2 endothelial anandamide receptor (Jarai et al., 1999; Mo et al., 2004; Offertaler et al., 2003), prevented anandamide-induced Ca^{2+} signaling in the nominal absence of extracellular Ca^{2+} (Fig.

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Table 1. Summary of the effect of tools used for the characterization of receptors involved in anandamide-triggered Ca^{2+} signaling in endothelial cells

Compound (Figure)	Characteristics	Concentration (μM)	Effect	References
LPI (suppl. Fig. S2G,H)	GPR55 agonist	10	Triggered Ca^{2+} signaling	(Oka et al., 2007)
HU210 (Fig. 2F, suppl. Fig. S2C)	CB_1R agonist	10	Prevented Ca^{2+} signaling upon AEA	(Felder et al., 1995; Fischbach et al., 2007)
O1602 (Fig. 2D,F,K, suppl. Fig. S2D)	e-aR/GPR55 agonist	10	Triggered Ca^{2+} signaling even in Ca^{2+} buffer	(Bukoski et al., 2002; Jarai et al., 1999; Johns et al., 2007)
O1618 (Fig. 2E)	e-aR/GPR55 antagonist (?)	10	Prevented Ca^{2+} signaling to AEA	(Jarai et al., 1999; Mo et al., 2004; Offertaler et al., 2003)
Rimonabant (Fig. 2B)	CB_1R and e-aR antagonist	1	Prevented Ca^{2+} signaling to AEA	(Jarai et al., 1999; Wagner et al., 1999)
AM251 (Fig. 2C)	CB_1R antagonist	10	Unmasked Ca^{2+} signaling to AEA in Ca^{2+} buffer	(Fischbach et al., 2007)
SR144528 (suppl. Fig. S2A)	CB_2R antagonist	1	No effect	(Rinaldi-Carmona et al., 1998)
SB366791 (suppl. Fig. S2B)	TRPV1R antagonist	10	No effect	(Boudaka et al., 2007)
GPR55 siRNA (Fig. 2H,I, suppl. Fig. S2H)	Knockdown of GPR55		Diminished Ca^{2+} signaling upon AEA, O1602, LPI	
GPR55 (Fig. 2J,K, suppl. Fig. S2H)	Overexpression of GPR55		Enhanced Ca^{2+} signaling to AEA, O1602, LPI	

2E). Moreover, in the presence of the CB_1R-specific agonist HU-210 (10 μM), activation of the e-aR with 10 μM O1602 failed to evoke Ca^{2+} signaling, while, after washout of HU-210, O1602 initiated Ca^{2+} signaling in the same cells (Fig. 2F). These data point to involvement of both anandamide receptors in endothelial cells in which CB_1R inhibits the signal cascade beyond e-aR, which yields intracellular Ca^{2+} mobilization and becomes overt in the nominal absence of external Ca^{2+}.

The 'atypical' non-CB_1 and CB_2 endothelial anandamide receptor (e-aR) was identified as G-protein-coupled receptor 55 (GPR55)

To identify the e-aR molecularly, the endothelial cells used for this study were tested for GPR55, a putative anandamide-binding protein (Baker et al., 2006; Ryberg et al., 2007). Using the respective primers, mRNA of GPR55 was found in endothelial cells (Fig. 2G). Hence, treatment with siRNA against GPR55, which was proved to reduce the expression of GPR55 by ~56% (supplementary material Fig. S2E), strongly diminished anandamide-triggered Ca^{2+} signaling in Ca^{2+}-free solution, as well as the effect of O1602 in the presence of extracellular Ca^{2+} (Fig. 2H,I, respectively), thus pointing to the involvement of GPR55 in anandamide-induced Ca^{2+} signaling in endothelial cells. Overexpression of GPR55 (supplementary material Fig. S2F) strongly elevated anandamide-triggered Ca^{2+} signaling in the nominal absence of extracellular Ca^{2+} (Fig. 2J). Consistent with this, the effect of O1602 (10 μM) on endothelial cytosolic free-Ca^{2+} was largely elevated in GPR55-overexpressing cells (Fig. 2K, Table 1).

Recently, GPR55 was described as a receptor of lysophosphatidylinositol [LPI (Oka et al., 2007)]. These data could be confirmed in endothelial cells in which the effect of 10 μM LPI was strongly reduced in cells treated with siRNA against GPR55 (supplementary material Fig. S2G), whereas overexpression of GPR55 yield enhanced Ca^{2+} signaling by LPI (supplementary material Fig. S2H).

The effect of anandamide on endothelial Ca^{2+} signaling was sensitive to divalent cations acting on extracellular bindings sites

Based on the unusual sensitivity of anandamide-triggered Ca^{2+} signaling to external Ca^{2+}, we tested whether or not the inhibitory effect of extracellular Ca^{2+} was due to Ca^{2+}-dependent chelating of anandamide in the medium. Using NMR spectroscopy, no evidence for a Ca^{2+}-anadamide complex was found (supplementary material Fig. S3A,B). Moreover, anandamide uptake and binding did not differ in the nominal absence or presence of extracellular Ca^{2+} (supplementary material Fig. S3C). The concentration-response curve of the inhibitory action of extracellular Ca^{2+} on anandamide-triggered intracellular Ca^{2+} signaling revealed an IC_{50} of 7.7 (6.7-8.8) μM (Fig. 3A). Similarly, Sr^{2+} and Ba^{2+} inhibited anandamide-triggered Ca^{2+} signaling in endothelial cells in a concentration-dependent manner (Fig. 3B). These results point to an extracellular Ca^{2+}-binding site being responsible for its inhibitory effect on anandamide-induced Ca^{2+} signaling.

Clustered αvβ3 and α5β1 integrins were involved in anandamide-induced Ca^{2+} signaling in human endothelial cells

Remarkably, the IC_{50} of extracellular Ca^{2+} to prevent anandamide-induced Ca^{2+} signaling was strikingly similar to that found for Ca^{2+}-mediated inhibition of integrin clustering (Leitinger et al., 2000). Therefore, involvement of clustered integrins in the transition of anandamide to evoke Ca^{2+} signaling was postulated. To challenge this hypothesis, clustering of integrins was initiated by Mn^{2+}, which increases the affinity of integrins for their ligand and can stimulate integrin clustering (Mould et al., 1998; Smith and Cheresh, 1990), prior to measurement of the effect of anandamide on endothelial Ca^{2+} concentration. Notably, in the presence of 70 μM Mn^{2+}, anandamide yielded strong Ca^{2+} elevation even in the presence of extracellular Ca^{2+} (Fig. 3C). In line with these results, inhibition of RhoA-associated kinase 1 and RhoA-associated kinase 2 (ROCK1 and ROCK2) that are essentially involved in the clustering of integrins (Rodriguez-Fernandez et al., 2001), by Y27632 [(R)-(+)-trans-N-(4-Pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide] [10 μM (Uehata et al., 1997)] strongly diminished anandamide-induced Ca^{2+} signaling in the absence of extracellular Ca^{2+} (Fig. 3D). Involvement of integrins was further confirmed by the inhibitory activities of fibronectin (20 μg/ml, supplementary material Fig. S3D), a large extracellular matrix protein ligand for endothelial integrins, and RGD, Arg-Gly-Asp peptide resembling the integrin-binding motif of fibronectin (Ruoslahti and Pierschbacher, 1987) (20 μg/ml; supplementary material Fig. S3E).

To further challenge the concept that integrins are involved in the process of anandamide-triggered Ca^{2+} signaling, the effects

