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Anandamide acts as an intracellular messenger amplifying Ca^{2+} influx via TRPV1 channels

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The endocannabinoid anandamide is able to interact with the transient receptor potential vanilloid 1 (TRPV1) channels at a molecular level. As yet, endogenously produced anandamide has not been shown to activate TRPV1, but this is of importance to understand the physiological function of this interaction. Here, we show that intracellular Ca^{2+} mobilization via the purinergic receptor agonist ATP, the muscarinic receptor agonist carbachol or the Ca^{2+}-ATPase inhibitor thapsigargin leads to formation of anandamide, and subsequent TRPV1-dependent Ca^{2+} influx in transfected cells and sensory neurons of rat dorsal root ganglia (DRG). Anandamide metabolism and efflux from the cell tonically limit TRPV1-mediated Ca^{2+} entry. In DRG neurons, this mechanism was found to lead to TRPV1-mediated currents that were enhanced by selective blockade of anandamide cellular efflux. Thus, endogenous anandamide is formed on stimulation of metabotropic receptors coupled to the phospholipase C/inositol 1,4,5-triphosphate pathway and then signals to TRPV1 channels. This novel intracellular function of anandamide may precede its action at cannabinoid receptors, and might be relevant to its control over neurotransmitter release.

Introduction

Anandamide was originally discovered as an endogenous partial agonist at the cannabinoid CB_1 receptor (Devane

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et al, 1992), that is, an endocannabinoid. Anandamide is formed by a Ca^{2+}-dependent and phosphodiesterase-catalysed hydrolysis of a membrane precursor, *N*-arachidonoyl-phosphatidylethanolamine (NAPE). Together with the other endocannabinoid 2-arachidonoylglycerol, it reduces neurotransmitter release and modulates synaptic plasticity through metabotropic cannabinoid CB_1 receptors (Piomelli, 2003). Recently, it has been shown that exogenous anandamide, unlike 2-arachidonoylglycerol, can activate transient receptor potential vanilloid 1 (TRPV1) ion channels at an intracellular site, which has been identified by site-directed mutagenesis studies (Caterina *et al*, 1997; Zygmunt *et al*, 1999; De Petrocellis *et al*, 2001; Jordt and Julius, 2002). TRPV1 belongs to the large family of TRP ion channels (Clapham, 2003), and can also be activated by the pungent compound of hot chilli peppers, capsaicin (CPS), and by the phorboid compound resiniferatoxin (RTX) (Szallasi and Blumberg, 1999). TRPV1 is most abundant in sensory fibres of the peripheral nervous system, but is found also in the CNS (Mezey *et al*, 2000; Roberts *et al*, 2004).

To date, it is unknown whether anandamide plays any TRPV1-mediated physiological function in either peripheral or central neurons. While in sensory neurons TRPV1 participates in inflammatory/thermal hyperalgesia (Caterina *et al*, 2000; Davis *et al*, 2000), the possible role of this channel in the CNS remains elusive, although several data point to its regulation of neurotransmitter release (Al-Hayani *et al*, 2001; Marinelli *et al*, 2002, 2003). Further insights into the biological significance of TRPV1 gating might be gained by looking at its cognate TRP receptors, for whose physiological role several theories have been proposed to date (Clapham, 2003). Since anandamide biosynthesis is Ca^{2+} dependent, and the localization of the biosynthetic and metabolic machinery of anandamide (Piomelli, 2003) as well as its suggested binding site on TRPV1 (Jordt and Julius, 2002) are intracellular, we hypothesize that this lipid may function as an intracellular Ca^{2+}-sensing messenger that amplifies intracellular Ca^{2+} levels via TRPV1. We have investigated here this hypothesis, which could open previously unexplored possibilities for the physiological role of both anandamide and TRPV1.

Results

Mobilization of intracellular Ca^{2+} induces anandamide formation

For anandamide to function as an endogenous intracellular messenger, Ca^{2+} mobilization from the ER should be sufficient to produce a transient rise in its levels. Therefore, in the first set of experiments, we have tested whether anandamide biosynthesis can be induced by emptying of Ca^{2+} stores in the ER by thapsigargin, which is a Ca^{2+}-ATPase inhibitor that blocks the re-uptake of Ca^{2+} in the ER. In an extracellular Ca^{2+}-free medium, thapsigargin at 1 μM produced a transient increase in intracellular anandamide levels in wild-type and hTRPV1-expressing human embryonic kidney (HEK)
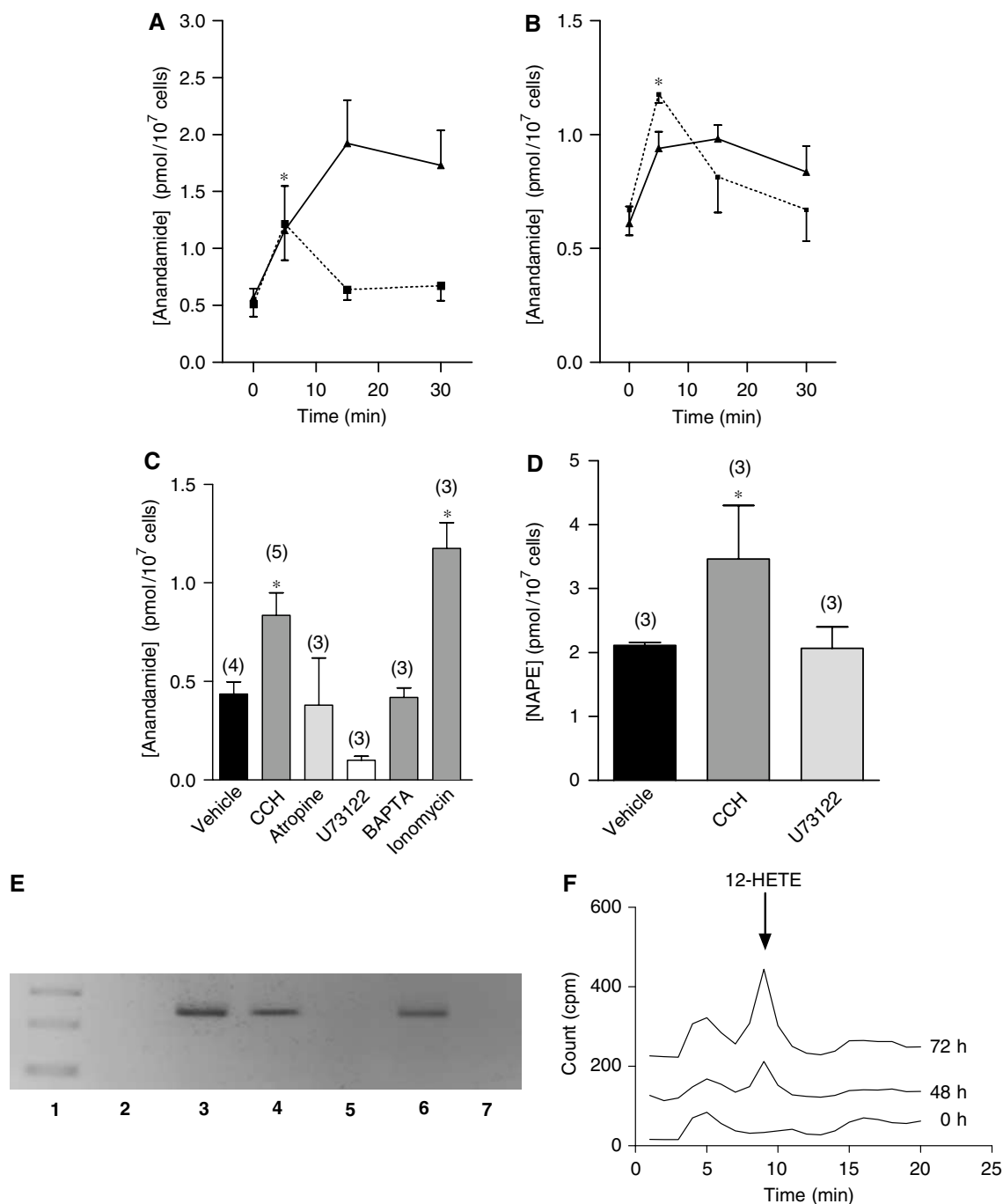
Figure 1 Mobilization of intracellular Ca^{2+} leads to anandamide formation. (**A, B**) Intra- and extracellular anandamide levels (dashed and solid lines, respectively; $n = 3$–7 per data point) after (A) thapsigargin (1 μM) or (B) carbachol (50 μM) stimulation. *Significantly different ($P < 0.05$ by ANOVA) versus 0 and 5 min. (**C**) Anandamide and (**D**) NAPE concentrations of hTRPV1-HEK293 cells after 30 min stimulation with carbachol (50 μM) in the presence of atropine (100 μM), U73122 (5 μM) or BAPTA/AM (10 μM). The effect of ionomycin (4 μM) in the presence of extracellular Ca^{2+} is also shown. (**E**) Expression of mRNA encoding the NAPE-specific PLD in HEK293 cells: lane 1, 100 bp DNA ladder, starting from 100 bp; lane 2, blank (no cDNA); lane 3, human genomic DNA; lane 4, cDNA from wild-type HEK293 cells; lane 5, nonretrotranscribed RNA from wild-type HEK293 cells; lane 6, cDNA from hTRPV1-HEK293 cells; lane 7, nonretrotranscribed RNA from wild-type hTRPV1-HEK293 cells. No PCR product was detected when the reverse transcriptase step was omitted (lanes 5 and 7). Similar data were obtained when using rat DRG neurons (not shown). (**F**) Reversed-phase HPLC chromatogram of lipid extracts of 100 μM [1-C^{14}]-AA incubation with hTRPV1 cell extracts after 0, 48 and 72 h of transient transfection with 12-lipoxygenase cDNA. 12-HETE (the reduced 12-lipoxygenase product of the putative endogenous ligand of TRPV1 receptors, 12-hydroperoxy-eicosatetraenoic acid) is not formed in nontransfected cells.

