Molecular Pharmacology Fast Forward. Published on December 7, 2005 as doi:10.1124/mol.105.015644
This article has not been copyedited and formatted. The final version may differ from this version.

MOL Manuscript # (15644)

Phosphorylation dependent desensitization of vanilloid receptor-1 (TRPV1) function in rat skeletal muscle arterioles and in CHO-TRPV1 cells by anandamide

Erzsébet Lizanecz, Zsolt Bagi, Enikő T. Pásztor, Zoltán Papp, István Édes, Noémi Kedei, Peter M. Blumberg, Attila Tóth

Division of Clinical Physiology, Institute of Cardiology, University of Debrecen, 4032 Debrecen, 22 Moricz Zs krt., Hungary (E.L., Z.B., E.T.P., Z.P., I.É., A.T.); Molecular Mechanisms of Tumor Promotion Section, Laboratory of Cellular Carcinogenesis and Tumor Promotion, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, 20892, USA (N.K., P.M.B., A.T.)

Molecular Pharmacology Fast Forward. Published on December 7, 2005 as DOI: 10.1124/mol.105.015644
This article has not been copyedited and formatted. The final version may differ from this version.

MOL Manuscript # (15644)

Running title: The endovanilloid anandamide as a desensitizer of the TRPV1

Corresponding author:

Attila Tóth, PhD

Division of Clinical Physiology, Institute of Cardiology, University of Debrecen

P. O. BOX 1, 4004, Debrecen, Hungary

Tel.: (36) 52-414-928

Fax: (36) 52-414-928

E-mail: atitoth@jaguar.unideb.hu

Number of text pages: 33

Number of tables: 1

Number of figures: 7

Number of references: 39

Number of words:

In the abstract: 245

In the introduction: 730

In the discussion: 1657

Abbreviations

CHO: Chinese hamster ovary cells; TRPV1 or VR1: vanilloid (capsaicin) receptor 1; CHO-TRPV1: CHO cells expressing the rat vanilloid receptor 1; DMEM: Dulbecco's Modified Eagle's Medium; DPBS: Dulbecco's Phosphate Buffered Saline; PMA: phorbol 12-myristate 13-acetate; PMSF: phenyl methyl sulfonyl fluoride

Abstract

It has been proposed that activation of TRPV1 affects the vasotone of resistance arteries. One of the endogenous activators of TRPV1 is anandamide. The effects of anandamide on TRPV1 responsiveness were tested on isolated, pressurized (80 mmHg) skeletal muscle (m. gracilis) arterioles (179±33 μm in diameter). We found that the TRPV1 agonist capsaicin (1 μM) elicited a substantial constriction in isolated arterioles (51±12%). In contrast, anandamide (0-100 μM) did not affect arteriolar diameter significantly (3±5%). Isolated vessels were also preincubated with anandamide (30 μM, for 20 min). This anandamide pre-treatment completely blocked capsaicin induced arteriolar constriction (response decreased to: 1±0.6%) and this inhibition was reversed by cyclosporin-A (100 nM, 5 min) treatment (constriction: 31±1%). An exogenous TRPV1 expressing cell line (CHO-TRPV1) was used to specifically evaluate TRPV1 mediated effects of anandamide. Determined by $^{45}Ca^{2+}$ uptake, the efficacy of anandamide in this system was 65±8% of that of capsaicin. Upon treatment of the cells with the protein phosphatase-2B inhibitor cyclosporin-A or the PKC activator PMA, anandamide was transformed to a full agonist. Anandamide treatment also caused a fast acute desensitization in these cells as measured by intracellular Ca^{2+} imaging. Application of cyclosporin-A or PMA reversed this desensitization. Our data suggest that anandamide may cause a complete (albeit phosphorylation dependent) desensitization of TRPV1 in skeletal muscle arterioles and in CHO-TRPV1 cells, which apparently transforms the ligand gated TRPV1 to a phosphorylation gated channel. This property of anandamide may provide a new therapeutic strategy to manipulate TRPV1 activity.

Molecular Pharmacology Fast Forward. Published on December 7, 2005 as DOI: 10.1124/mol.105.015644
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

The vanilloid receptor 1 (TRPV1) is a non-selective cation channel, structurally belonging to the transient receptor potential family (TRP) of ion channels. In the periphery, TRPV1 is primarily expressed in sensory C and A-δ fibers (Caterina et al., 1997) and acts as a ligand-, proton- and heat-activated molecular integrator of nociceptive stimuli (Szallasi and Blumberg, 1999; Di Marzo et al., 2002a, b; Ross, 2003). Activation of TRPV1 leads to central (pain) and to local 'sensory-efferent' effects (Szolcsanyi, 2000). These include the release of vasoactive agents (such as calcitonin gene related peptide, CGRP) and subsequent vasorelaxation (Zygmunt et al., 1999).