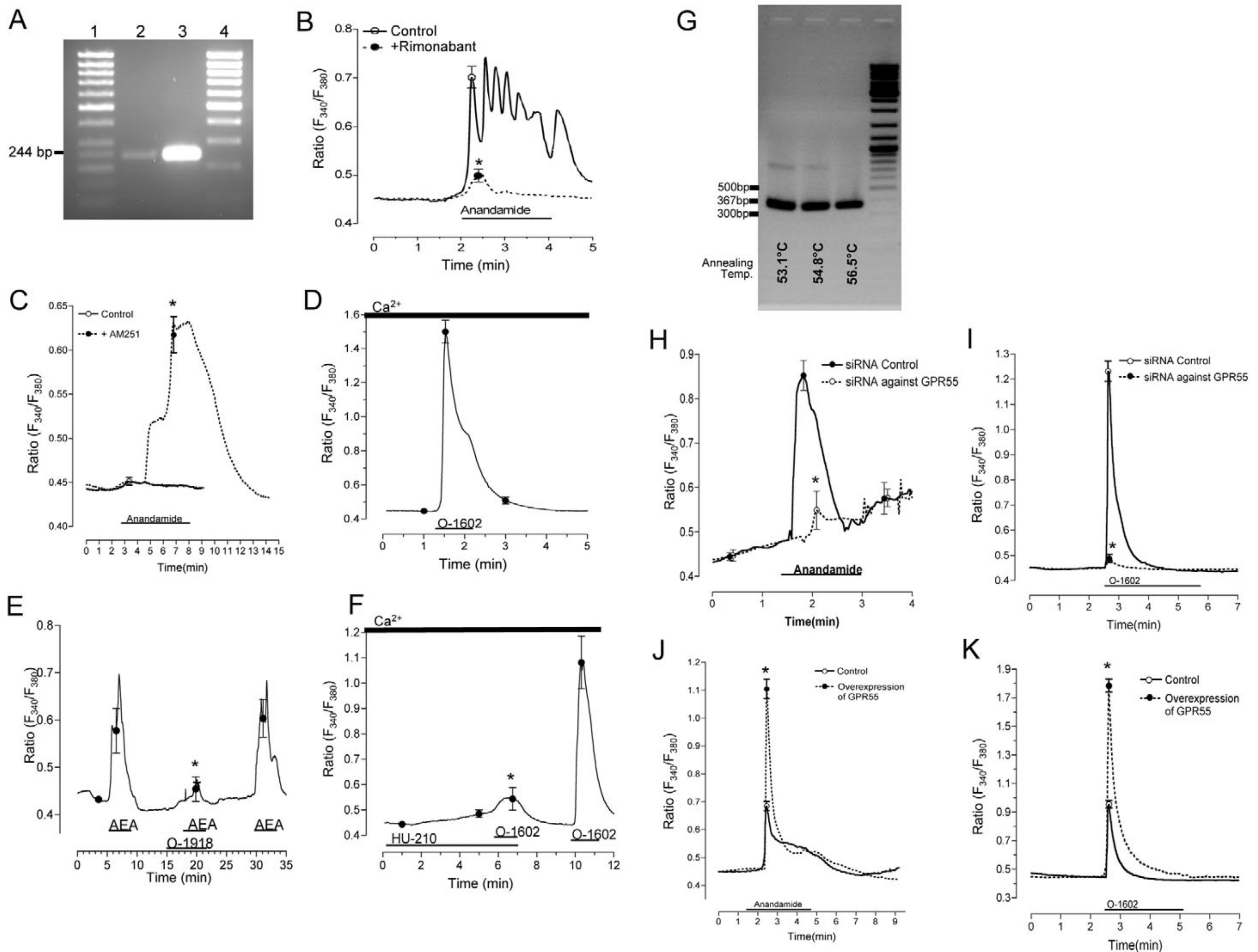

Fig. 2. 'Atypical' endothelial anandamide receptors are identified as GPR55 that mediates anandamide-initiated Ca^{2+} signaling, while activation of CB_1 receptors prevent anandamide-induced Ca^{2+} signaling. (A) Representative RT-PCR on CB_1R in a human endothelial cell line (lane 2) in comparison with human brain as positive control (lane 3). (B) In Ca^{2+}-free solution, the effect of 10 μM anandamide on cytosolic free-Ca^{2+} concentration was analyzed in the absence (*n*=19) or presence of the CB_1R antagonist rimonabant (1 μM) (*n*=28). (C) In the presence of the CB_1R antagonist AM251 (10 μM), 10 μM anandamide triggered Ca^{2+} signaling even in the presence of extracellular Ca^{2+} (*n*=59). (D) In the presence of 2 mM extracellular Ca^{2+}, the effect of the e-aR agonist O1602 (10 μM) on cytosolic free-Ca^{2+} concentration was tested (*n*=17). (E) In the absence of extracellular Ca^{2+}, the effect of O1918 (10 μM), an inhibitor of the e-aR, on anandamide (10 μM, AEA) -induced Ca^{2+} signaling was assessed (*n*=8). (F) The effect of the CB_1R agonist HU-210 (10 μM) on O1602 (e-aR agonist, 10 μM) -induced Ca^{2+} signaling was tested in the presence of 2 mM extracellular Ca^{2+} (*n*=10). (G) Representative RT-PCR on GPR55 in the human endothelial cell line using various annealing temperatures. (H) Pretreatment with siRNA against GPR55 (*n*=49; control, *n*=48) on anandamide (10 μM) -induced cytosolic Ca^{2+} signaling was monitored in nominally Ca^{2+}-free solution. (I) Pretreatment with siRNA against GPR55 (*n*=62; control, *n*=18) on O1602 (10 μM) -induced cytosolic Ca^{2+} signaling in Ca^{2+}-containing solution. (J) Consequences of GPR55 overexpression on anandamide (AEA, 10 μM) -induced Ca^{2+} signaling in the nominal absence of extracellular Ca^{2+} (*n*=43; control, *n*=42). (K) Consequences of GPR55 overexpression on O1602 (10 μM) -induced Ca^{2+} signaling in the nominal absence of extracellular Ca^{2+} (*n*=21; control, *n*=25). Cytosolic free-Ca^{2+} concentrations were recorded using fura-2. **P*<0.005 versus the absence of the respective receptor blocker/activator.

of the crosslinking antibody against αvβ3 integrin [LM609, 2 μM (Charo et al., 1990; Cheresh, 1987)] and two function-blocking antibodies against the β1 [JB1A, 2 μM (Mohri et al., 1996)] and the β3 subunits [B3A, 2 μM (Vignali et al., 1990)]. During preincubation for 15 minutes in Ca^{2+}-containing medium, none of the integrin-targeting antibodies affected basal endothelial Ca^{2+} levels, while all prevented the effect of anandamide in the absence of extracellular Ca^{2+} (Fig. 3E-G). In line with these findings, B3A also prevented O1602-induced Ca^{2+} signaling in Ca^{2+}-containing buffer (Fig. 3H). These data point to the involvement of αvβ3 and α5β1 integrins in GPR55-mediated Ca^{2+} signaling by anandamide and O1602 in endothelial cells.

Anandamide induced diverse tyrosine phosphorylation patterns depending on the presence of extracellular Ca^{2+}

As tyrosine kinases are known to represent early downstream targets of integrin-mediated signaling, the effect of anandamide on the activity of endothelial tyrosine kinases [using the FRET-based sensor Picchu 936X (Kurokawa et al., 2001; Schaeffer et al., 2003)] and the patterns of tyrosine phosphorylation were assessed in the

Table 2. Summary of the effect of tools used for the characterization of signaling cascades downstream of endothelial anandamide receptors

Compound (Figure)	Characteristics	Concentration	Effect
PP1/PP2 (Fig. 4, suppl. Fig. S4B)	Src family kinase inhibitors	10 μM	Prevented AEA and O-1602-triggered Ca^{2+} signaling; inhibited protein phosphorylation in response to AEA
Wortmannin (Fig. 5A,B, suppl. Fig. S5)	PI3K inhibitor	0.1 μM	Prevented Ca^{2+} signaling upon AEA (Ca^{2+}-free buffer) as well as that in response to O-1602 in Ca^{2+}-containing buffer
U73122 (Fig. 5C,D)	PLC inhibitor	10 μM	Reduced Ca^{2+} signaling upon AEA (Ca^{2+}-free buffer) as well as that in response to O-1602 in Ca^{2+}-containing buffer
LFM-A13 (Fig. 6C,D)	Bmx/Etk inhibitor	10 μM	Prevented Ca^{2+} signaling upon AEA (Ca^{2+}-free buffer) as well as that in response to O-1602 in Ca^{2+}-containing buffer
Bmx/Etk siRNA (Fig. 6B)	Knockdown of Bmx/Etk		Diminished Ca^{2+} signaling upon AEA (Ca^{2+}-free buffer) as well as that in response to O-1602 in Ca^{2+}-containing buffer
Piceatannol (Fig. 7A)	Syk inhibitor	5 μM	Unmasked Ca^{2+} signaling to AEA in Ca^{2+}-containing buffer
Syk siRNA (Fig. 7B)	Knockdown of Syk		Unmasked Ca^{2+} signaling to AEA in Ca^{2+}-containing buffer
PTX (Fig. 7C)	Inhibiton of $G\alpha_{i/o}$	400 ng/ml	Unmasked Ca^{2+} signaling to AEA in Ca^{2+}-containing buffer

presence and absence of extracellular Ca^{2+}. Notably, anandamide yielded fast activation of PP1-sensitive tyrosine kinases under any conditions indicated by increase in the FRET signal of Picchu 936X (Fig. 4A,B) but not the inactive mutant Picchu 938X. Although this is a rather general sensor for several tyrosine kinases and, thus, no conclusion can be drawn on the actual kinase(s) involved, it uniquely reveals the kinetics of tyrosine phosphorylation upon anandamide. However, corresponding western blot analyses revealed markedly different effects of anandamide on protein tyrosine phosphorylation, depending on extracellular Ca^{2+}. In the presence of extracellular Ca^{2+} a fast, strong and long lasting phosphorylation of a ~60 kDa band occurred in response to anandamide stimulation, while phosphorylation of a 70-80 kDa band reached its maximum 5 minutes after agonist stimulation (supplementary material Fig. S4A). By contrast, in the absence of external Ca^{2+} (i.e. under conditions of anandamide-induced Ca^{2+} signaling) the 70-80 kDa band appeared much earlier, while the ~60 kDa band was delayed compared with the presence of extracellular Ca^{2+} (supplementary material Fig. S4A). Identical findings were obtained in the presence of 70 μM Mn^{2+} in Ca^{2+}-containing buffer.

Tyrosine phosphorylation upon anandamide in presence and absence of extracellular Ca^{2+} was prevented by 10 μM PP1 [1-(1,1-Dimethylethyl)-1-(4-methylphenyl)-1H-pyrazolo[3,4-d]pyrimidin-