293 cells (Figure 1A), as measured by mass spectrometry. As the amounts of anandamide produced in the presence of extracellular Ca^{2+} (1.43 ± 0.30 pmol/10^7 cells; $n = 4$) were not higher than those formed in its absence

(1.73 ± 0.31 pmol/10^7 cells; $n = 7$), these data indicate that Ca^{2+} release from intracellular Ca^{2+} stores is sufficient to enhance anandamide levels. The peak of intracellular anandamide occurred within 5 min from stimulation, when

it reached approximately a concentration of 0.2 μM, and was followed by a time-dependent release of anandamide into the extracellular medium.

Activation of metabotropic $G_{q/11}$-coupled receptors results in mobilization of intracellular Ca^{2+} via the phospholipase (PLC)/inositol 1,4,5-triphosphate (IP_3) pathway. To test whether this pathway can induce anandamide biosynthesis, we have stimulated HEK293 cells, which endogenously express $G_{q/11}$-coupled muscarinic receptors, with the nonselective muscarinic receptor agonist carbachol, in the absence of extracellular Ca^{2+}. Transiently increased levels of intracellular anandamide, peaking at 5 min, followed by its release into the medium, were again observed (Figure 1B). This effect was abolished by pretreatment of the cells with the nonselective muscarinic receptor antagonist atropine and the PI-selective PLC inhibitor U73122, and by blocking intracellular Ca^{2+} with BAPTA/AM (Figure 1C). Furthermore, also ionomycin enhanced anandamide levels in the presence of extracellular Ca^{2+} and in the absence of carbachol (Figure 1C). The increased anandamide levels were paralleled by enhanced levels of its biosynthetic precursor (Di Marzo et al, 1994), NAPE (Figure 1D), indicating that the higher concentration of anandamide results from de novo biosynthesis. Accordingly, using reverse transcriptase–polymerase chain reaction (RT–PCR), the Ca^{2+}-dependent enzyme catalysing NAPE conversion into anandamide, the NAPE-specific phospholipase D (PLD; Okamoto et al, 2004), was found to be constitutively and abundantly expressed in both wild-type and transfected HEK293 cells (Figure 1E). Thus, these data indicate that mobilization of intracellular Ca^{2+} leads to a transient formation of intracellular anandamide.

It is important to note that other known endogenous ligands of TRPV1, that is, N-arachidonoyldopamine and lipoxygenase products of arachidonic acid (Hwang et al, 2000; Huang et al, 2002), were not detected in cell extracts of thapsigargin-stimulated HEK293 cells, using a very sensitive isotope dilution liquid chromatography mass spectrometric method (detection limit ~30 fmol). By contrast, when HEK293 cells, which do not express constitutively any lipoxygenase activity, were transiently transfected with 12-lipoxygenase cDNA, oxygenated derivatives of arachidonic acid were detected (Figure 1F).

Mobilization of intracellular Ca^{2+} leads to TRPV1 activation

To establish whether the 'transients' of intracellular anandamide described above have any functional consequences at the cellular level, we used a Fura-2-AM-based fluorescence method with a Ca^{2+} reconstitution protocol (Figure 2A). We tested whether stimulation of muscarinic receptors leads to TRPV1 activation in hTRPV1-HEK293 cells. In an extracellular Ca^{2+}-free medium, carbachol dose-dependently released Ca^{2+} from the ER (Figure 2B) in a way blocked by the nonselective muscarinic receptor antagonist, atropine, the selective IP_3 receptor antagonist, xestospongin C, and the inhibitor of PI-selective PLC, U73122, but not by its inactive analogue U73343 (not shown). Thus, carbachol activates the PLC/IP_3 pathway through muscarinic receptors. It is well known that mobilization of intracellular Ca^{2+} from the ER results in extracellular Ca^{2+} influx (Venkatachalam et al, 2002; Zitt et al, 2002; Clapham, 2003; Putney, 2003). Indeed, upon reconstitution of 2.5 mM Ca^{2+} in the extracel-

lular medium, we observed an immediate rise in intracellular Ca^{2+} levels. This increase in intracellular Ca^{2+} was dependent on the dose of carbachol (Figure 2A–C) and was not observed when the cells were pretreated with atropine and U73122 (Figure 2C). Although significant Ca^{2+} entry was observed also in the absence of any stimuli (Figure 2A, lower trace), likely due to leaky cells, the amount of stimulus-induced Ca^{2+} entry was still very strong. In fact, even after correction for leaky cells (which accounted for ~19% of the total Ca^{2+} entry and about 36–40% of the carbachol-induced Ca^{2+} entry), the stimulus-to-vehicle ratio was still >2 and the maximum change in fluorescence ratio ($\Delta F_{340/380}$) upon addition of extracellular Ca^{2+} was 1.34 ± 0.15 for 50 μM carbachol (see Materials and methods). Importantly, with 10 μM xestospongin C, a selective IP3 receptor inhibitor, we observed a significant ($P < 0.05$) inhibition of 50 μM carbachol-induced Ca^{2+} influx (Figure 2C). Furthermore, when both atropine (1 μM) and U73122 (1 μM) were given to cells after the carbachol-induced peak of intracellular calcium, they did not significantly affect carbachol-induced Ca^{2+} influx (89.8 ± 4.3 and $88.1 \pm 4.7\%$ of control, $N = 3$, $P > 0.05$). This indicates that a brief stimulation by muscarinic receptors and the PLC/IP_3 pathway of intracellular Ca^{2+} mobilization are both necessary and sufficient to induce extracellular Ca^{2+} entry.

Three lines of evidence suggest that the TRPV1 channel is involved in this carbachol-induced influx of extracellular Ca^{2+}. Firstly, carbachol-induced extracellular Ca^{2+} influx was significantly less pronounced in wild-type cells than in hTRPV1-HEK293 cells (Figure 2D). It is noteworthy that basal Ca^{2+} levels were not significantly different in wild-type HEK293 cells from hTRPV1-expressing HEK293 cells: $F_{340/380} = 1.26 \pm 0.03$ ($n = 7$) versus $F_{340/380} = 1.36 \pm 0.09$ ($n = 12$), respectively. Secondly, when TRPV1 was desensitized by pretreatment with its prototypical ligand CPS, before stimulation with carbachol, extracellular Ca^{2+} influx was reduced to levels similar to those observed in wild-type cells (Figure 2D), without affecting carbachol-induced Ca^{2+} transient (see below). Conversely, CPS-induced Ca^{2+} influx was reduced when the cells were pretreated with carbachol (see also below) (Figure 2B). Finally, we found that SKF96365, a nonselective TRPV1 antagonist, completely abolished carbachol-induced Ca^{2+} entry in hTRPV1-HEK293 cells. Furthermore, two other structurally different TRPV1 antagonists, that is, 5'-iodo-RTX (I-RTX) and capsazepine (CPZ), were able to attenuate carbachol-induced Ca^{2+} influx (Figure 2D). This attenuation was not observed in wild-type cells (Figure 2E), thereby excluding nonselective effects of the antagonists.