Compared to the well characterized effects of TRPV1 upon stimulation by exogenous substances (Szallasi and Blumberg, 1999), relatively little is known about its endogenous ligands. So far, anandamide (N-arachidonoyl-ethanolamide) (Zygmunt et al., 1999), NADA (N-arachidonoyl-dopamine) (Huang et al., 2002) and different lipoxygenase products (Hwang et al., 2000) have been identified as endogenous agonists of TRPV1. Among them, anandamide was originally identified as an endocannabinoid activating the G-protein-coupled metabotropic cannabinoid CB1 receptor (Devane et al., 1992).

The reported potencies of anandamide to activate TRPV1 and CB1 receptors are in the same range (from 10 nM to 10 μM) (Di Marzo et al., 2002b; Ross, 2003). Interestingly, the potency and efficacy of anandamide is affected by its metabolism. Ross et al (Ross et al., 2001) reported only low efficacy of anandamide at TRPV1 receptors overexpressed in CHO cells which was greatly enhanced by inhibition of fatty acid amide hydrolase.

Molecular Pharmacology Fast Forward. Published on December 7, 2005 as DOI: 10.1124/mol.105.015644
This article has not been copyedited and formatted. The final version may differ from this version.

As has been pointed out earlier (Ross, 2003), the partial activation of TRPV1 may evoke partial inhibition for other endogenous ligands. Such ligands could be N-arachidonyl dopamine (NADA) (Huang et al., 2002) or other yet unidentified endogenous ethanolamides (Szolcsanyi, 2000). Previously, it has also been suggested that stimulation of TRPV1 by anandamide leads to desensitization to subsequent agonist challenge (tachyphylaxis) (Smart et al., 2001; Tognetto et al., 2001).

Both anandamide and capsaicin causes vasodilation of mesenteric (this report, besides others), hepatic, basilar and meningeal arterioles (Zygmunt et al., 1999; Ralevic et al., 2001; Harris et al., 2002; Akerman et al., 2004; O'Sullivan et al., 2004) through the stimulation of cannabinoid and TRPV1 receptors, respectively. In these studies, anandamide-mediated vasorelaxation was mainly (Zygmunt et al., 1999; Ralevic et al., 2001; Harris et al., 2002; Akerman et al., 2004) or partly (O'Sullivan et al., 2004) attributed to the activation of TRPV1. In mesenteric arteries TRPV1 activation produces vasodilation due to CGRP release from the sensory nerve terminals innervating the blood vessels (Zygmunt et al., 1999). However, it has also been reported that activation of TRPV1 can cause vasoconstriction in rat (Scotland et al., 2004) and canine (Porszasz et al., 2002) mesenteric arteries or in coronary vessels (Szolcsanyi et al., 2001). In these latter cases TRPV1 mediated SP (Scotland et al., 2004) or endothelin (Szolcsanyi et al., 2001) release was suggested as possible mechanisms. Moreover, it was also reported, that *in vivo* anandamide treatment (0.075 – 3 mg/kg) caused a transient vasoconstriction in renal, mesenteric and hindquaters vessels in conscious rats (Gardiner et al., 2002). This latter constriction lasted less than 60 s and was not mediated by cannabinoid receptors. In contrast, there is little known about the functional role of TRPV1 in skeletal muscle

Molecular Pharmacology Fast Forward. Published on December 7, 2005 as DOI: 10.1124/mol.105.015644
This article has not been copyedited and formatted. The final version may differ from this version.

arterioles. Additionally, it is not addressed, what are the vascular and cellular effects of agonists if they are present at high concentrations for a longer period of time (acute desensitization).

Here we present evidence that functional TRPV1 is expressed in skeletal muscle arterioles and its stimulation by capsaicin leads to robust vasoconstriction in isolated vessels possessing spontaneous myogenic tone. In contrast, anandamide stimulation was without acute effects on the same vessels but caused complete desensitization of TRPV1 receptors to capsaicin. This anandamide-mediated desensitization of TRPV1 was reversed by calcineurin (protein phosphatase 2B) inhibition. To investigate the underlying mechanisms, a TRPV1 overexpressing cell line was used. In this system, anandamide caused a fast and strong acute desensitization of TRPV1 which was modulated by PKC and protein phosphatase 2B in the continuous presence of anandamide.

Our data suggest that anandamide can be an endogenous desensitizer of TRPV1, in addition to being a partial agonist of this receptor. We propose a mechanism for the indirect gating of TRPV1 by reversible phosphorylation in the continuous presence of anandamide and suggest that this behavior, in general, represents a potential therapeutic approach for modulating TRPV1 activity by partial agonists.

Molecular Pharmacology Fast Forward. Published on December 7, 2005 as DOI: 10.1124/mol.105.015644
This article has not been copyedited and formatted. The final version may differ from this version.

Materials and methods

Isolation of skeletal muscle arterioles and measurement of vessel diameter

Wistar rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg). Using microsurgical instruments and an operating microscope, a gracilis muscle arteriole (~ 0.5 mm in length) running intramuscularly was isolated and transferred into an organ chamber containing two glass micropipettes filled with physiological salt solution (PSS) composed of (in mmol/L): 110 NaCl, 5.0 KCl, 2.5 $CaCl_2$, 1.0 $MgSO_4$, 1.0 KH_2PO_4, 5.0 glucose and 24.0 $NaHCO_3$ equilibrated with a gas mixture of 10% O_2 and 5% CO_2, balanced with nitrogen, at pH 7.4. Vessels were cannulated on both ends and micropipettes were connected with silicone tubing to an adjustable PSS-reservoir. Inflow and outflow pressures were set to 80 mmHg. Temperature was set at 37^{o}C by a temperature controller. The internal arteriolar diameter at the midpoint of the arteriolar segment was measured by videomicroscopy (Bagi et al., 2005). Animal experiments were approved by the University of Debrecen, Medical and Health Science Center and were in accordance with the standards established by the National Institutes of Health.