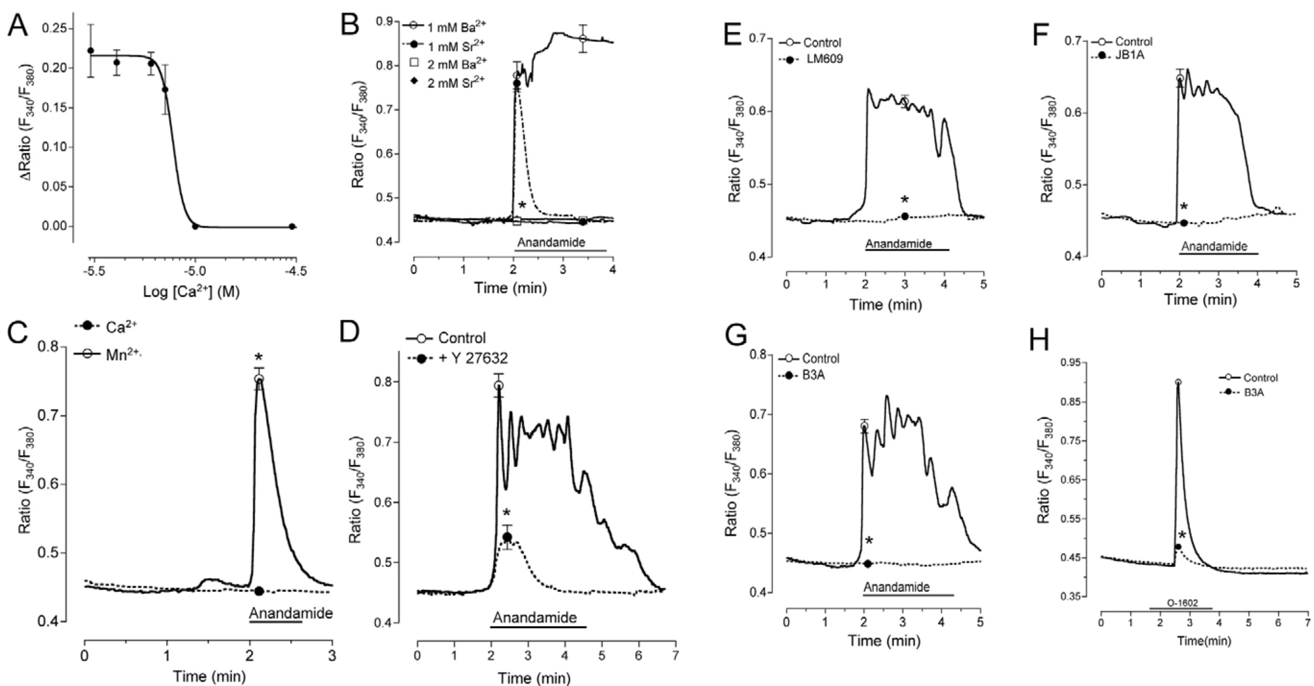

Fig. 3. Anandamide/GPR55-induced Ca^{2+} signaling is linked to integrins. (A,B) The sensitivity of anandamide (10 μM) -triggered cytosolic Ca^{2+} signaling to various concentrations of extracellular Ca^{2+} (n=10), Ba^{2+} (n=24) and Sr^{2+} (n=31) was tested. (C) Comparison between the effect of 10 μM anandamide in Ca^{2+}-containing buffer in the absence (n=20) or presence of 70 μM Mn^{2+} (n=40). (D) The effect of 10 μM anandamide on cytosolic free-Ca^{2+} concentration was monitored in the nominal Ca^{2+}-free solution in the absence (n=25) or presence of the ROCK inhibitor Y27632 (10 μM, n=29). (E-G) The effects of antibodies (2 μM) against αvβ3 integrin (LM609) (control, n=34; LM609, n=30) and the β1 (JB1A) (control, n=28; JB1A, n=33) and β3 subunits (B3A) (control, n=38; B3A, n=30) on cytosolic Ca^{2+} signaling induced by 10 μM anandamide was assessed in nominally Ca^{2+}-free solution. (H) The effect of function-blocking β3-integrin antibody B3A on O1602 (10 μM) -initiated Ca^{2+} signaling in the presence of extracellular Ca^{2+} (control, n=65; B3A, n=84). Cytosolic free-Ca^{2+} concentrations were recorded using fura-2. *P<0.0001 versus the absence of the respective compound.

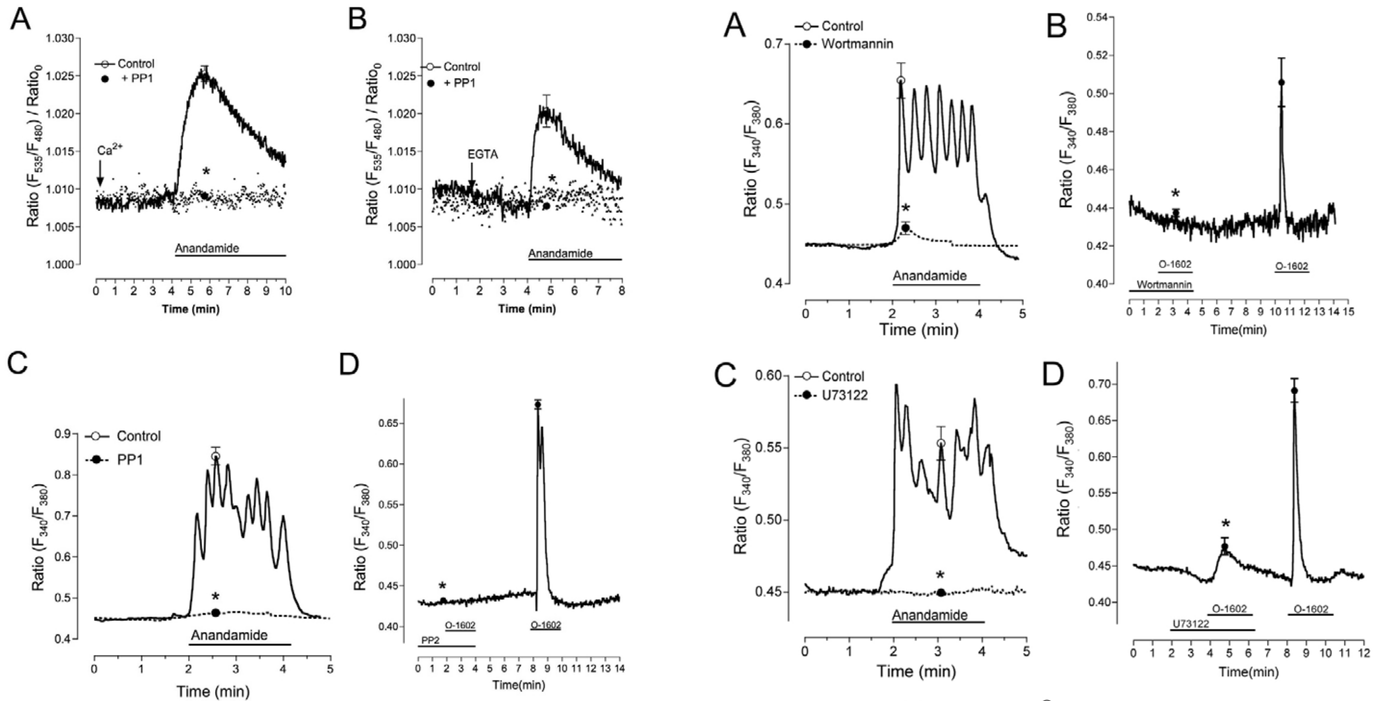

Fig. 4. Anandamide initiates tyrosine phosphorylation. (A,B) The effect of anandamide (10 μM) -induced activation of tyrosine kinases was visualized in single endothelial cells in the presence (A) and absence (B) of extracellular Ca^{2+}. As indicated, 10 μM PP1 was added to the media (*n*=4 under each condition). Tyrosine kinase activity was monitored by measuring the excitation ratio of F_{535}/F_{480} nm (FRET) at 440 nm excitation. (C) The effect of 10 μM anandamide on cytosolic free-Ca^{2+} concentration was tested in nominally Ca^{2+}-free solution in the absence (*n*=24) or presence of 10 μM PP1 (*n*=25). (D) In Ca^{2+}-containing solution, Ca^{2+} intracellular Ca^{2+} signaling was initiated by 10 μM O1602 in the presence of 10 μM PP2, followed by an additional stimulation with 10 μM O1602 after washout of PP2 as indicated (*n*=14). For visualization of tyrosine kinase activity in single endothelial cells, cells were transiently transfected with Picchu 936X. Cytosolic free-Ca^{2+} concentration was recorded using fura-2. *$P<0.001$ versus the absence of PP1.

4-amine; a potent inhibitor of Src-family tyrosine kinases (Hanke et al., 1996)] (supplementary material Fig. S4B), which also abolished anandamide-triggered Ca^{2+} signaling in Ca^{2+}-free solution (Fig. 4C), pointing to the activation of Src-family kinase(s) under all conditions. This assumption was further supported by the inhibitory effect of 10 μM PP2 [3-(4-chlorophenyl) 1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine; a potent inhibitor of Src-family tyrosine kinases (Hanke et al., 1996)] on O1602-induced Ca^{2+} signaling in Ca^{2+}-containing solution (Fig. 4D, Table 2).

PI3K, Bmx/Etk and PLC represented downstream targets of integrin-dependent anandamide/GPR55-induced signal transduction in endothelial cells

At 0.1 μM, the selective inhibitor of phosphoinositide 3 kinase (PI3K) wortmannin (Table 2) diminished tyrosine phosphorylation of the 70-80 kDa band (supplementary material Fig. S5) and abolished anandamide-triggered Ca^{2+} signaling in nominally Ca^{2+}-free solution (Fig. 5A) and in the presence of 70 μM Mn^{2+} in Ca^{2+} containing buffer (data not shown). Notably, wortmannin was less active in reducing the 70-80 kDa band in the presence of extracellular Ca^{2+}, suggesting two different proteins between 70-80 kDa: a wortmannin-sensitive one becoming phosphorylated in