Similar results were obtained when thapsigargin (1 μM), instead of carbachol, was used to mobilize intracellular Ca^{2+} in hTRPV1-HEK293 (Figure 2F and H). This is in line with the finding that thapsigargin can indirectly produce inward currents in part via TRPV1 (Liu et al, 2003). In fact, pretreatment of cells with the TRPV1 antagonists I-RTX and CPZ and with SKF96365 significantly reduced thapsigargin-induced Ca^{2+} influx after extracellular Ca^{2+} reconstitution. A high concentration (5 μM) of U73122, given to cells before thapsigargin, affected thapsigargin-induced Ca^{2+} influx ($43.5 \pm 3.6\%$ of control, $N = 3$, $P < 0.05$), but not thapsigargin-induced Ca^{2+} mobilization ($83.5 \pm 8.6\%$ of control, $N = 3$, $P > 0.05$), in agreement with its capability of directly interacting with
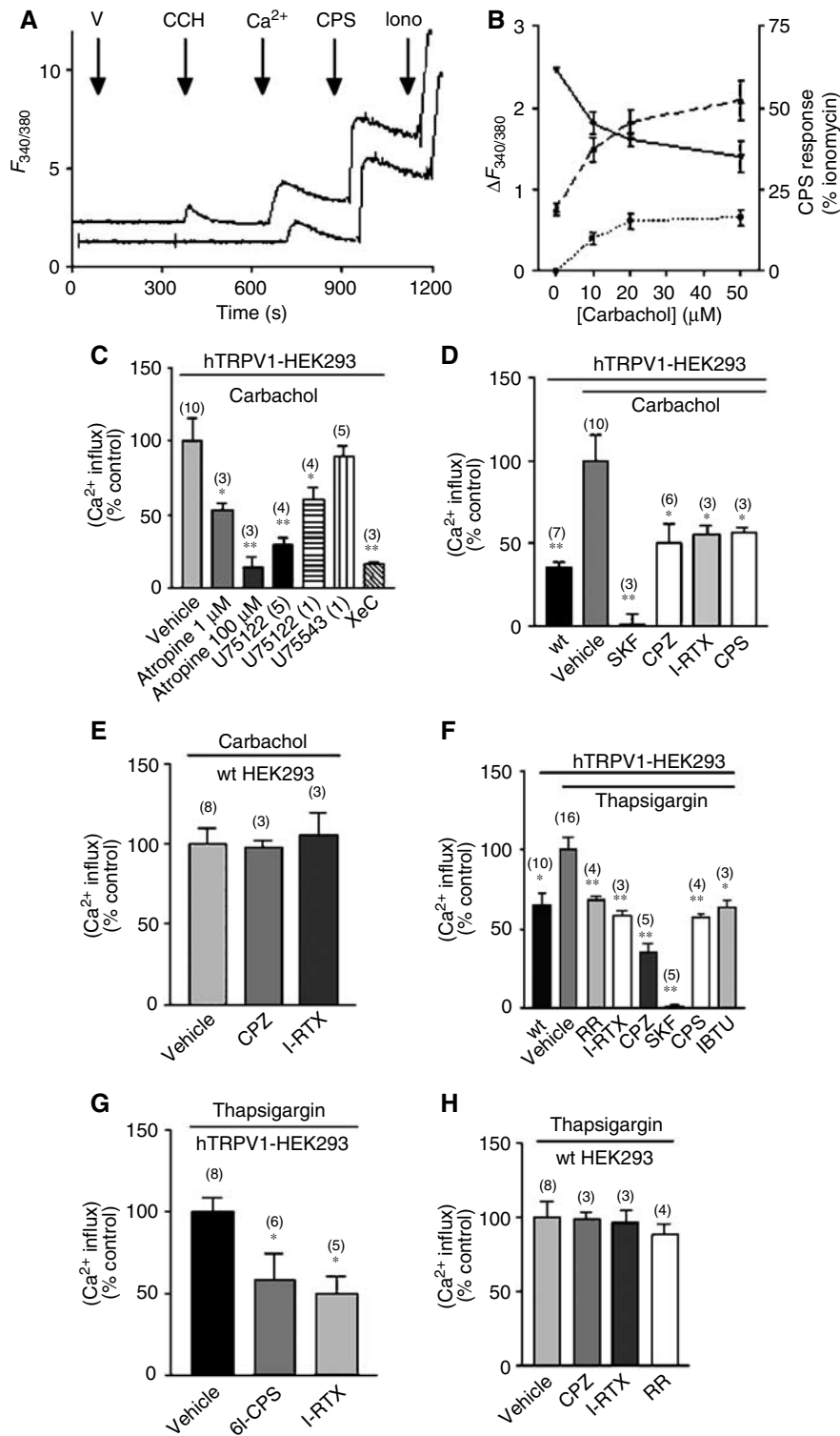
Figure 2 Mobilization of intracellular Ca^{2+} leads to TRPV1 activation. (**A**) Typical fluorescence trace of a Ca^{2+} reconstitution experiment. Arrows indicate time point of compound addition (V, vehicle; carbachol (upper trace, CCH, $50 \mu M$, or vehicle, lower trace); $CaCl_2$ (2.5mM); CPS ($0.1 \mu M$) and ionomycin (Iono, $4 \mu M$). (**B**) Carbachol releases Ca^{2+} from the ER (punctuated line) ($n = 7–12$ for each data point), followed by extracellular Ca^{2+} influx (dashed line) upon Ca^{2+} reconstitution. CPS ($0.1 \mu M$) stimulation induces Ca^{2+} influx (solid line, right Y-axis). (**C**) Effects of pretreatment with atropine (1 and $100 \mu M$), U73122 (5 and $1 \mu M$), U73343 ($1 \mu M$), xestospongin (XeC, $10 \mu M$), or (**D**) of 10 min pretreatment with CPS ($0.1 \mu M$) CPZ ($10 \mu M$), 5-iodo-resiniferatoxin (I-RTX, 1 nM), SKF96365 (SKF, $30 \mu M$) on $50 \mu M$ carbachol-induced Ca^{2+} entry in hTRPV1-HEK293 ($\Delta F_{340/380}$ ($50 \mu M$ CCH) $= 1.34 \pm 0.15$); wt, wild-type cells. (**E**, **H**) TRPV1 antagonists do not reduce calcium influx in wt HEK293 cells. (**F**) Thapsigargin ($1 \mu M$) induced extracellular Ca^{2+} entry in hTRPV1-expressing HEK293 cells ($\Delta F_{340/380}$ ($1 \mu M$ Thps) $= 0.96 \pm 0.07$) and wt HEK293 cells. The effects of CPZ ($10 \mu M$), ruthenium red (RR, $10 \mu M$), IBTU ($2.5 \mu M$), 5'-iodo-resiniferatoxin (I-RTX, 10 nM), SKF96365 (SKF, $30 \mu M$) and desensitization with $0.1 \mu M$ CPS are also shown. (**G**) In the presence of extracellular Ca^{2+}, thapsigargin ($1 \mu M$) increases intracellular Ca^{2+} levels. The effects of selective hTRPV1 antagonists 5-iodo-resiniferatoxin (I-RTX, 10 nM) and 6-iodo-nordihydrocapsaicin (6I-CPS, 10 nM) are shown.

channels involved in extracellular Ca^{2+} influx after Ca^{2+} mobilization from intracellular stores. These data reinforce the hypothesis that the mobilization of intracellular Ca^{2+} from the ER is sufficient to activate TRPV1. When Ca^{2+} was present in the extracellular medium since the beginning, and during thapsigargin stimulation, similar results were obtained (Figure 2G), thus possibly suggesting that the presence of leaky cells did not strongly bias the data obtained above with the Ca^{2+} reconstitution protocol. Altogether, these data indicate that TRPV1 is not only a receptor-operated channel, but is also gated following intracellular Ca^{2+} mobilization. Thus, in addition to sensitization or activation by removal of its inhibition by phosphatidylinositolbisphosphate via PLC-mediated hydrolysis (Chuang et al, 2001) or by protein kinase C-catalysed phosphorylation following PLC-mediated diacylglycerol release (Premkumar and Ahern, 2000; Tominaga et al, 2001), TRPV1 can also be activated by increases of intracellular Ca^{2+}.