CHO-TRPV1 cell culturing

The selected stable CHO cell clone expressing TRPV1 (Tet-Off induced CHO-TRPV1 cells) (Szallasi et al., 1999) was cultured in maintaining media (F12 supplemented with 10% FBS (USA sourced), 25 mM HEPES, pH 7.5, 250 µg/ml geneticin (all from Life Technologies Inc., Rockville, MD, USA) and 1 mg/L tetracycline

Molecular Pharmacology Fast Forward. Published on December 7, 2005 as DOI: 10.1124/mol.105.015644
This article has not been copyedited and formatted. The final version may differ from this version.

(Calbiochem, La Jolla, CA, USA). For $^{45}Ca^{2+}$ uptake, CHO-TRPV1 cells were plated in 24-well plates to yield a cell density of 20-40% confluency. The next day, the media was changed to remove the tetracycline in order to induce TRPV1 expression. Experiments were performed approximately 48 h after induction. For intracellular Ca^{2+} concentration measurements, CHO-TRPV1 cells were plated on 25 mm round glass coverslips in maintaining media (F12 supplemented with 10% FBS, 25 mM HEPES, pH 7.5, 250 µg/ml geneticin and 1 mg/L tetracycline). The next day, the media was changed to inducing media (maintaining media without tetracycline but containing 1 mM sodium butyrate) to induce TRPV1 expression. Experiments were done approximately 24 hours after induction.

$^{45}Ca^{2+}$ uptake experiments

Experiments were performed as described previously (Toth et al., 2004). Briefly, CHO-TRPV1 cells were used immediately upon removal from the CO_2 incubator or were preincubated with 100 nM PMA, 100 nM CY-A or with 100 nM PMA and 100 nM CY-A applied together. For the $^{45}Ca^{2+}$ uptake assay, cells were incubated for 5 min at 37 °C in a total volume of 500 µl of serum-free DMEM (Life Technologies Inc., Rockville, MD, USA) containing 1.8 mM $CaCl_2$ in the presence of 0.25 mg/ml bovine serum albumin (BSA, Sigma, St. Louis, MO, USA), 1 µCi/ml $^{45}Ca^{2+}$ (ICN, Costa Mesa, CA, USA), and increasing concentrations of anandamide. Maximal response was determined by the response caused by 300 nM capsaicin on the same plate. Immediately after the incubation, extracellular $^{45}Ca^{2+}$ was removed by washing the cells three times with cold DPBS (Life Technologies Inc., Rockville, MD, USA) containing 1.8 mM $CaCl_2$. Then,

Molecular Pharmacology Fast Forward. Published on December 7, 2005 as DOI: 10.1124/mol.105.015644
This article has not been copyedited and formatted. The final version may differ from this version.

400 µl RIPA buffer (50 mM Tris-Cl pH 7.4; 150 mM NaCl; 1% Triton X-100; 0.1% SDS; 1% sodium deoxycholate) was added to each well in order to lyse the cells. Plates were shaken slowly for 20 min. Then, 300 µl of cell lysate was transferred from each well into a scintillation vial and radioactivity was determined by scintillation counting. For each data point in each experiment, four wells were assayed. Data from these experiments were analyzed by computer fit to the Hill equation. Each experiment was performed at least three times.

Ca^{2+} imaging

Experiments were performed as described earlier (Toth et al., 2003). Briefly, CHO-TRPV1 cells were transferred to DPBS containing 1 mg/ml BSA and 5 µM fura2-AM (Molecular Probes, Eugene, OR, USA) for 2 hours at room temperature. The cells were then kept in maintaining media at room temperature until the measurements, which were carried out in DPBS. The fluorescence of individual cells was measured with an InCyt Im2 fluorescence imaging system (Intracellular Imaging Inc., Cincinnati, OH, USA). The cells within a field were illuminated alternately at 340 and 380 nm. Emitted light >510 nm was measured. Data were analyzed with the Incyt 4.5 software and further processed with Excel (Microsoft) and GraphPad Prism 2.0 (Graphpad Software Inc.) software.

Immunohistochemistry

Frozen skeletal muscle (m. gracilis) tissue samples were embedded in Tissue-Tek O.C.T compound (Electron Microscopy Sciences, Hatfield, PA, USA). Cryostat sections

Molecular Pharmacology Fast Forward. Published on December 7, 2005 as DOI: 10.1124/mol.105.015644
This article has not been copyedited and formatted. The final version may differ from this version.