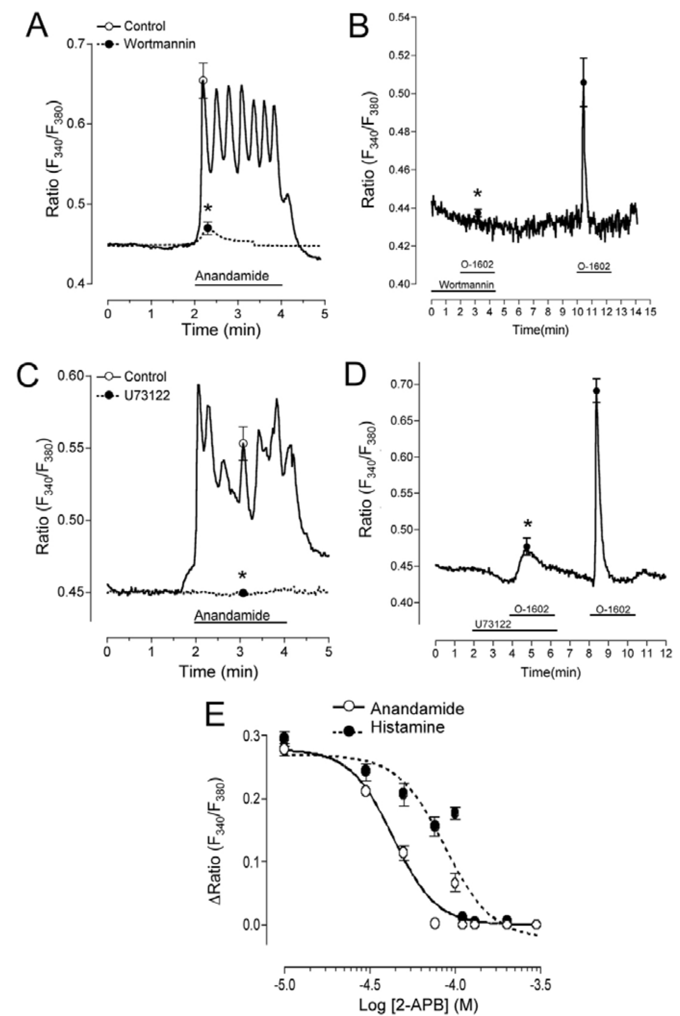

Fig. 5. PI3K is involved in anandamide/GPR55-evoked Ca^{2+} signaling. (A) The effect of 10 μM anandamide on cytosolic free-Ca^{2+} concentration was tested in the nominal Ca^{2+}-free solution in the absence (*n*=33) or presence (*n*=38) of 0.1 μM wortmannin. (B) In the presence of extracellular Ca^{2+}, intracellular Ca^{2+} signaling was initiated by 10 μM O1602 in the presence of 0.1 μM wortmannin followed by an additional stimulation with 10 μM O1602 after washout of the PI3K inhibitor as indicated (*n*=14). (C) The effect of the PLC inhibitor U73122 (2 μM, *n*=26; control, *n*=30) on anandamide (10 μM) -trigged cytosolic Ca^{2+} signaling was tested in Ca^{2+}-free buffer. (D) In Ca^{2+}-containing buffer, intracellular Ca^{2+} signaling was initiated by 10 μM O1602 in the presence of 1 μM U73122 followed by an additional stimulation with 10 μM O1602 after washout of the PLC inhibitor as indicated (*n*=14). (E) Concentration-response relationship of 2APB on cytosolic Ca^{2+} signaling induced by either 100 μM histamine (*n*=4) or 10 μM anandamide (*n*=6) in nominally Ca^{2+}-free solution. Cytosolic free-Ca^{2+} concentrations were recorded in single cells using fura-2. *$P<0.001$ versus the absence of the inhibitor.

the absence of extracellular Ca^{2+} (or the presence of 70 μM Mn^{2+}); and a wortmannin-insensitive protein being phosphorylated in the presence of extracellular Ca^{2+}. In line with the inhibitory potential of wortmannin on anandamide-induced Ca^{2+} signaling in nominal Ca^{2+}-free/Mn^{2+}-containing solutions, this PI3K inhibitor prevented Ca^{2+} signaling in response to O1602 in the presence of extracellular Ca^{2+} (Fig. 5B, Table 2).

To test the involvement of phospholipase C (PLC) activation in the integrin/PI3K-dependent Ca^{2+} recruitment by anandamide, U73122 [(1-[6-[[(17β)-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-

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1H-pyrrole-2,5dione (Bleasdale et al., 1990), Table 2], an inhibitor of PLC (Aschner et al., 1997) was used. Cytosolic Ca^{2+} elevations induced by anandamide in the absence of external Ca^{2+} (Fig. 5C) or O1602 in Ca^{2+}-containing buffer (Fig. 5D) were prevented by 2 μM U73122. Moreover, the rather unspecific and membrane permeable Ins(1,4,5)P_3-receptor antagonist 2APB [2-aminoethoxydiphenylborate (Maruyama et al., 1997; Koganezawa and Shimada, 2002)] prevented endothelial Ca^{2+} elevation in response to 10 μM anandamide and 10 μM histamine at comparable potency with IC_{50} values of 43 (30-60) and 87 (50-152) μM, respectively (Fig. 5E). These data suggest that anandamide-triggered Ca^{2+} signaling is due to PLC-mediated formation of Ins(1,4,5)P_3 that, in turn, elevates cytosolic Ca^{2+} concentration by intracellular mobilization from the ER.

One potential candidate for bridging PI3K activation to PLC stimulation is bone marrow kinase, X-linked/epithelial and endothelial tyrosine kinase (Bmx/Etk) (Qiu and Kung, 2000), a member of the Tec family (Takesono et al., 2002). Immunoprecipitation revealed a 3.2±0.5-fold (n=4) increase in phosphorylation of Bmx/Etk upon anandamide in the absence but not presence of extracellular Ca^{2+} (Fig. 6A). Accordingly, siRNA against Bmx/Etk (efficiency shown in supplementary material Fig. S6A) diminished anandamide-induced Ca^{2+} signaling in the absence of external Ca^{2+} or in the presence of 70 μM Mn^{2+} in Ca^{2+}-containing solution (Fig. 6B). Moreover, 10 μM of the selective Tec family inhibitor LFM-A13 [2-cyano-N-(2,5-dibromophenyl)-3-hydroxy-2-butenamide, Table 2] (Chau et al., 2002) prevented anandamide-triggered Ca^{2+} signaling in the absence of external Ca^{2+} or (Fig. 6C) in the presence of Mn^{2+} in Ca^{2+}-containing solution. These data are in line with phosphorylation of the 70-80 kDa band upon anandamide stimulation (supplementary material Fig. S5) representing Bmx/Etk that, in turn, activates PLCγ leading to intracellular Ca^{2+} mobilization via the generation of Ins(1,4,5)P_3. Consistent with this, inhibition of Bmx/Etk by 10 μM LFM-A13 also prevented O1602 (10 μM)-triggered Ca^{2+} signaling in Ca^{2+} containing solution (Fig. 6D).

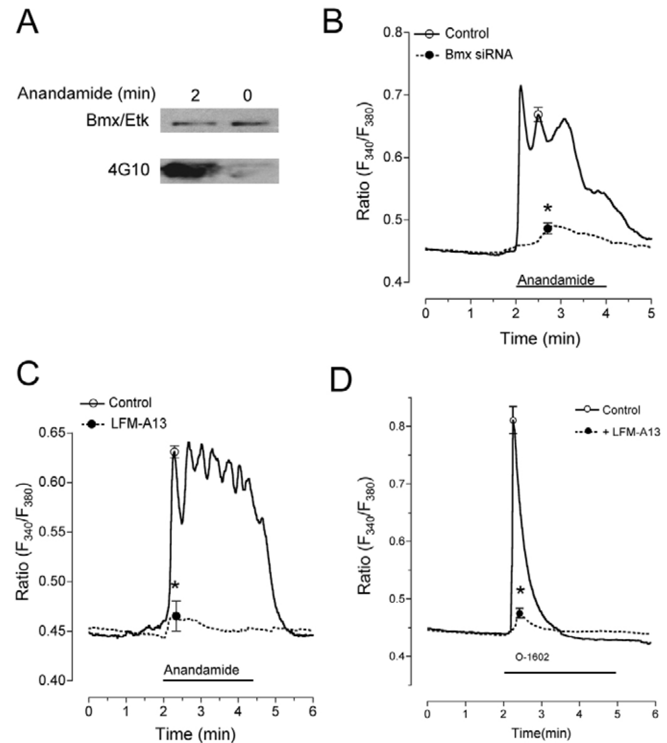

Fig. 6. Bmx/Etk is the downstream target for PI3K upon GPR55 stimulation by anandamide. (A) Phosphorylation of Bmx/Etk upon stimulation with 10 μM anandamide in the nominal absence of extracellular Ca^{2+} was tested by immunoprecipitation using anti-Bmx/Etk antibody (upper blot) and subsequent staining with the anti-phosphotyrosine antibody 4G10 (lower blot). (B,C) The effects of the Tec-family (Bmx/Etk) kinase inhibitor LFM-A13 (10 μM, n=46; control, n=30) or pretreatment with siRNA against Bmx/Etk (n=6; control, n=27) on anandamide (10 μM) -induced cytosolic Ca^{2+} signaling were monitored in nominally Ca^{2+}-free solution. (D) The effect of the Tec-family (Bmx/Etk) kinase inhibitor LFM-A13 (10 μM, n=75; control, n=71) on O1602 (10 μM) -trigged cytosolic Ca^{2+} signaling in Ca^{2+}-containing buffer was tested. Standard immunoprecipitation was applied. In experiments with siRNA, cells were transiently transfected with a vector encoding approved siRNA against Bmx/Etk. Experiments were performed 48 hours after transfection. Cytosolic free-Ca^{2+} concentrations were recorded using fura-2. *P<0.0001 versus the absence of the inhibitor.

In the presence of external Ca^{2+}, activation of Syk via $G\alpha_{i/o}$ exhibited inhibitory action on PI3K-Bmx-PLCγ pathway and prevented anandamide/GPR55-induced Ca^{2+} signaling

Based on the western blot (supplementary material Fig. S5) that revealed a wortmannin-insensitive tyrosine phosphorylation at 70-80 kDa in the presence but not absence of extracellular Ca^{2+}, a negative-feedback mechanism against the PI3K-Bmx-PLCγ signaling via tyrosine phosphorylation [possibly via spleen tyrosine kinase (Syk)] was hypothesized. RT-PCR of the endothelial cell line used proved the expression of Syk in its long form (Wang et al., 2003) (supplementary material Fig. 6B), which matches the 70-80 kDa band in western blots (supplementary material Fig. S5). To further challenge the concept of inhibitory action of Syk on PI3K-Bmx-PLCγ signaling, the effect of the Syk inhibitor piceatannol [3,4,3′,5′-tetrahydroxy-trans-stilbene (Geahlen and McLaughlin, 1989; Ashikawa et al., 2002)] was investigated. In cells that only weakly responded to anandamide in the presence of external Ca^{2+}, this agonist yielded strong Ca^{2+} signaling in the presence of 5 μM piceatannol (Fig. 7A, Table 2). These findings were further supported by the induction of Ca^{2+} signaling by anandamide despite the presence of extracellular Ca^{2+} (Fig. 7B) in cells, which were transiently transfected with siRNA against Syk (supplementary material Fig. S6A). To verify the signaling mechanisms by which endothelial CB_1R triggers activation of Syk, $G\alpha_{i/o}$ protein, which has been reported to be coupled to this cannabinoid receptor (Mukhopadhyay et al., 2002), was inhibited by ADP ribosylation with pertussis toxin. Inhibition of $G\alpha_{i/o}$ protein by pertussis toxin (Table 2) (400 ng/ml, 3 hours) yielded anandamide-induced Ca^{2+} signaling even in the presence of extracellular Ca^{2+} (Fig. 7C), thus pointing to $G\alpha_{i/o}$-mediated activation of Syk that, in turn, inhibits PI3K-Bmx-PLCγ-Ca^{2+} signaling (see Fig. 10).