The TRPV1 channels activated by intracellular Ca^{2+} mobilization are likely located on the plasma membrane

Apart from the plasma membrane, a population of TRPV1 receptors was described to be present also on the ER of cells overexpressing this protein (Liu et al, 2003; Turner et al, 2003). Although our calcium fluorescence technique does not allow us to distinguish between these two populations of TRPV1 channels, four lines of evidence strongly suggest that only the plasma membrane population gates Ca^{2+} following Ca^{2+} mobilization by thapsigargin: (1) In the absence of extracellular Ca^{2+}, neither CPS (up to $20 \mu M$) nor anandamide (up to $50 \mu M$) released intracellular Ca^{2+} in hTRPV1-HEK293 cells (not shown); we checked with $1 \mu M$ thapsigargin or $50 \mu M$ carbachol the status of the intracellular stores after addition of CPS ($20 \mu M$) or anandamide ($50 \mu M$), and in both cases the two former compounds were still capable of strongly releasing intracellular Ca^{2+} (thapsigargin after CPS $\Delta F_{340/380} = 0.25 \pm 0.02$, $n = 3$, thapsigargin after anandamide $\Delta F_{340/380} = 0.24 \pm 0.02$, $n = 3$, thapsigargin alone $\Delta F_{340/380} = 0.27 \pm 0.04$; $n = 6$; carbachol after CPS $\Delta F_{340/380} = 0.28 \pm 0.04$, $n = 3$, carbachol after anandamide $\Delta F_{340/380} = 0.25 \pm 0.03$, $n = 3$, carbachol alone $\Delta F_{340/380} = 0.32 \pm 0.04$; $n = 6$). (2) Only RTX at $4 \mu M$ and CPS at $100 \mu M$, that is, at doses that are 100-fold higher than those sufficient for TRPV1 activation at the plasma membrane, mobilized intracellular Ca^{2+}. However, pretreatment with $1 \mu M$ thapsigargin reduced RTX-induced intracellular Ca^{2+} release by 75% (from $\Delta F_{340/380} = 0.17 \pm 0.03$, $n = 7$ to $\Delta F_{340/380} = 0.04 \pm 0.01$, $n = 6$). This suggests that the RTX-sensitive Ca^{2+} pool in the ER is overlapping with the thapsigargin-sensitive pool to a great extent. Therefore, emptying the calcium stores with thapsigargin also largely depletes the RTX-sensitive store and cannot lead to its activation. (3) Ruthenium red, a cell-impermeable nonselective TRPV1 antagonist, significantly reduced thapsigargin-induced extracellular Ca^{2+} influx in hTRPV1-HEK293, but not wild-type, cells (Figure 2F). (4) IBTU, a specific TRPV1 antagonist selective for the plasma membrane population of TRPV1 channels (Toth et al, 2004), significantly reduced thapsigargin-induced extracellular Ca^{2+} influx ($-35.9 \pm 3.8\%$, $n = 3$; Figure 2F), but not RTX-induced mobilization of intracellular Ca^{2+} in the absence of extracellular Ca^{2+} ($+0.33 \pm 1.9\%$, $N = 3$, not shown), in hTRPV1-HEK293 cells. IBTU also significantly

reduced carbachol-induced extracellular Ca^{2+} influx by $32.2 \pm 4.1\%$ ($P < 0.05$, $n = 5$, not shown).

Formation of intracellular anandamide following calcium mobilization causes TRPV1 activation and calcium influx

Formation of intracellular anandamide, described in the first section of this paper, and Ca^{2+} entry mediated by plasma membrane TRPV1, described in the previous section, both induced by carbachol- and thapsigargin-induced mobilization of intracellular Ca^{2+} stores, might be two independent events. However, two observations indicate that the transient peak of intracellular anandamide observed here causes Ca^{2+} entry by acting at TRPV1: (1) Exogenously added anandamide induces Ca^{2+} influx via TRPV1 in hTRPV1-HEK293 cells ($EC_{50} = 261 \pm 13$ nM; $n = 3$, which is in the range of the intracellular concentrations produced by thapsigargin and carbachol stimulation); this effect is blocked by CPZ and I-RTX and is not observed in wild-type HEK293 cells (not shown). (2) Anandamide (100 nM), when injected into the cell, is very efficacious at inducing TRPV1-mediated plasma membrane currents in neurons expressing high levels of this channel (Evans et al, 2004). These latter observations suggest that this lipid induces elevated intracellular Ca^{2+} by activating a predominant population of TRPV1 on the plasma membrane.

If anandamide acts as an intracellular messenger at TRPV1, modulation of its concentration in the cell should affect TRPV1 activity, and hence TRPV1-mediated Ca^{2+} entry. Intracellular anandamide can be inactivated through two concurrent processes, that is, (1) its extrusion from the cell, which is mediated by a specific membrane mechanism selectively inhibited by some synthetic fatty acid amides, and (2) intracellular enzymatic hydrolysis (Piomelli, 2003). Preincubation of cells with two different classes of selective inhibitors of anandamide transport through the plasma membrane with no direct activity at TRPV1, VDM11 and OMDM-1 (De Petrocellis et al, 2000; Ortar et al, 2003) significantly enhanced Ca^{2+} influx in hTRPV1-HEK293 cells (Figure 3A and D). This was not observed in wild-type cells (Figure 3B and C) nor when thapsigargin or carbachol were omitted (not shown), thus demonstrating that anandamide is acting only through TRPV1 and not any other endogenously expressed Ca^{2+}-permeable channel. Importantly, VDM11 concomitantly increased the percentage of intracellular anandamide levels after 30 min of stimulation from 28 ± 5 to $63 \pm 5\%$ ($n = 3$, $P < 0.05$). These data indicate that the efflux of anandamide from the cell is tonically limiting its actions on TRPV1.