(thickness 10 μm) were placed on adhesive slides and fixed in acetone for 10 min. The slices were blocked with normal goat sera (1.5% in PBS, Sigma, St. Louis, MO, USA) for 20 min and stained with anti-capsaicin receptor antibody (raised in rabbit, Calbiochem, La Jolla, CA, USA) at 1:100 dilution in the blocking buffer. Then, the slices were incubated with a biotin-labeled anti-rabbit antibody (1:200, Vector Laboratories, Burlingame, CA, USA) and the immunocomplexes were visualized by a Vector VIP substrate (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. Finally, the slices were also stained with methyl green to visualize the nuclei.

Molecular Pharmacology Fast Forward. Published on December 7, 2005 as DOI: 10.1124/mol.105.015644
This article has not been copyedited and formatted. The final version may differ from this version.

Results

The vasoactive effects of anandamide and capsaicin were measured in resistance arterioles (132-223 μm in diameter at 80 mmHg) of skeletal muscle (m. gracilis) of the rat. The isolated arteries were mounted in a perfusion myograph system and developed spontaneous myogenic tone without the use of any vasoactive agent (myogenic constriction: 25 ± 4%, diameter in the presence of Ca^{2+} was 179 ± 33 μm and in the absence of Ca^{2+} was 234 ± 20 μm at 80 mmHg, n = 15, $p<0.01$). The functionality of the endothelium was tested at the beginning of each experiment by administration of acetylcholine (10^{-7} M, 95 ± 13% dilation, n = 15), while smooth muscle functions were checked by addition of norepinephrine (10^{-7} M, 31 ± 14% constriction, n = 15) (Table 1).

First, the effects of TRPV1 stimulation were tested on the isolated vessels. In this series of experiments, 1 μM capsaicin caused 58 ± 8 % constriction (n=5) which was blocked by the competitive TRPV1 antagonist capsazepine (10 μM, constriction was 2 ± 5 %, n=5, $p<0.01$) suggesting that the capsaicin effects were TRPV1 specific (Fig. 1A). To investigate the desensitization of TRPV1 in this system in separate experiments, isolated vessels were incubated with 1 μM capsaicin for 20 min, followed by 40 min regeneration and the vessels were tested again with 1 μM capsaicin. According to the results, the maximal constriction was 51 ± 12 % (n=5, Fig 1B, capsaicin before) and it did not decrease significantly after the 20 min incubation with capsaicin, followed by a 40 min regeneration period (maximal response is 35 ± 7 %, n=5, Fig 1B, capsaicin after, p=0.29). To further pursue the specificity of TRPV1 specific constriction in this system

Molecular Pharmacology Fast Forward. Published on December 7, 2005 as DOI: 10.1124/mol.105.015644
This article has not been copyedited and formatted. The final version may differ from this version.

immunohistochemistry was also performed (Fig. 1C) and revealed a strong TRPV1- like immunostaining in the smooth muscle layer of skeletal muscle vessels.

The TRPV1 mediated vasoconstriction in skeletal muscle arteries was in marked contrast to the vasodilatation observed on mesenteric vessels earlier. Therefore, mesenteric arterioles were also tested to support the experimental conditions and design of this study, to measure the vascular effects of TRPV1 stimulation, and to confirm earlier data. Indeed, we found a concentration dependent dilation of mesenteric vessels evoked by both capsaicin and anandamide although the efficacy of capsaicin was significantly higher (maximal dilatation is 96.3 ± 2.1 % for capsaicin and 23.6 ± 3.3 % for anandamide, $p<0.01$, $n=5$). This capsaicin and anandamide mediated dilation (without apparent vasoconstriction) is in accordance with earlier reports (Zygmunt et al., 1999; Ralevic et al., 2001; Harris et al., 2002; Akerman et al., 2004; O'Sullivan et al., 2004).

Effects of anandamide on the skeletal muscle vessel diameter were tested by cumulative dose-response measurements. Following 1 min of incubation no significant changes in vessel diameter were detected over a range of 1 nM to 100 μM anandamide compared to the control (response to 100 μM is shown in Fig. 2, dilatation: 3 ± 5 %). It was also suggested previously that anandamide can desensitize the vanilloid receptor-1 (TRPV1) (Smart et al., 2001; Tognetto et al., 2001; Di Marzo et al., 2001a; Helyes et al., 2003; Toth et al., 2005). We therefore tested the effects of 30 μM anandamide (for 20 min followed by a 40 min regeneration period) on the responsiveness of TRPV1 in this system (Fig. 2). 1 μM capsaicin alone caused a significant constriction of these vessels (constriction is 51 ± 12 % $n = 5$, $p = 0.018$). Capsaicin-induced constriction was completely abolished by anandamide (30 μM) pretreatment (constriction decreased to 1 ±

Molecular Pharmacology Fast Forward. Published on December 7, 2005 as DOI: 10.1124/mol.105.015644
This article has not been copyedited and formatted. The final version may differ from this version.