Clustered β1 integrin released binding to CB_1 receptor upon anandamide stimulation

To explore how integrin-clustering shields anandamide-induced PI3K-Bmx-PLCγ-Ca^{2+} signaling from the inhibitory effect of CB_1R-$G\alpha_{i/o}$-Syk signaling, interaction of integrins with CB_1R was investigated by immunoprecipitation. Under conditions of unclustered integrins (i.e. in the presence of external Ca^{2+}), the binding of CB_1R to β1 integrin was not affected by anandamide (normalized CB_1R ratio without versus with anandamide 1: 1.03±0.04, n=4, n.s.; Fig. 7D). By contrast, if integrin clustering was facilitated by Mn^{2+}, anandamide yielded detachment of the CB_1R from β1 integrin (CB1R was normalized to β1 integrin; CB_1R ratio without anandamide versus

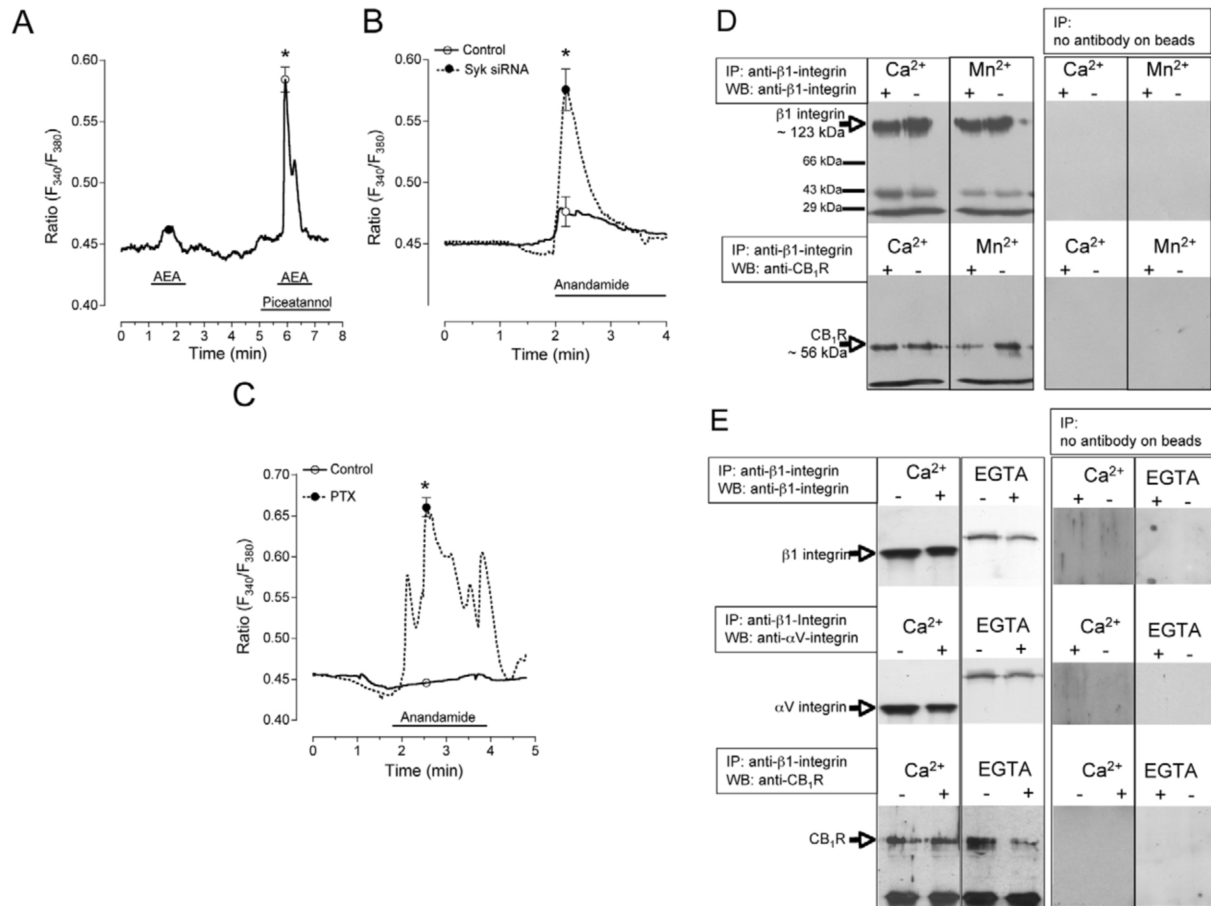

Fig. 7. Syk is the mediator of CB_1R-originated repression of anandamide-induced Ca^{2+} signaling. (A,B) The inhibition of Syk by either its inhibitor piceatannol (5 μM, *n*=24) or siRNA against Syk (*n*=7; control, *n*=4) allowed strong cytosolic Ca^{2+} elevation in response to 10 μM anandamide in the presence of 2 mM extracellular Ca^{2+}. (C) The effect of the G_i protein inhibitor pertussis toxin (400 ng/ml for 3 hours, *n*=8; control, *n*=8) on anandamide (10 μM) -induced Ca^{2+} signaling in the presence of 2 mM extracellular Ca^{2+} was verified in human endothelial cells. (D) Immunoprecipitation using anti-β1-integrin antibody and subsequent staining with either anti-β1-integrin (upper blot) or anti-CB_1R (lower blot). Endothelial extracts were harvested from cells under basal conditions (-) and after stimulation with 10 μM anandamide (+) in the presence of 2 mM extracellular Ca^{2+} (Ca^{2+}) or 2 mM extracellular Ca^{2+} plus 70 μM Mn^{2+} (Mn^{2+}). Right panels shows precipitation with beads that were not preloaded with anti-β1-integrin were used. (E) Immunoprecipitation using anti-β1-integrin antibody and subsequent staining with either anti-β1-integrin (upper blot), anti-αV-integrin (middle blot) or anti-CB_1R (lower blot). Endothelial extracts were harvested from cells under basal conditions (-) and after stimulation with 10 μM anandamide (+) in the and absence (EGTA) of extracellular Ca^{2+}. Right panels shows precipitation with beads that were not preloaded with anti-β1-integrin were used. Standard immunoprecipitation was applied. In experiments with siRNA, cells were transiently transfected with a vector encoding approved siRNA against Syk 48 hours prior to the experiments. Cytosolic free-Ca^{2+} concentrations were recorded using fura-2. *P<0.0001 versus the absence of the inhibitor.

with anandamide 1: 0.31±0.13, *n*=4, P<0.05; Fig. 7D). Identical results were obtained in Ca^{2+}-free solution (CB1R was normalized to β1 integrin; CB_1R ratio without anandamide versus with anandamide 1: 0.30±0.21, *n*=3, P<0.05; Fig. 7E). Although under conditions of clustered integrins the binding of the CB_1R to β1 integrin was affected by anandamide, it did not alter the localization/ distribution of CB_1R (supplementary material Fig. S6C).

Thus, under conditions of clustered integrins, anandamide yielded detachment of the CB_1R from β1 integrin, which, in turn, may preserve anandamide-induced signaling pathways via e-aR leading to cytosolic Ca^{2+} elevation.

Depending on integrin clustering, anandamide triggered either nuclear accumulation of NFκB or NFAT while MAPK activation occurred under all conditions

To investigate transcriptional consequences of the two distinct signaling pathways initiated by anandamide via either CB_1R or e-aR, the nuclear recruitment of nuclear factor κB (NFκB) and nuclear factor of activated T-cells (NFAT) upon anandamide stimulation was investigated under conditions of clustered and unclustered integrins. In the presence of extracellular Ca^{2+} (i.e. unclustered integrins), anandamide evoked nuclear accumulation of the p65 subunit of NFκB (Fig. 8A) but not NFAT (Fig. 9A), whereas if integrins were clustered by Mn^{2+} in the presence of external Ca^{2+}, anandamide failed to stimulate p65 translocation (Fig. 8B) but evoked strong nuclear accumulation of NFAT (Fig. 9B).

In contrast to the selective activation of the transcription factors NFκB and NFAT, Erk1 and Erk2 were activated by anandamide under all circumstances. Notably, Erk1 and Erk2 activation in response to anandamide was slightly faster (+42±26% after 2 minutes) but less pronounced (maxima +430±88% after 45 minutes; *n*=3) under conditions of unclustered integrins (Fig. 9C) than if integrin clustering was evoked by Mn^{2+} (–5±17% after 2 minutes

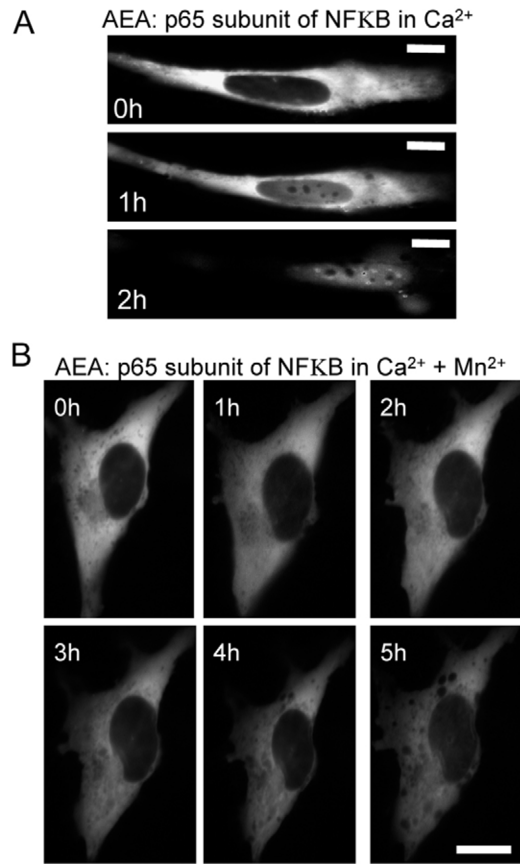

Fig. 8. NFκB is activated independently of anandamide-triggered Ca^{2+} signals. The effect of 10 μM anandamide on the (trans)location of GFP-tagged p65 subunit of NFκB was visualized in endothelial cells in buffer containing 2 mM Ca^{2+} in the absence (A) or presence (B) of 70 μM Mn^{2+}. Images of endothelial cells that were transiently transfected with p65-GFP were taken 48 hours after transfection using a conventional fluorescence microscope. Representative images of at least 10 cells out of three experiments on alternate days are shown. Scale bars: 10 μm.