HEK293 cells possess also a fatty acid amide hydrolase (FAAH) that degrades anandamide to arachidonic acid and ethanolamine (Cravatt et al, 1996). To further support our hypothesis that anandamide, produced following store emptying, tonically activates TRPV1, and to rule out the contribution of arachidonic acid metabolites in this process (Hwang et al, 2000; Watanabe et al, 2003), we used an inhibitor of FAAH, methyl-arachidonoyl-fluoro-phosphonate (MAFP). This compound also inhibits the Ca^{2+}-dependent phospholipase A_2, the enzyme mostly responsible for arachidonic acid mobilization. MAFP increased carbachol- and thapsigargin-induced extracellular Ca^{2+} influx in hTRPV1-HEK293 cells (Figure 3A and D), but not in wild-type cells
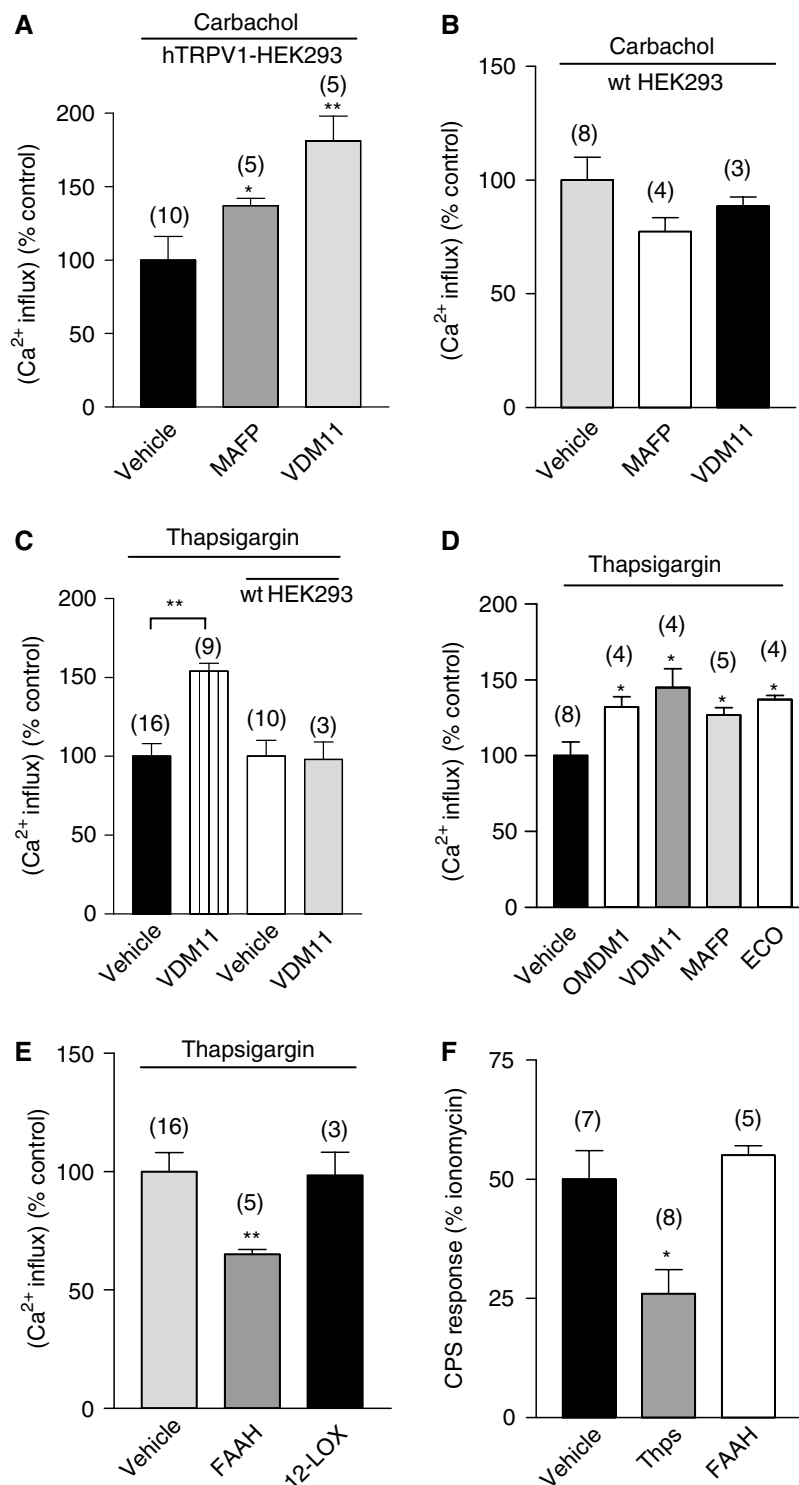
Figure 3 Anandamide metabolism and efflux from cell limit TRPV1 activation. (**A–D**) Carbachol- and thapsigargin-induced Ca^{2+} influx ($\Delta F_{340/380}$ (50 μM CCH) = 1.34 ± 0.15 and $\Delta F_{340/380}$ (1 μM Thps) = 0.96 ± 0.07) in hTRPV1-HEK293 cells is enhanced by pretreatment with inhibitors of anandamide metabolism MAFP (10 μM) and econazole (ECO, 10 μM) and by anandamide transport inhibitors VDM11 (4 μM) and OMDM1 (10 μM), but (B, C) not in wild-type cells. In panel D, extracellular Ca^{2+} was present during thapsigargin treatment. (**E**) Transient overexpression of FAAH in hTRPV1-HEK293 cells leads to a reduction of thapsigargin-induced extracellular Ca^{2+} influx. Transient expression of 12-lipoxygenase (12-LOX) does not influence thapsigargin-induced Ca^{2+} influx. (**F**) Thapsigargin-induced desensitization of TRPV1 receptor, as measured by Ca^{2+} influx upon a stimulation with CPS, is abolished when FAAH is transiently overexpressed.

(Figure 3B), and did not enhance Ca^{2+} influx in hTRPV1-HEK293 cells in the absence of thapsigargin or carbachol (not shown). Finally, when the cells were pretreated with the cytochrome P450 inhibitor econazole, there was even

an enhancement of thapsigargin-induced Ca^{2+} influx (Figure 3D). These data argue against the involvement of arachidonic acid metabolites in TRPV1-mediated Ca^{2+} entry, and again indicate that anandamide is tonically and

selectively activating TRPV1 and not any other endogenously expressed TRP channel.

To further demonstrate that anandamide, and not any other putative TRPV1 ligand (e.g. lipoxygenase metabolites or N-arachidonoyldopamine), acts as an intracellular messenger at TRPV1, we used also nonpharmacological approaches. First, we reduced anandamide levels either by overexpression of FAAH or by elimination of arachidonic acid, the ultimate biosynthetic precursor of anandamide. Transient overexpression of rat FAAH into hTRPV1-HEK293 cells increased anandamide hydrolysis from 180 ± 56 to 3385 ± 102 pmol/min/mg protein ($n=3$, $P<0.05$) and concomitantly decreased thapsigargin-induced Ca^{2+} influx to the same level as in wild-type HEK293 cells (compare Figure 3E with Figure 2F). The CPS response in the thapsigargin-stimulated, FAAH-transfected cells was comparable to vehicle-stimulated cells (Figure 3F). This indicates that the thapsigargin-induced desensitization of TRPV1 was absent when anandamide degradation was upregulated. Cells cultured for 2 days in the presence of 10 μM CP-24879, a mixed Δ^5/Δ^6-desaturase inhibitor that blocks arachidonic acid formation from essential fatty acid precursors (Obukowicz et al, 1998), showed a strongly reduced thapsigargin-induced Ca^{2+} influx from $\Delta F_{340/380} = 0.96 \pm 0.08$ to 0.24 ± 0.02 ($n=4$, $P<0.05$). At the same time, these cells, when stimulated for 15 min with thapsigargin (1 μM), produced significantly lower intracellular amounts of anandamide with respect to cells cultured for 2 days with vehicle (0.6 ± 0.2 versus 1.4 ± 0.3 pmol/10^7 cells, $P<0.05$, $n=3$). Finally, hTRPV1-HEK293 cells transiently transfected with human 12-lipoxygenase (Figure 1E), although potentially capable of producing compounds active at TRPV1 (Figure 1F), did not exhibit an increased thapsigargin-induced Ca^{2+} influx (Figure 3E). Altogether, these findings indicate that anandamide, and not other putative endogenous TRPV1 agonists, mediates thapsigargin- and carbachol-induced Ca^{2+} influx via TRPV1.

Intracellular anandamide leads to TRPV1 activation in rat sensory neurons

To study whether the formation of anandamide induced by intracellular Ca^{2+} mobilization also leads to endogenous activation of TRPV1 in a native system, we have used neonatal primary sensory neurons from dorsal root ganglia (DRG). These cells constitutively express high levels of TRPV1 and synthesize anandamide in a Ca^{2+}-dependent manner (Tognetto et al, 2001; Trevisani et al, 2002; Ahluwalia et al, 2003a, b). As in hTRPV1-HEK293 cells, mobilization of intracellular Ca^{2+} by thapsigargin in rat sensory neurons induced the formation of anandamide whose intracellular concentration was significantly enhanced by pretreatment with VDM11 (Figure 4A). We found, using single-cell Ca^{2+} fluorescence imaging, that thapsigargin-induced extracellular Ca^{2+} entry in rat DRG neurons can be reduced by over 50% by CPZ or I-RTX (Figure 4B). Furthermore, thapsigargin-induced extracellular Ca^{2+} entry was strongly enhanced by VDM11 and MAFP (Figure 4B). Thus, these data indicate that endogenously formed anandamide can activate TRPV1 in rat primary sensory neurons.