0.6 %, n = 5). The inhibitory effect of anandamide on TRPV1 was reversed by the calcineurin (protein phosphatase 2B) inhibitor cyclosporin-A (CY-A, 100 nM) (response to 1 μM capsaicin was 31 ± 1 %, n = 4, p = 0.014 for constriction, but no significant difference from capsaicin alone, p = 0.12). Furthermore, anandamide caused a significant vasoconstriction (maximal constriction is 7 ± 2%, n=4, p=0.01) when applied after 100 nM CY-A (5 min pretreatment). CY-A alone had no effects on vessel diameter (1 to 1000 nM, n = 5, data not shown).

To explore the possible mechanisms leading to the phosphorylation-dependent anandamide mediated desensitization of TRPV1 in skeletal muscle arterioles we used an exogenous TRPV1 expressing cell line (CHO-TRPV1) (Toth et al., 2003, 2004, 2005). $^{45}Ca^{2+}$ uptake experiments (Fig. 3) indicated that anandamide is a partial agonist in this system (with a maximal effect of 65 ± 8 %, n = 7 of that induced by 300 nM capsaicin). This partial agonism was converted to full agonism by the activation of PKC with PMA (100 nM, 98 ± 14 %, n = 3, p = 0.059), by inhibition of phosphatase 2B (calcineurin) using cyclosporine-A (CY-A, 100 nM, 145 ± 14 % n = 5, P<0.001), or by the simultaneous application of PMA and CY-A (137 ± 13 %, n = 3, p = 0.001). Changes in the apparent K_d values were also observed in parallel with the maximal effects (K_d values: anandamide alone: 30 ± 6 μM, n = 7; PMA: 9 ± 4 μM, n = 5, p = 0.027; CY-A: 11 ± 5 μM, n = 5, p = 0.047; CY-A and PMA applied together: 11 ± 3 μM, n = 3, p = 0.117). The role of the order of PMA and CY-A treatments were not studied. Motivated by the somewhat low potency of anandamide in our system, we also tested the effect of the fatty acid amide hydrolase inhibitor PMSF (0.5 mM) and found a moderate

Molecular Pharmacology Fast Forward. Published on December 7, 2005 as DOI: 10.1124/mol.105.015644
This article has not been copyedited and formatted. The final version may differ from this version.

sensitization of TRPV1 to anandamide in the presence of PMSF (81.0 ± 4.8 % efficacy and 10.5 ± 2.8 μM potency, n=4).

To test the anandamide mediated desensitization specifically on TRPV1 receptors, CHO-TRPV1 cells, which do not express cannabinoid receptors, were preincubated with anandamide (1-100 μM for 15 min at 37 °C), then $^{45}Ca^{2+}$ uptake was initiated by 50 nM capsaicin (the half maximally effective dose of capsaicin in this system). Anandamide inhibited the capsaicin induced $^{45}Ca^{2+}$ uptake in a dose dependent manner (Fig. 4), with an apparent IC_{50} value of 21 ± 2 μM (n = 3). This IC_{50} is close to the K_d determined in the previous assays (Fig. 3, K_d = 30 ± 6 μM, n = 7).

Next, we characterized the anandamide mediated cellular effects on TRPV1 receptors at the level of the intracellular Ca^{2+} concentrations of CHO-TRPV1 cells. Anandamide alone (30 μM) caused a transient elevation of intracellular Ca^{2+} concentration (Fig. 5A), but this elevation was lower than that induced by capsaicin (data not shown). Preincubation of the cells with PMA (100 nM, 15 min, Fig. 5B) sensitized the cells to anandamide (30 μM) and desensitization was not prominent (n = 3). Similar effects were detected in case of CY-A (100 nM, Fig. 5C).

In addition to receptor sensitization, the resensitization characteristics of TRPV1 were also tested, using similar experimental protocol to that used with the isolated rat vessels. CHO-TRPV1 cells were treated with 30 μM anandamide and at 15 min buffer (control, Fig. 6A) or PMA (100 nM, Fig. 6B) or CY-A (100 nM, Fig. 6C) was added in the continued presence of anandamide. Both PMA and CY-A were able to resensitize TRPV1 which had been desensitized by anandamide (n = 3).

Discussion

Here, we found that anandamide alone had no effect on the vasotone of skeletal muscle arterioles, in contrast with the profound vasodilation observed in case of mesenteric vessels. This result could arise from at least three different mechanisms. First, anandamide induced stimulation of cannabinoid or TRPV1 receptors might have low efficacy to achieve vasoactive responses in skeletal muscle arterioles. Second, the simultaneous stimulation of cannabinoid and TRPV1 receptors may cause opposite effects in this system. Third, there could be no functional receptors present in the preparations to be activated. This latter explanation can be excluded since capsaicin caused a significant vasoconstriction, indicating the presence of functional TRPV1 receptors in our preparations. Furthermore, pretreatment of skeletal muscle arterioles with CY-A revealed an anandamide mediated constriction. These data suggest that anandamide induces dephosphorylation of the receptor (suppressing its activity), which could be reversed using a phosphatase inhibitor.