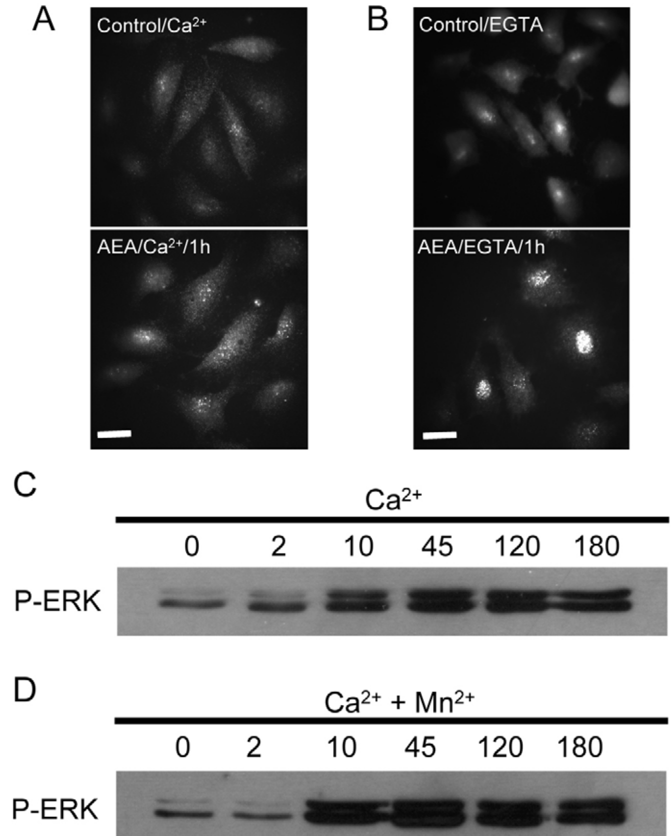

Fig. 9. Anandamide-induced Ca^{2+} signal subsequently activates NFAT, Erk1 and Erk2. (A,B) The effect of 10 μM anandamide on the (trans)location of GFP-tagged NFAT was visualized in endothelial cells in the presence (A) or absence (B) of 2 mM extracellular Ca^{2+}. (C,D) Time dependency of the effect of 10 μM anandamide on Erk1 and Erk2 phosphorylation in endothelial cells in 2 mM Ca^{2+}-containing buffer in the absence (C) or presence (D) of 70 μM Mn^{2+}. Images of endothelial cells that were transiently transfected with NFAT-GFP were taken 48 hours after transfection using a conventional fluorescence microscope. Data presented are representative for at least eight cells out of three experiments on alternate days. Scale bars: 10 μm. For assessment of Erk1 and Erk2 activation, standard western blotting was applied.

and maxima +730±185% after 45 minutes; n=3) (Fig. 9D) or in the absence of extracellular Ca^{2+} (data not shown).

Discussion

Our data demonstrate that two distinct receptors for anandamide exist in human endothelial cells, which were characterized as CB_1R and GPR55. Both receptors trigger distinct signaling pathways, whereby one exhibits negative feedback on the other, depending on the status of integrin configuration. As illustrated in Fig. 10A, the presence of extracellular Ca^{2+} anandamide binding to CB_1R results in G_i protein-mediated activation of Syk and its subsequent signal transduction pathway, resulting in activation of NFκB. Additionally, Syk inhibits PI3K that represents a key signaling protein in the transduction of GPR55-originated signaling. However, once integrins are clustered (Fig. 10B), either by removal of extracellular Ca^{2+} or addition of Mn^{2+}, Syk does not further inhibit GPR55-triggered signaling. Subsequently, the latter causes intracellular Ca^{2+} mobilization from the ER via a PI3K-Bmx-PLCγ pathway and, in turn, activation of Ca^{2+}-activated NFAT (Fig. 10B). Notably, although the activation of transcription factors varies depending on the signaling pathway, Erk1 and Erk2 are activated under all circumstances (Fig. 10). Although the physiological consequences of these concomitant and interrelated signaling pathways in response to anandamide in endothelial cells are unclear and need to be further explored, the crucial role of integrin-clustering may point to a circumstance-dependent bivalent contribution of this endocannabinoid to cell stimulation, proliferation, angiogenesis and/or migration under conditions such like inflammation, permeability or hypoxia.

Receptor(s) involved in anandamide-induced endothelial cell activation

Endocannabinoids interact with two cannabinoid receptors identified by molecular cloning: CB_1R, which is expressed predominantly in the brain (Matsuda et al., 1990) but is also present in peripheral tissues (Shire et al., 1995), and CB_2R, expressed mainly by cells of the immune system (Munro et al., 1993) but also calf pulmonary aortic endothelial cells (Zoratti et al., 2003). In human and murine vasculature, the actions of endogenous cannabinoids have been widely interpreted as being mediated by CB_1R, although there is a

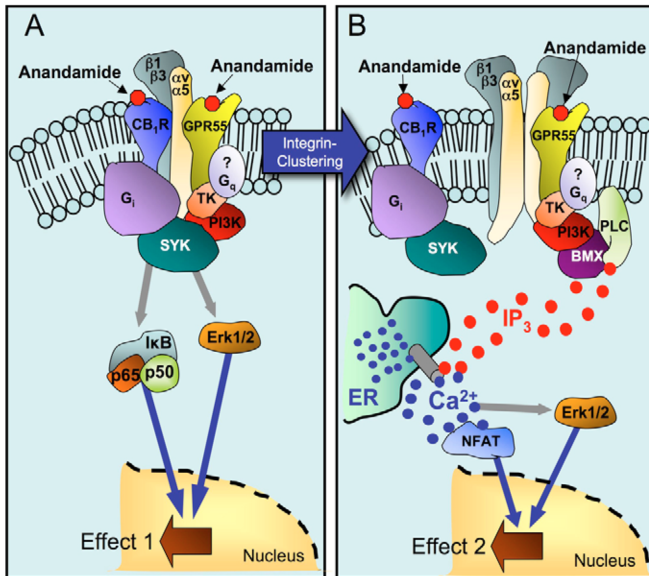

Fig. 10. Schemes of the two signaling cascades activated by anandamide in endothelial cells. (A) Anandamide-induced Ca^{2+} signaling under conditions of unclustered integrins originates from CB_1R that is linked to β1 integrin and stimulates Syk via $G\alpha_{i/o}$, subsequently resulting in the activation of NFκB, Erk1 and Erk2. Additionally, Syk inhibits PI3K and, thus, the downstream signaling of the GPR55 is prevented. (B) Once integrins are clustered, CB_1R uncouples from β1 integrin and, thus, no negative feedback on PI3K via Syk occurs. Consequently, GPR55-initiated signaling becomes promoted, resulting in the activation of a PI3K-Bmx-PLCγ cascade that triggers in the production of IP_3 and subsequent intracellular Ca^{2+} mobilization, yielding activation of NFAT, Erk1 and Erk2. Based on the data presented, involvement of $G_{q/s}$ in GPR55-triggered signaling cannot be not confirmed or excluded at this stage. Abbreviations: α1β3, α1β3 integrin; αvα5, αvα5 integrin; Bmx/Etk, bone marrow kinase, X-linked/epithelial and endothelial tyrosine kinase; CB_1R, cannabinoid 1 receptor; ER, endoplasmic reticulum; Erk1/2, extracellular signal regulated kinases 1 and 2; GPR55, G-protein-coupled receptor 55 (e-aR, 'atypical' endothelial anandamide receptor); IP_3, Ins(1,4,5)P_3; G_i, G_i protein; G_q, G_q ($G_{\alpha13}$) protein; NFAT, nuclear factor of activated T-cells; NFκB, nuclear factor κB; p50, nuclear factor κB p50; p65, nuclear factor κB, subunit 3; PI3K, phosphoinositide 3-kinase; PLC, phospholipase C; Syk, spleen tyrosine kinase; Tk, tyrosine kinase.

growing amount of evidence, particularly in isolated heart and blood vessel preparations, that another cannabinoid receptor, referred as e-aR may exist (Offertaler et al., 2003).

Our findings that, despite the presence of CB_1R, its very selective synthetic agonist HU210 [(6aR)-trans-3-(1,1-dimethylheptyl)-6a,7,10,10a-tetrahydro-1hydroxy-6,6-dimethyl-6H-dibenzo[b,d]pyran-9-methanol] (Felder et al., 1995) failed to stimulate intracellular Ca^{2+} signaling while the e-aR agonist O1602 (Bukoski et al., 2002; Jarai et al., 1999) evoked a strong Ca^{2+} signal in Ca^{2+} containing solution, indicate that anandamide-triggered Ca^{2+} signaling is mediated by e-aR in the human umbilical vein-derived endothelial cell line used in this study. However, anandamide failed to induce a Ca^{2+} signal in the presence of extracellular Ca^{2+}. Accordingly, this points to a repressive effect of CB_1R-originated signal transduction on signaling pathways beyond e-aR. This hypothesis was further confirmed by our findings that activation of CB_1R with HU210 strongly diminished the O1602-initiated Ca^{2+} signal. Notably, caution is advised on the specificity of CBR antagonist/agonists, and, thus, additional efforts to characterize involved receptors were undertaken.