ATP has been shown to induce TRPV1-mediated inflammatory hyperalgesia through activation of the $G_{q/11}$-coupled P_2Y receptors in primary DRG sensory neurons (Tominaga et al, 2001). Here, we show that anandamide contributes to ATP-mediated Ca^{2+} influx in adult rat DRG neurons (Figure 4C and D). In the absence of extracellular Ca^{2+}, ATP mobilized intracellular Ca^{2+} and significantly enhanced intracellular anandamide levels, which were even further increased by blocking its transport out of the neurons with VDM11 (Figure 4C). Upon reconstitution of Ca^{2+}, the subsequent Ca^{2+} entry (Figure 4D) could be significantly attenuated by CPZ (-28%) and I-RTX (-93%), and by desensitization with CPS (-77%) or RTX (-90%). This latter process has recently been shown to be specific for TRPV1-expressing neurons (Karai et al, 2004). ATP-induced Ca^{2+} entry was again strongly enhanced by VDM11 ($+74\%$). This latter effect was also blocked by CPZ (not shown). When DRG neurons were pretreated with U73122, the enhanced Ca^{2+} influx by both ATP and VDM11 was inhibited (Figure 4D). Accordingly, anandamide levels were also reduced in ATP-stimulated neurons pretreated with U73122 (Figure 4C). Thus, the production of intracellular anandamide and its subsequent activation of TRPV1 are dependent on a functionally active PLC/IP$_3$ pathway.

Patch-clamp experiments in DRG neurons confirm that TRPV1 is the channel involved in intracellular anandamide actions

To further verify anandamide-induced TRPV1 channel activation upon store depletion, we carried out whole-cell patch-clamp experiments to measure ion currents in DRG neurons. Neurons were divided into TRPV1-expressing or nonexpressing by testing the response to CPS. A 1 s test pulse of 500 nM CPS was applied, followed by a longer application (30–60 s); TRPV1-expressing cells always exhibited a response >100 pA to the 1 s pulse of CPS, and in nonexpressing cells the response was <5 pA to both CPS exposures. The CPS test was performed at the end of the experiment. Thapsigargin induced a slowly developing inward current detectable only in CPS-responsive, TRPV1-expressing rat and mouse DRG neurons (Figure 5A). In the presence of the same concentration of VDM11 shown here to enhance anandamide levels in DRG neurons (Figure 4A), this current was significantly elevated, whereas in nonTRPV1-expressing neurons no significant current was activated ($P<0.01$, $n=18$; Figure 5A and D). VDM11 per se did not produce any measurable current (not shown). Both CPZ and I-RTX totally erased this current (Figure 5B and D). The I–V plot of the cation current evoked by thapsigargin + VDM11 was typical of TRPV1 (Caterina et al, 1997), exhibiting a clearly outwardly rectifying current and a reversal potential near 0 mV (2.2 ± 1.1 mV, $n=4$; Figure 5C). Furthermore, in DRG neurons prepared from TRPV1$-/-$ mice, no current was induced with thapsigargin + VDM11 (Figure 5A and D). These data, taken together, strongly suggest that, in DRG neurons, the enhanced intracellular anandamide levels produced following intracellular Ca^{2+} release gate uniquely the plasma membrane population of TRPV1 and no other channel.

Discussion

We have described here for the first time a cellular process in which TRPV1 is activated by endogenously formed anandamide, thereby revealing a role for anandamide as an intracellular messenger participating in Ca^{2+} signal amplification. According to the mechanism proposed here, anandamide
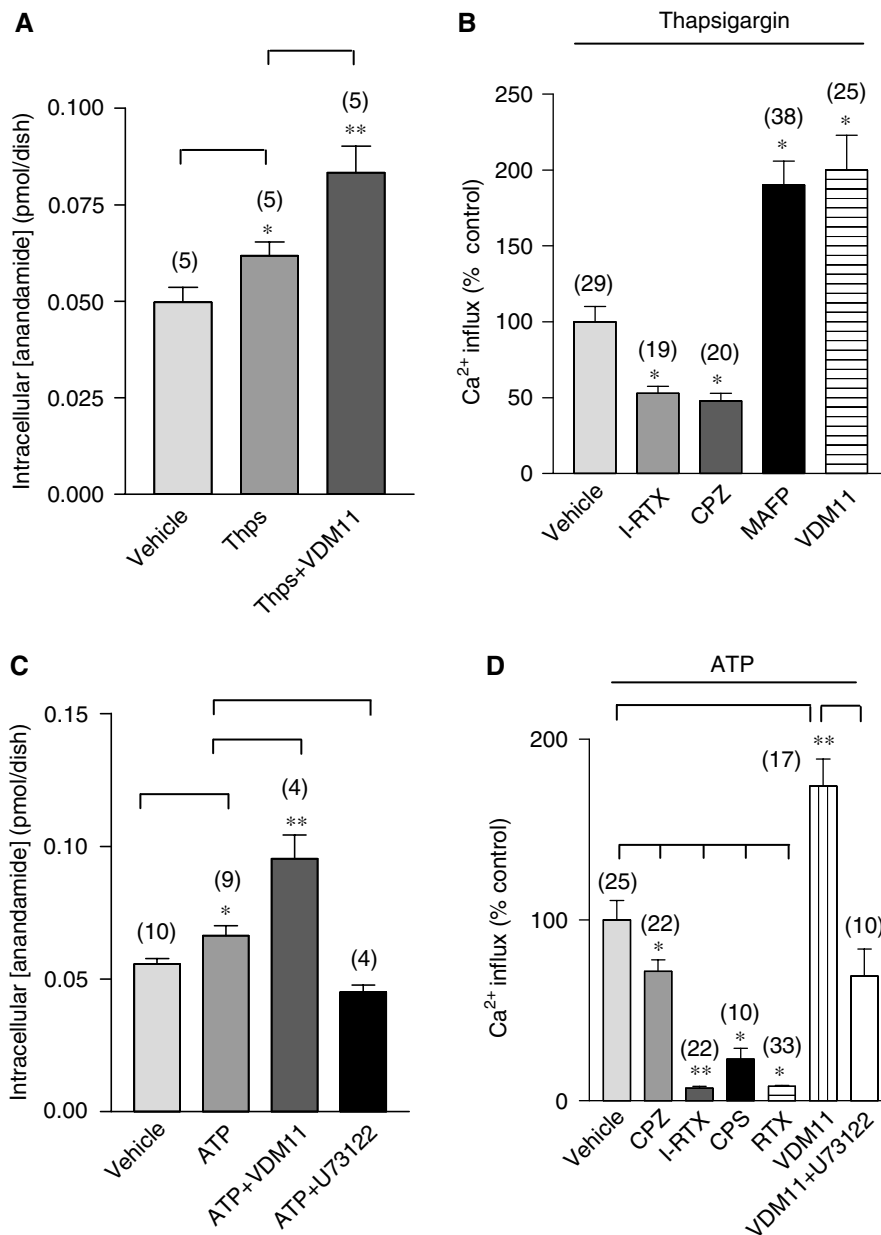
Figure 4 Mobilization of intracellular Ca^{2+} leads to anandamide formation and subsequent TRPV1 activation in sensory neurons of rat DRG. Intracellular levels of anandamide in rat DRG neurons (25 000 neurons/dish) upon (**A**) thapsigargin (1 μM) treatment for 30 min of neonatal neurons, and (**C**) ATP (100 μM) treatment (15 min) of adult neurons. In both cases, an extracellular Ca^{2+}-free medium was used, but otherwise the conditions were as described (Ahluwalia *et al*, 2003b). In panel C, the effects of anandamide transport inhibitor VDM11 (4 μM) and PLC inhibitor U73122 (5 μM) are also shown. (**B**) Thapsigargin (1 μM)-induced extracellular Ca^{2+} influx in neonatal rat DRG neurons. The effects of CPZ (10 μM), I-RTX (10 nM), MAPF (10 μM) and VDM11 (4 μM) are shown. (**D**) ATP (100 μM)-induced extracellular Ca^{2+} entry can be attenuated by pretreatment with CPZ (10 μM), I-RTX (10 nM), CPS (10 μM) and RTX (10 nM), and enhanced by pretreatment with VDM11 (4 μM) in adult rat DRG neurons. The effect of VDM11 is not observed when ATP-induced Ca^{2+} release from ER is blocked by U73122 (5 μM). At these doses, CPZ, MAFP and VDM11 did not affect Ca^{2+} entry in the absence of ATP.

is formed following the activation of the PLC/IP_3 pathway and, prior to its hydrolysis or release from cells, it induces TRPV1-mediated Ca^{2+} influx (Figure 6).