The observed efficacy (65 ± 8 % relative to that of capsaicin) and potency (30 ± 6 μM) of anandamide on TRPV1 is somewhat lower than that reported by others. In particular, Di Marzo et al. (Di Marzo et al., 2001b) determined a potency of anandamide of 800±200 nM at CB1 receptors from rat brain and 350±91 nM at TRPV1 receptors overexpressed in HEK cells. One explanation of these differences may be that fatty acid amide hydrolase activities are different in these systems. Indeed, Ross et al. (Ross et al., 2001) reported very similar efficacy of anandamide to this work (10 μM anandamide had 17.8±3.9% efficacy in their system, compared to 21.6 ± 2.6% determined here) using TRPV1 expressed in CHO cells similarly to us. Additionally, we confirmed that

Molecular Pharmacology Fast Forward. Published on December 7, 2005 as DOI: 10.1124/mol.105.015644
This article has not been copyedited and formatted. The final version may differ from this version.

application of PMSF was able to sensitize TRPV1 expressing cells to anandamide as likewise described by Ross et al. (Ross et al., 2001), although we found only a moderate sensitization compared to their measurements. Further possibilities may also contribute to the differences in the potency and efficacy of anandamide, including different levels of TRPV1 expression, differences in protein phosphorylation (Vellani et al., 2001), anandamide transporters (De Petrocellis et al., 2001) and other factors (Di Marzo et al., 2001a; Ross, 2003). Some of these other factors (PKC activation and protein phosphatase 2B inhibition) were also confirmed to be effective in this work.

The tissue concentration of anandamide usually ranges between 4 to 200 nM, but there are also data suggesting that much higher concentrations (more than 2 μM in the supernatant) could be synthesized by stimulated cultured primary neurons (Ahluwalia et al., 2003). In this study we did not attempt to determine what concentration of anandamide is present physiologically. Instead, we accepted that anandamide is a physiological regulator of TRPV1 and tested our system to make sure we were using a high enough concentration of anandamide to have effects on TRPV1. Additionally, we have also shown that in our CHO-TRPV1 system the potency of anandamide is most likely to be underestimated (due to effects of PKC activation, calcineurin and fatty acid amide hydrolase inhibition).

Our data suggest that the observed low efficacy of anandamide compared to capsaicin on TRPV1 can possibly be related to the desensitization of TRPV1. Indeed, it has been published that stimulation of TRPV1 leads to desensitization to subsequent agonist challenge (well known as tachyphylaxis) in the case of anandamide (Smart et al., 2001; Tognetto et al., 2001; Di Marzo et al., 2001b; Helyes et al., 2003). However, in

Molecular Pharmacology Fast Forward. Published on December 7, 2005 as DOI: 10.1124/mol.105.015644
This article has not been copyedited and formatted. The final version may differ from this version.

contrast with the others we used a model of acute desensitization instead of tachyphylaxis. The physiological difference between the two models is that in the case of tachyphylaxis we expect repeated bursts of release of anandamide, while in case of acute desensitization we expect the continuous presence of the anandamide. This behavior (acute desensitization) is not limited to anandamide since other endogenous TRPV1 ligands, such as NADA (Toth et al., 2003) or N-oleoyldopamine (Szolcsanyi et al., 2004) were also reported to cause acute desensitization, although without data about the role of phosphorylation.

One of the prominent mechanisms of TRPV1 desensitization is receptor dephosphorylation. It was suggested that PKC is one of the kinases involved in the phosphorylation of TRPV1 (Vellani et al., 2001; Bhave et al., 2003; Mandadi et al., 2004), while calcineurin was suggested as the phosphatase (Docherty et al., 1996). It has also been shown that changes in the phosphorylation state of TRPV1 could reverse the desensitization (tachyphylaxis) caused by repeated applications of 100 nM capsaicin (Mandadi et al., 2004). Taking in account that some of the PKC isozymes and the calcineurin are calcium dependent enzymes, an intriguing possibility is that the actual activities of these kinases and phosphatase are most probable depends on the level of TRPV1 activation (intracellular Ca^{2+} concentration) and their Ca^{2+} sensitivity. In arterioles, activation of PKC led to TRPV1 independent vascular effects, therefore we used the calcineurin inhibitor cyclosporin-A (CY-A), which was without vasoactive effects when applied alone. Treatment with CY-A, indeed, reversed the desensitization of TRPV1 as evaluated with capsaicin.

Molecular Pharmacology Fast Forward. Published on December 7, 2005 as DOI: 10.1124/mol.105.015644
This article has not been copyedited and formatted. The final version may differ from this version.

To further investigate the possible cellular mechanisms, we used a heterologous TRPV1 expression system, in which TRPV1 of the rat was expressed in CHO cells and tested the effects of phosphorylation on the acute desensitization of TRPV1. The advantage of this system is that TRPV1 mediated effects could be specifically observed, since there is no endogenous expression of cannabinoid or TRPV1 receptors in the CHO cells. Both the potency and the efficacy of anandamide in this system were found to be similar to that which was reported earlier (Ross, 2003). Moreover, the partial efficacy allowed the investigation of phosphorylation in regulating the responsiveness of TRPV1 to anandamide. Our data support the idea that both the potency and efficacy of anandamide are controlled by phosphorylation. The acute desensitization of TRPV1 by anandamide was strong enough to cause reduced capsaicin mediated responses. Results of intracellular Ca^{2+} imaging experiments suggested that the partial efficacy of anandamide was the reason for the fast acute TRPV1 desensitization. This desensitization was blocked (Fig. 5) and reversed (Fig. 6) by PKC activation and calcineurin inhibition. Moreover, a higher $^{45}Ca^{2+}$ uptake was found in case of CY-A compared to control or PMA treatments. This observation suggests that there might be multiple physiologically relevant phosphorylation sites in TRPV1, additionally to the PKC sites, being sensitive to dephosphorylation catalyzed by calcineurin