However, in the absence of external Ca^{2+}, anandamide initiated strong cytosolic Ca^{2+} signaling. Notably, this signal was insensitive to the CB_2R antagonist SR144528 but was prevented by rimonabant, an inhibitor of CB_1R as well as of e-aR (Offertaler et al., 2003). Importantly, as O1918, the antagonist of the e-aR (Jarai et al., 1999; Mo et al., 2004; Offertaler et al., 2003), prevented anandamide-induced Ca^{2+} signaling in the absence of external Ca^{2+}, these data indicate that in the nominal absence of extracellular Ca^{2+} the CB_1R-originated suppressive effect is uncoupled from the e-aR pathway, and, thus, allows the generation of a Ca^{2+} signal. Hence, using RT-PCR and siRNA, the putative anandamide receptor GPR55 (Brown, 2007; Hiley and Kaup, 2007; Ryberg et al., 2007) was identified as the e-aR. Hence, overexpression of GPR55 strongly increased Ca^{2+} signaling upon anandamide and O1602 treatment in Ca^{2+}-free and Ca^{2+}-containing buffer, respectively. However, the potency of anandamide was less in this study than previously reported (Ryberg et al., 2007), which may be due to the different cell models (i.e. transfected HEK 293 cells versus endothelial cells). Hence, lysophosphatidylinositol, a recently described agonist of the GPR55 (Oka et al., 2007), was found to trigger Ca^{2+} signaling via constitutive GPR55 in endothelial cells. Accordingly, there are several questions arising: first, how does the e-aR/GPR55 trigger Ca^{2+} signaling; second, what is the molecular mechanism of the CB_1R-mediated suppressive effect on anandamide-induced Ca^{2+} signaling; third, why and how is the Ca^{2+} signal via e-aR/GPR55 shielded from CB_1R-mediated suppression in the absence of extracellular Ca^{2+}; and, fourth, what are the physiological consequences of both signaling pathways for transcriptional control in endothelial cells?

GPR55 interacts with integrins and triggers Ca^{2+} signaling via PI3K and Bmx

In the absence of extracellular Ca^{2+}, integrins might be involved in the transition of GPR55-originated signaling that yields intracellular Ca^{2+} mobilization from the ER (Fig. 10B). In particular, the inhibitory properties of the crosslinking antibody against αvβ3 integrin [LM609 (Charo et al., 1990; Cheresh, 1987)] and the functional-blocking antibodies against the β1 [JB1A (Mohri et al., 1996)] and β3 subunits [B3A (Vignali et al., 1990)] on anandamide/O1602-induced Ca^{2+} signaling fuel the hypothesis that, upon anandamide/O1602 stimulation, GPR55 interacts with αvβ3 and α5β1 integrins, which is prerequisite to transmitting outside-in signaling towards intracellular downstream targets (Fig. 10A). This conclusion is further supported by the inhibitory effects of fibronectin (Rupp and Little, 2001) or its integrin-binding motif RGD (Bar-Shavit et al., 1991; Ruoslahti, 2003; Ruoslahti and Pierschbacher, 1987) on anandamide-/O1602-induced Ca^{2+} signaling. The importance of integrin clustering was additionally confirmed by our results that the inhibition of ROCK, which is required for clustering of the integrins (Rodriguez-Fernandez et al., 2001), by Y27632 (Uehata et al., 1997) strongly diminished anandamide-induced Ca^{2+} signaling.

Anandamide-induced Ca^{2+} signaling in Ca^{2+}-free medium was sensitive to the inhibition of PI3K, PLC and the Ins(1,4,5)P_3 receptor. Thus, the involvement of PI3K upstream of PLC activation and Ins(1,4,5)P_3-mediated intracellular Ca^{2+} mobilization can be assumed (Fig. 10B). The 70-80 kDa protein Bmx/Etk, a unique tyrosine kinase of the Tec family in endothelial and epithelial cells (Takesono et al., 2002), was identified as mediator between PI3K and PLC. PI3K-induced activation of Bmx/Etk has been described in prostate cancer cells and in breast cancer cells (Bagheri-Yarmand

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et al., 2001; Qiu et al., 1998). In support of this, the Tec family inhibitor LFM-A13 (Chau et al., 2002; Mahajan et al., 1999), as well as Bmx/Etk-targeted siRNA inhibited anandamide- and O1602-induced Ca^{2+} signaling. Our data that the wide-range tyrosine kinase inhibitors PP1 and PP2 (Liu et al., 1999) abolished anandamide- and O1602-induced Ca^{2+} signaling, and the enhanced tyrosine phosphorylation lead us to suggest that Bmx/Etk undergoes tyrosine phosphorylation by Src family kinases.

Overall, our data point to anandamide-triggered Ca^{2+} signaling as the apparent result of a signaling cascade starting with anandamide binding to GPR55 that clusters with $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins to transmit outside-in signaling, leading to PI3K-mediated activation/translocation of Bmx/Etk. After Bmx/Etk phosphorylation by a member of the Src family (Stephens et al., 2001), it activates PLCγ (Qin and Chock, 2001) that, in turn, instigates Ins(1,4,5)P_3-triggered intracellular Ca^{2+} mobilization from the ER (Fig. 10B).

CB_1-receptor mediated suppression on anandamide-triggered Ca^{2+} signaling is mediated via $G\alpha_{i/o}$ and Syk

Notably, inhibition of $G\alpha_{i/o}$ by pertussis toxin unmasked intracellular Ca^{2+} signaling upon anandamide in the presence of extracellular Ca^{2+}, pointing to the involvement of $G\alpha_{i/o}$ downstream of CB_1R (Howlett et al., 2002) in a negative-feedback loop that prevents intracellular Ca^{2+} signaling upon anandamide under these conditions (Fig. 10A).

A potential executor of the inhibitory effects on GPR55-PI3K-Bmx signaling downstream of CB_1R and $G\alpha_{i/o}$ is Syk. This tyrosine kinase with splicing variants of ~72 kDa and ~68 kDa (Wang et al., 2003) inhibits activation of PI3K in breast cancer cells (Mahabeleshwar and Kundu, 2003) and is expressed in endothelial cells (Inatome et al., 2001). Accordingly, the long form of Syk could be found in the human endothelial cell line used for this study. Moreover, selective inhibition of Syk by piceatannol (Ashikawa et al., 2002) and the respective siRNA unmasked anandamide-triggered Ca^{2+} signaling in the presence of extracellular Ca^{2+}. Additionally, wortmannin-resistant tyrosine phosphorylation of a 70-80 kDa protein was found in response to anandamide in the presence but not absence of extracellular Ca^{2+}. These data indicate that endothelial Syk indeed executes repression of anandamide-triggered Ca^{2+} signaling downstream to CB_1R and $G\alpha_{i/o}$ (Fig. 10A).

Integrins shield GPR55-triggered signaling from CB_1R-controlled suppression

Neither NMR spectroscopy nor anandamide uptake experiments provided evidence for a Ca^{2+}-anandamide chelate to prevent binding/uptake of free anandamide and, thus, Ca^{2+} signaling in the presence of extracellular Ca^{2+}. Therefore, we speculated that the interaction of extracellular Ca^{2+} with cell membrane constituents is responsible for the observed inhibitory effect of extracellular Ca^{2+} on anandamide-induced Ca^{2+} signaling in endothelial cells. Hence, the existence of a rather specific Ca^{2+}-binding site was further supported by the findings that the Ca^{2+} surrogates Sr^{2+} and Ba^{2+} also prevented anandamide-induced Ca^{2+} signaling in a concentration-dependent manner. Notably, the IC_{50} value of Ca^{2+} (~8 μM) to prevent anandamide-triggered Ca^{2+} signaling is strikingly similar to that found for Ca^{2+}-induced inhibition of integrin clustering (Leitinger et al., 2000; Leitinger et al., 1999). Consequently, the involvement of these dynamically regulated transmembrane heteromultimers in unmasking GPR55-triggered Ca^{2+} signaling in the absence of extracellular Ca^{2+} was postulated. Clustered integrins (i.e. in the absence of extracellular Ca^{2+}) may protect GPR55-initiated signaling against the repressive effects of CB_1R-originated pathways. In line with this assumption, Mn^{2+}, a potent activator of integrin clustering (Mould et al., 1998; Smith and Cheresh, 1990; Thamilselvan et al., 2003), allowed anandamide-induced Ca^{2+} signaling even in the presence of extracellular Ca^{2+}, while Mn^{2+} did not induce any Ca^{2+} signal by itself.

In addition, binding of CB_1R to the β1-integrin subunit was released upon anandamide only if integrins were clustered, which further supports the concept that clustering of integrins shields anandamide-induced PI3K-Bmx-PLCγ-Ca^{2+} signaling from the inhibitory effect of CB_1R activation (Fig. 10). Besides CB_1R, phospho-Syk was also found to interact directly with the cytoplasmic domain of β1-, β2- and β3-integrin subunits (Woodside et al., 2002).

Accordingly, the interaction of CB_1R and Syk with inactive (i.e. unclustered) β1 integrin might either disturb the cytoplasmic integrin rearrangement necessary to transmit the GPR55-initiated signal to its downstream target or allow Syk-mediated inhibition of PI3K owing to the proximity of both proteins (Fig. 10A). However, once integrins cluster either by activation of ROCK, extracellular Mn^{2+} or removal of extracellular Ca^{2+}, CB_1R and Syk are released from β1 integrin and no further suppression of PI3K occurs, thus allowing GPR55-originated Ca^{2+} signaling to be transmitted (Fig. 10B). Though the involvement of a $G_{\alpha 13}$ in GPR55-triggered Ca^{2+} signaling (Ryberg et al., 2007) seem unlikely in the cells used, owing to the involvement of integrins, it cannot be excluded.

In contrast to anandamide that binds to CB_1R and GPR55, activation of GPR55 only by either O1602 or LPI yielded Ca^{2+} signaling independently of extracellular Ca^{2+}, thus indicating that the negative feedback by CB_1R needs to be activated in order to achieve inhibition of the GPR55-originated pathway.