Although our findings indicate that anandamide behaves as a Ca^{2+} influx factor in cells expressing TRPV1 receptors and stimulated with intracellular Ca^{2+}-store-emptying stimuli, they do not allow to suggest that the phenomenon we have described represents a store-operated calcium entry (SOCE) mechanism (Venkatachalam *et al*, 2002; Zitt *et al*, 2002; Clapham, 2003; Putney, 2003). In fact, it has been suggested

that TRP channels, including TRPV1 (Liu *et al*, 2003), may be activated by the depletion of intracellular Ca^{2+} stores, via either store-channel coupling or a diffusible messenger, to gate extracellular Ca^{2+} to replenish these stores (Venkatachalam *et al*, 2002; Zitt *et al*, 2002; Putney, 2003; Bolotina, 2004; Nilius, 2004; Penner and Fleig, 2004). However, we did not investigate the relationship between store depletion and TRPV1 activation, but only between the latter and the increased cytoplasmic Ca^{2+} concentrations that follow store depletion. Therefore, further studies are
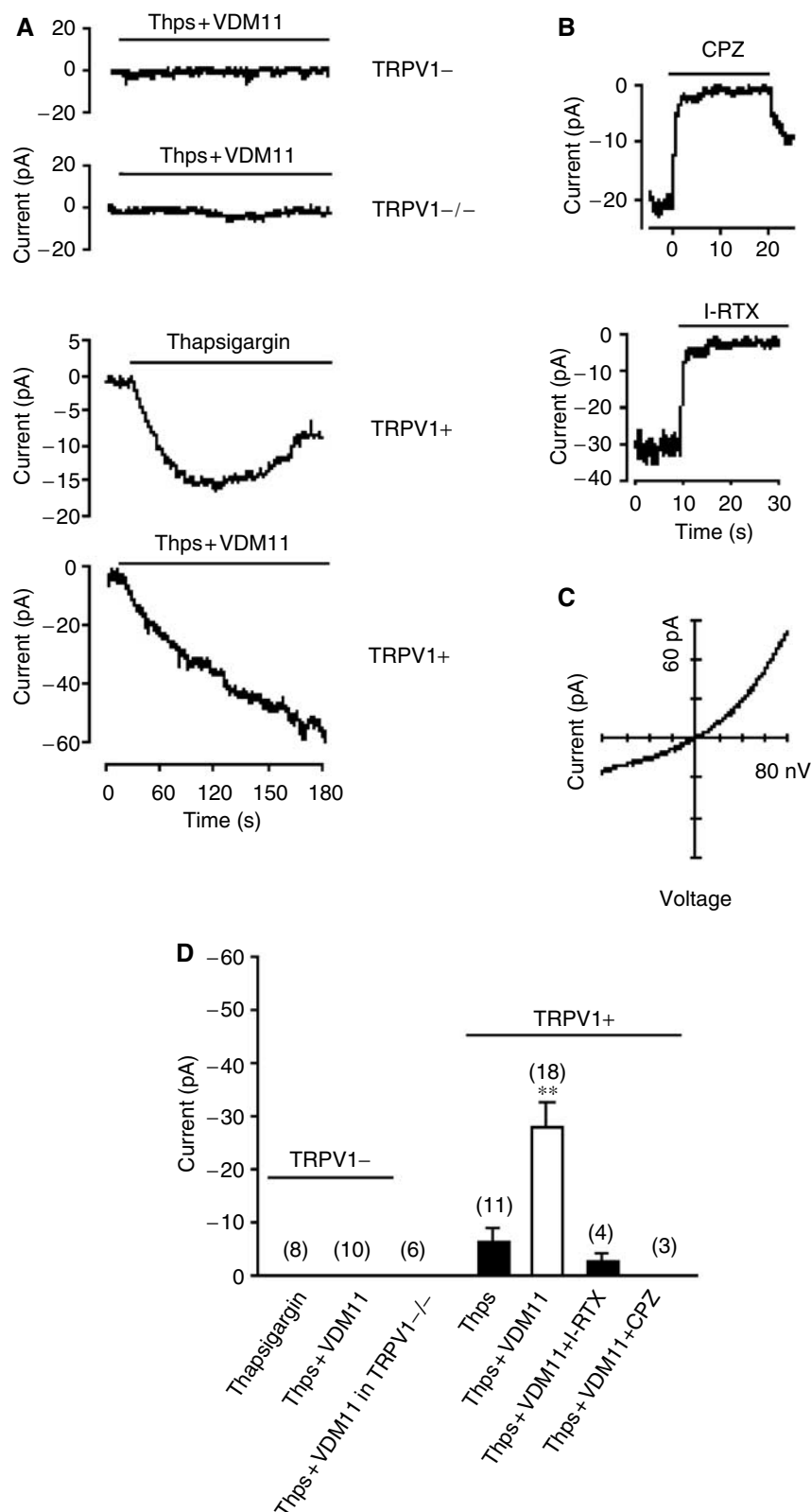
Figure 5 Mobilization of intracellular Ca^{2+} by thapsigargin and concomitant blockade of anandamide efflux induce a TRPV1-mediated current in sensory neurons of DRG. (**A**) Thapsigargin generates an inward current in TRPV1-expressing rat DRG neurons pretreated with VDM11 (4 μM) (bottom panel), but not in non-TRPV1-expressing neurons or in neurons from TRPV1−/− mice (two upper traces). Thapsigargin *per se* elicited a much weaker current (middle panel). VDM11 alone induced no detectable current (not shown). The thapsigargin + VDM11-induced current exhibited the *I–V* relationship shown in (**C**), and was blocked by CPZ (10 μM) (**B**, upper panel) and I-RTX (10 nM) (**B**, lower panel). (**D**) Summary of the effects obtained in patch-clamp experiments. The thapsigargin-induced current was significantly larger in TRPV1-expressing neurons exposed to VDM11 than in neurons either not exposed to VDM11 or exposed to VDM11 but not expressing TRPV1 ($P<0.01$), and was blocked by CPZ and I-RTX. Mean capacitance of neurons treated with thapsigargin + VDM11 was 34 ± 3 pF, normalized current was 0.8 ± 0.1 pA/pF and capacitance range was 20–60 pF.
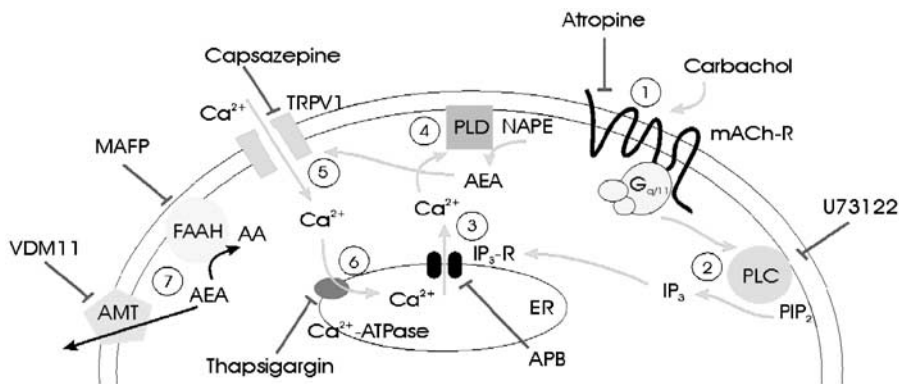
Figure 6 Anandamide as an intracellular messenger inducing Ca^{2+} influx via TRPV1. Stimulation of G-protein-coupled receptors (1) coupled to $G_{q/11}$ activates the PLC/IP$_3$ pathway (2) and results in Ca^{2+} mobilization from the ER (3). The increase in intracellular Ca^{2+} activates the biosynthetic pathway of anandamide (AEA) (4), which, alone or together with concomitant sensitizing stimuli, signals to TRPV1 (5) to gate Ca^{2+} entry in the cytoplasm (6). Anandamide action is terminated either by its extrusion from the cell or hydrolysis by FAAH (7).

necessary to investigate whether or not the mechanism described here is related to SOCE. On the other hand, our findings do resemble the actions of cytochrome metabolites of arachidonic acid, which have been proposed to act as calcium influx factors (Rzigalinski et al, 1999) and to activate TRPV4 (Watanabe et al, 2003).