Based on our observations and those of others, here we propose a mechanism for the regulation of TRPV1 responsiveness upon stimulation with anandamide (Fig. 7). According to this model, we suggest three different phases: resting activated and desensitized. In the resting phase there are no ligands present and TRPV1 is inactive. The steady state level of phosphorylation determines the sensitivity of TRPV1 to anandamide.

Molecular Pharmacology Fast Forward. Published on December 7, 2005 as DOI: 10.1124/mol.105.015644
This article has not been copyedited and formatted. The final version may differ from this version.

The phosphorylation of TRPV1 is a function of the apparent phosphatase (e.g. protein phosphatase 2B, calcineurin) and kinase (e.g. PKC) activities. If TRPV1 phosphorylation is enhanced (e.g. by activation of PKC or inhibition of calcineurin) TRPV1 sensitivity to anandamide is higher. The second phase is the activation of the TRPV1 by anandamide. At the appearance of anandamide the channel activity increases and the intracellular Ca^{2+} concentration rises. This activation is, however, followed by a rapid desensitization (third phase). During this acute desensitization period (in the continuous presence of the ligand) the activity of the receptor and the intracellular Ca^{2+} concentrations are apparently regulated by reversible phosphorylation: dephosphorylation keeps the TRPV1 desensitized (and the intracellular Ca^{2+} concentration low), while phosphorylation sensitizes the TRPV1. In this desensitized period, the TRPV1 behaves as a phosphorylation gated channel until anandamide is present in sufficient concentration. However, when anandamide concentration decreases, the channel will be closed independently from its phosphorylation state.

These observations could be of physiological importance if anandamide or anandamide like substances (e.g. drugs with similar properties) are continuously present and bound to the TRPV1. In this case TRPV1 is silenced by acute desensitization but can be activated by phosphorylation alone (Premkumar and Ahern, 2000). Such anandamide like substances might be able to silence TRPV1 activity without the irritative effects of the full agonists (like capsaicin) providing an improved therapeutic strategy to control disorders caused by TRPV1 hypersensitivity. Indeed, some partial agonists/antagonists of TRPV1 have been identified (Wang et al., 2003) as part of the extensive medicinal

Molecular Pharmacology Fast Forward. Published on December 7, 2005 as DOI: 10.1124/mol.105.015644
This article has not been copyedited and formatted. The final version may differ from this version.

chemistry effort directed at TRPV1. Like anandamide, these compounds showed enhanced efficacy upon activation of PKC.

Finally, these data add to the complexity of the physiological effects of anandamide. The role of anandamide as an endogenous activator of TRPV1 is under discussion (Szolcsanyi, 2000; Zygmunt et al., 2000; Smart and Jerman, 2000; Di Marzo et al., 2001a; Ross, 2003; Wang et al., 2003). Our data presented here add to the range of possibilities, suggesting that anandamide is able to desensitize TRPV1 *in vivo*. This desensitization might be modulated by CB1 receptors, when TRPV1 and CB1 receptors are expressed in the same cells (Helyes et al., 2003), or might be mediated by the stimulation of TRPV1 itself (CHO-TRPV1 data, presented here).

In summary, the physiological effects of anandamide on the vasculature are most probably mediated by both TRPV1 and CB1 receptors in general. It is therefore possible that anandamide has dual actions at CB1 and TRPV1 receptors: in the short term anandamide activates the CB1 receptors and TRPV1 receptors simultaneously, while at longer times it can desensitize the TRPV1 receptors and render them to be a metabotropic (phosphorylation dependent) receptor. In most cases both TRPV1 and CB1 stimulation lead to vasodilatation, which makes it complicated to determine the relative contributions of these receptors to the vasodilatation. However, it is shown here that TRPV1 stimulation leads to vasoconstriction in the case of skeletal muscle arterioles. Investigating the effects of anandamide on these vessels, we found a complete desensitization of TRPV1. Our data suggest that anandamide is an endogenous partial activator of TRPV1, but at least in some cases (as in the case of the skeletal muscle

arterioles) it can function as a desensitizer of TRPV1, which apparently transforms the ligand gated TRPV1 to a phosphorylation gated channel.

References

Ahluwalia J, Yaqoob M, Urban L, Bevan S and Nagy I (2003) Activation of Capsaicin-Sensitive Primary Sensory Neurones Induces Anandamide Production and Release. J Neurochem **84**: 585-591.

Akerman S, Kaube H and Goadsby P J (2004) Anandamide Acts As a Vasodilator of Dural Blood Vessels in Vivo by Activating TRPV1 Receptors. Br J Pharmacol **142**: 1354-1360.