Transcription factor activation upon anandamide depends on the signaling pathway activated

Syk induces tyrosine phosphorylation of IκBα, causing dissociation, phosphorylation and nuclear translocation of the p65 subunit of NFκB (Takada et al., 2003). This is in line with the data indicating that, in the presence of extracellular Ca^{2+}, anandamide triggered nuclear translocation of p65 (Fig. 10A). However, under conditions of clustered integrins, anandamide failed to evoke the translocation of p65. The lack of NFκB activation under these conditions is consistent with reports pointing to the importance of Ca^{2+} oscillation for the activation of this transcription factor (Hu et al., 1999). By contrast, nuclear translocation of Ca^{2+}/calcineurin activated NFAT (Clipstone and Crabtree, 1992; Loh et al., 1996) upon stimulation with anandamide was exclusively obtained under conditions of clustered integrins (Fig. 10B). These findings are in agreement with our previous report that intracellular Ca^{2+} mobilization activates NFAT in human endothelial cells (Bochkov et al., 2002). Notably, although the contribution of NFκB and NFAT to gene expression and regulation of cell function often overlaps, there are considerable differences as NFκB is frequently referred to as being involved in endothelial cell apoptosis (von Albertini et al., 1998) and the initiation of inflammatory processes (Badrichani et al., 1999), while NFAT has been implicated in angiogenesis (Qin et al., 2006) and cell growth (Li et al., 2005).

Interestingly, anandamide-activated mitogenic signaling results in phosphorylation of Erk1 and Erk2 under all conditions, which is consistent with frequent reports that integrins and cannabinoid receptors induce mitogen-activated protein kinase (MAPK)

signaling pathways (Liu et al., 2000). The phenomenon of Erk1 and Erk2 phosphorylation under the various conditions (i.e. with and without Ca^{2+} signaling) might be related to the activation of different pathways, resulting in phosphorylation of Erk1 and Erk2. Consistently, Syk stimulates Erk1 and Erk2 via the Ras-MAPK pathway (Kawakami et al., 2003) but also Ca^{2+} that is known to influence cell proliferation and differentiation (Berridge et al., 2000a; Berridge et al., 2000b) stimulates the Ras-MAPK pathway (Cullen and Lockyer, 2002).

In addition to such alternative activation of distinct transcription factors, the observed GPR55-triggered Ca^{2+} signaling may also mediate Ca^{2+}-sensitive cell functions such as activation of nitric oxide synthase, formation of prostaglandins or endothelium-derived hyperpolarizing factor (Graier et al., 1994), proliferation (Munaron, 2002), or permeability (Tiruppathi et al., 2006). Accordingly, depending on the status of integrins, anandamide initiates alternate processes in endothelial cells that may even lead to opposite cell functions (e.g. apoptosis versus cell proliferation).

Although both signaling cascades and their mutual interrelation could be described in herein, important questions still remain to be answered. What are the events that favor one of the two alternative signaling pathways? What are the physiological and/or pathological consequences of the respective signaling cascades? Particularly in view of the known initiators of integrin clustering such as inflammation (Staunton et al., 2006), adhesion (Shattil and Newman, 2004) and migration (Lindbom and Werr, 2002), the physiological implication of the dual signaling induced by anandamide in endothelial cells urges its investigation.

Materials and Methods

Chemicals, supplies and materials

Fura-2/AM was from Molecular Probes Europe (Leiden, Netherlands). ^{3}H-anandamide was obtained from APB (Vienna, Austria). Anandamide (arachidonylethanolamide), R-(+)-methandamide, U73122, 2APB, LMF-A13, HU210 and piceatannol were purchased from Tocris Cookson (Northpoint, Avonmouth, Bristol, UK). O1602, O1918 and AM251 were from Cayman Europe (Tallinn, Estonia). SR144528 and rimonabant were kind gifts from Sanofi-Recherche (Monpellier, France). Bradykinin, arachidonic acid, ATP and lysophosphatidylinositol were from Sigma Chemicals (St Louis, MO, USA). RNeasy Mini kit and the EndoFree Plasmid Maxi kit were purchased from Qiagen (VWR, Vienna, Austria). RQ1 RNase-free DNase I and TransFas were from Promega (Mannheim, Germany). RNAguard and random hexamer primers were from APB. First-strand buffer, moloney murine leukemia virus reverse transcriptase, cell culture media and media substitutes were obtained from Life Technologies (Gibco, Invitrogen, Vienna, Austria). Fetal calf serum was from PAA (Linz, Austria). DyNAzyme II DNA polymerase was from Finnzymes Oy (Vienna, Austria). Primers were synthesized at MWG Biotech (Ebersberg, Germany). Restriction enzymes and T4 DNA ligase were purchased at New England BioLabs (Frankfurt, Germany) or Promega. The expression vectors pcDNA 3 and pBudCE4.1 were obtained from Invitrogen (Vienna, Austria). The dNTPs and all other chemicals were from Roth (Karlsruhe, Germany). The CB1R antibody (H-150) was from Santa Cruz Biotechnology (Heidelberg, Germany). Antibodies for β1 integrin (JB1A) and αV integrin (SAM-1) were obtained from Biomedica/Chemicon (Millipore, Vienna, Austria) and Protein G Sepharose 4 Fast Flow for immunoprecipitation was from GE Healthcare (Vienna, Austria).

Cell culture and transfection

The human umbilical vein derived endothelial cell line, EA.hy926 (Edgell et al., 1983) at over 45 passages was grown in DMEM containing 10% FCS and 1% HAT. In addition, we have performed experiments in short-term cultured human umbilical vein endothelial cells and human uterine artery endothelial cells (Trenker et al., 2007). If necessary, dishes were coated with 20 μg/ml fibronectin (Short et al., 1998). Transient transfection was performed using Transfast according to the manufacturer's protocol.

Reverse transcriptase-PCR

Total RNA was isolated using the RNeasy Mini Kit according to the manufacturer's protocol and subjected to RT-PCR as described previously (Zoratti et al., 2003). The identity of all PCR products was verified on TAE agarose gels and was further confirmed by automated sequencing of the isolated PCR.

Reduction of gene expression by siRNA

The siRNA against human Bmx/Etk was selected at position 1482 bp of the human mRNA and ligated into pSuppressor using the *Sal*I/*Xba*I sites. For identification of siRNA-expressing cells, the U6 promotor and Bmx/Etk or Syk siRNA were cloned into pBudCE4.1 encoding mtDsRed (Malli et al., 2003) via *Kpn*I/*Xho*I at the second multicloning site. The siRNA against human Syk in pSuppressor was purchased by Imgenex. siRNA against GPR55 was from Quiagen (Hs_GPR55_5_HP). The efficiency of siRNAs and of an appropriate negative control was approved by real-time PCR (supplementary material Fig. S2E; see Fig. 4A).

Western blot analysis

EA.hy926 cells were scraped in lysis buffer and disrupted by three cycles of freezing/thawing. Equal amounts of total protein were subjected to western blot and detected by enhanced chemiluminescence as previously described (Schaeffer et al., 2003). If required, membranes were stripped with 0.1 M glycine (pH 2.5), and re-probed.

Immunoprecipitation

Cell lysates were incubated with 2-5 μg (200 μg/ml) of specific antibody overnight. The obtained immune complexes were precipitated with Protein G Sepharose 4 Fast Flow, according to the manufacturer's protocol and subjected to western blot analyses.

Immunohistochemistry

Fixed and permeabilized cells grown on glass cover were incubated overnight at 4°C with anti-NFAT antibody. After incubation with Alexa Fluor 488 secondary antibody (2 μg/ml), the glass cover slips were sealed and fluorescence was visualized using deconvolution microscopy.

Measurement of tyrosine kinase activity

Tyrosine kinase activity was monitored in single endothelial cells using a probe for tyrosine phosphorylation of the CrkII adaptor protein [Picchu-936X (Kurokawa et al., 2001)], as previously described (Schaeffer et al., 2003).

Cytosolic free Ca^{2+} measurements

Cytosolic free-Ca^{2+} was measured using Fura2/AM as previously described (Frieden et al., 2002; Malli et al., 2005; Trenker et al., 2007). $[Ca^{2+}]_{cyto}$ was expressed as $(F_{340}/F_{380})/F_0$.

FRET measurement of endoplasmic free-Ca^{2+} concentration and kinase activity

Tyrosine kinase activity was monitored in single endothelial cells using a probe for tyrosine phosphorylation of the CrkII adaptor protein [Picchu-936X (Kurokawa et al., 2001)] as previously described (Schaeffer et al., 2003). For the assessment of the intraluminal free-Ca^{2+} concentration of the ER, cells were transiently transfected with $Cam4_{er}$ and measurements were performed as described previously (Frieden et al., 2002; Malli et al., 2003; Malli et al., 2005). FRET-based sensors were excited at 440 nm (440AF21, Omega Optical, Brattleboro, VT, USA) and emission was collected simultaneously at 535 and 480 nm using an optical beam splitter (Dual-View MicroImager, Optical Insights, Visitron Systems, Puchheim, Germany). Kinase activity or alterations in ER Ca^{2+} were expressed as $(F_{535}/F_{480})/F_0$.

We thank Beatrix Petschar and Anna Schreilechner for their excellent technical assistance, R. Tsien (University of California, San Diego, CA) for $Cam4_{er}$, M. Matsuda and A. Miyawaki (Riken, Wako, Saitama, Japan) for providing the Picchu-936X constructs, Tullio Pozzan (University Padova, Padova, Italy) for mtDsRed, and C. J. S. Edgell (University of North Carolina, Chapel Hill, NC) for the EA.hy926 cells. The expression vector for p65-GFP was a gift from J. Schmid (Medical University of Vienna, Vienna, Austria). We also thank Andrew Irving (University of Dundee, UK) for the 3xHA-GPR55 cDNA. This work was supported by the Austrian Funds (P16860-B09, P20181-B05 and SFB F3010-B05) and by the Franz Lanyar Foundation at the Medical University of Graz. The Institute of Medical Molecular Biology and Biochemistry was supported by the infrastructure program (UGP4) of the Austrian ministry of education, science and culture. Nariman Balenga was supported by a PhD Fellowship in PhD Program Molecular Medicine at the Medical University of Graz.

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