Finally, our findings might be physiologically relevant to spontaneous neurotransmitter release and short-term synaptic plasticity in neurons (Emptage et al, 2001). The recent reports that the threshold for TRPV1 gating by ligands can be sensibly decreased following cell depolarization (Ahern and Premkumar, 2002), and that, conversely, even low concentrations of ligands can cause gating of TRPV1 at negative, as opposed to positive, membrane potentials (Voets et al, 2004), may also be relevant to these phenomena.

Materials and methods

Animals
Adults and newborn albino Sprague–Dawley rats ($\sim$300 g) were used (Pampaloni, Italy). All experiments complied with the national guidelines and were approved by the regional ethics committee.

Cell culture and transfection
The hTRPV1-HEK293 and wild-type cells were grown as reported (De Petrocellis et al, 2001). Transient transfections of hTRPV1-HEK293 cells with pcDNA3 vectors containing 12- or 15-lipoxygenase or FAAH were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. In some experiments, the amount of polyunsaturated fatty acids in hTRPV1-HEK293 cells was reduced by adding 10 μM CP-24879 (Cayman Chemicals) to the medium for 2 days to block biosynthesis of arachidonic acid (Obukowicz et al, 1998). It is noteworthy that serum levels were stepwise reduced in 4 days to 0% before CP-24879 incubation, because it is a source of exogenous polyunsaturated fatty acids.

Anandamide and N-arachidonoyldopamine quantification
Confluent hTRPV1-HEK293 cells or adult rat DRG neurons were washed with phosphate-buffered saline without Mg^{2+}/Ca^{2+} and incubated with thapsigargin (1 μM), carbachol (50 μM) or ATP (100 μM) for indicated time in Hanks' balanced salt solution ($+$2.5 mM EGTA, pH 7.4) without $CaCl_2$ at room temperature. Atropine and U73122 were added to the cells 5 min before stimulation. In some experiments cells were loaded with BAPTA/AM prior to stimulation. Incubation was terminated by removing the buffer. Lipids were extracted from the cells with Bligh and Dyer method and anandamide, released in the incubation buffer, was

recovered by solid phase extraction. Reversed-phase HPLC with isotope dilution mass spectrometry, using d^8-anandamide (10 pmol) and d^8-N-arachidonoyldopamine (50 pmol) as internal standard, was applied to quantify anandamide and N-arachidonoyldopamine as described (Di Marzo et al, 2001; Huang et al, 2002).

RT–PCR analysis
The expression of mRNAs for NAPE-PLD in HEK cells, HEK-hVR1 cells and rat DRG neurons was examined by RT–PCR. The PCR primers for NAPE-PLD were selected on the basis of the sequence of the NAPE-PLD mRNA (NCBI accession number AB112352). The NAPE-PLD sense and antisense primers were 5′-TGGACTGGTGGG AGGAG-3′ (nt 689–705) and 5′-GGTTCATAAGCTCCGATGGG-3′ (nt 919–938), respectively. The expected size of the amplicon was 249 bp for NAPE-PLD.

Enzyme assays
Analyses of the activity of FAAH and 12- and 15-lipoxygenase in cell homogenates and PLD with lipid extracts to determine NAPE levels were conducted as described (Di Marzo et al, 2001; Veldhuis et al, 2003).

Measurement of changes in $[Ca^{2+}]_i$
Measurement of intracellular Ca^{2+} fluorescence in cultured rat DRG was made as previously described (Trevisani et al, 2002; Liu et al, 2003). Cells were loaded with Fura-2-AM (3 μM) in Ca^{2+}-free buffer solution composed of (mM) KCl 5.4, $MgSO_4$ 0.4, NaCl 135, D-glucose 5, HEPES 10 with BSA 0.1%, 2.5 EGTA and 0.1% bovine serum albumin, for 40 min at 37°C, at pH 7.4. The dye was excited at 340 and 380 nm to indicate relative $[Ca^{2+}]_i$ changes by the F_{340}/F_{380} ratio. Changes in fluorescence were monitored after a 10 min stabilization, and in a 0–300 s time interval after neuron stimulation and over 300 s after Ca^{2+} addition. Ca^{2+} fluorescence spectrofluorometry with hTRPV1-HEK293 and wild-type cells was performed in a quartz cuvette with Fura-2-AM or Fluo-3 (in case Ca^{2+} was present during thapsigargin incubation) as described (De Petrocellis et al, 2001). The Ca^{2+} reconstitution protocol was as follows: 300 s pretreatment with vehicle or compound as specified, then store-depleting agent, then 300 s post-treatment (in the case of CPZ), followed immediately by $CaCl_2$ (2.5 mM), then CPS (0.1 μM) and finally ionomycin (4 μM). Ca^{2+} influx was calculated as the maximum increase in F_{340}/F_{380} ratio upon addition of extracellular Ca^{2+} minus the nonselective Ca^{2+} influx as measured with a blank (no agonist/thapsigargin added) to correct for leaky cells. The Ca^{2+} signal in HEK293 cells was calibrated previously (Bisogno et al, 2001) and normalized to total Ca^{2+} influx in Figures 2C–H, 3 and 4. After correction for leaky cells, the maximum change in fluorescence ratio ($\Delta F_{340/380}$) upon addition of extracellular Ca^{2+} was 1.34 ± 0.15 and 0.96 ± 0.07 for 50 μM carbachol and 1 μM thapsigargin, respectively.

Whole-cell patch-clamp experiments

All recordings were made from the somata of small-diameter DRG neurons (capacitance range was 20–60 pF), at ambient room temperature (20–24°C), with the whole-cell patch-clamp technique using an Axon 200B patch-clamp amplifier and pCLAMP software (Axon Instruments). Patch pipettes of resistance 1.5–2 MΩ were prepared from borosilicate glass using a Sutter Instruments P-87 horizontal micropipette puller (Novato, CA, USA). Pipette capacitance was compensated electronically before breaking into the whole-cell mode, and the series resistance after establishing the whole-cell mode (which ranged from 4 to 7 MΩ), calculated from the time constant of current decay in response to a voltage step, was routinely checked to ensure that it did not increase in the course of an experiment, but was not compensated given the small size of currents recorded. Only one recording was performed on each culture dish in order to ensure that data were not obtained from cells that had been inadvertently exposed to test treatments.

Recordings were performed in an extracellular medium of the following composition (mM): 140 NaCl, 10 CaCl$_2$, 1 MgCl$_2$, 4 KCl, 10 Hepes and 4 glucose, neutralized to pH 7.4 with NaOH. Ultrapure water (MilliQ) was used in the preparation of all solutions. Intracellular solution contained the following (mM): 135 KCl, 1.6 MgCl$_2$, 0.1 EGTA, 2.5 MgATP, 0.2 Li$_2$GTP and 10 Hepes, neutralized to pH 7.3 with NaOH. Given the slow onset and small size of currents measured, a large number of recordings were performed in order to select only cells with particularly stable holding currents. Holding potential was set at –80 mV, as this increased the stability of the holding current. Experimental protocol after obtaining a whole-cell recording involved first exposure to a control or VDM11-containing solution, followed by application of thapsigargin. At the end of each experiment, a pulse of 500 nM CPS was applied to the recorded neuron to test for functional expression of TRPV1 (see Results).

Data analysis

Data are given as mean ± s.e. Number of experiments or neurons (*n*) is given in brackets. Statistical analysis was performed with Student's test or a one-way ANOVA, when appropriate followed with Dunnet's or Bonferroni's *post hoc* test. Results were considered statistically significant if $P < 0.05$ (*) or $P < 0.01$ (**).

Acknowledgements

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