Bagi Z, Erdei N, Toth A, Li W, Hintze T H, Koller A and Kaley G (2005) Type 2 Diabetic Mice Have Increased Arteriolar Tone and Blood Pressure: Enhanced Release of COX-2-Derived Constrictor Prostaglandins. Arterioscler Thromb Vasc Biol **25**: 1610-1616.

Bhave G, Hu H J, Glauner K S, Zhu W, Wang H, Brasier D J, Oxford G S and Gereau R W (2003) Protein Kinase C Phosphorylation Sensitizes but Does Not Activate the Capsaicin Receptor Transient Receptor Potential Vanilloid 1 (TRPV1). Proc Natl Acad Sci USA **100**: 12480-12485.

Caterina MJ, Schumacher M A, Tominaga M, Rosen T A, Levine J D and Julius D (1997) The Capsaicin Receptor: a Heat-Activated Ion Channel in the Pain Pathway. Nature **389**: 816-824.

Devane WA, Hanus L, Breuer A, Pertwee R G, Stevenson L A, Griffin G, Gibson D, Mandelbaum A, Etinger A and Mechoulam R (1992) Isolation and Structure of A Brain Constituent That Binds to the Cannabinoid Receptor. Science **258**: 1946-1949.

Di Marzo V, Bisogno T and De Petrocellis L (2001a) Anandamide: Some Like It Hot. Trends Pharmacol Sci **22**: 346-349.

Di Marzo V, Blumberg P M and Szallasi A (2002a) Endovanilloid Signaling in Pain. Curr Opin Neurobiol **12**: 372-379.

Di Marzo V, De Petrocellis L, Fezza F, Ligresti A and Bisogno T (2002b) Anandamide Receptors. Prostag Leukotr Ess **66**: 377-391.

Di Marzo V, Lastres-Becker I, Bisogno T, De Petrocellis L, Milone A, Davis J B and Fernandez-Ruiz J J (2001b) Hypolocomotor Effects in Rats of Capsaicin and Two Long Chain Capsaicin Homologues. Eur J Pharmacol **420**: 23-131.

Docherty RJ, Yeats J C, Bevan S and Boddeke H W (1996) Inhibition of Calcineurin Inhibits the Desensitization of Capsaicin-Evoked Currents in Cultured Dorsal Root Ganglion Neurones From Adult Rats. Pflug Arch Eur J Phy **431**: 828-837.

Gardiner SM, March J E, Kemp P A and Bennett T (2002) Complex Regional Haemodynamic Effects of Anandamide in Conscious Rats. Br J Pharmacol **135**: 1889-1896.

Harris D, McCulloch A I, Kendall D A and Randall M D (2002) Characterization of Vasorelaxant Responses to Anandamide in the Rat Mesenteric Arterial Bed. J Physiol **539**: 893-902.

Helyes Z, Nemeth J, Than M, Bolcskei K, Pinter E and Szolcsanyi J (2003) Inhibitory Effect of Anandamide on Resiniferatoxin-Induced Sensory Neuropeptide Release in Vivo and Neuropathic Hyperalgesia in the Rat. Life Sci **73**: 2345-2353.

Huang SM, Bisogno T, Trevisani M, Al Hayani A, De Petrocellis L, Fezza F, Tognetto M, Petros T J, Krey J F, Chu C J, Miller J D, Davies S N, Geppetti P, Walker J M and Di Marzo V (2002) An Endogenous Capsaicin-Like Substance With High Potency at Recombinant and Native Vanilloid VR1 Receptors. Proc Natl Acad Sci USA **99**: 8400-8405.

Hwang SW, Cho H, Kwak J, Lee S Y, Kang C J, Jung J, Cho S, Min K H, Suh Y G, Kim D and Oh U (2000) Direct Activation of Capsaicin Receptors by Products of Lipoxygenases: Endogenous Capsaicin-Like Substances. Proc Natl Acad Sci USA **97**: 6155-6160.

Mandadi S, Numazaki M, Tominaga M, Bhat M B, Armati P J and Roufogalis B D (2004) Activation of Protein Kinase C Reverses Capsaicin-Induced Calcium-Dependent Desensitization of TRPV1 Ion Channels. Cell Calcium **35**: 471-478.

O'Sullivan SE, Kendall D A and Randall M D (2004) Heterogeneity in the Mechanisms of Vasorelaxation to Anandamide in Resistance and Conduit Rat Mesenteric Arteries. Br J Pharmacol **142**: 435-442.

Porszasz R, Porkolab A, Ferencz A, Pataki T, Szilvassy Z and Szolcsanyi J (2002) Capsaicin-Induced Nonneural Vasoconstriction in Canine Mesenteric Arteries. Eur J Pharmacol **441**: 173-175.

Premkumar LS and Ahern G P (2000) Induction of Vanilloid Receptor Channel Activity by Protein Kinase C. Nature **408**: 985-990.

Ralevic V, Kendall D A, Jerman J C, Middlemiss D N and Smart D (2001) Cannabinoid Activation of Recombinant and Endogenous Vanilloid Receptors. Eur J Pharmacol **424**: 211-219.

Ross RA (2003) Anandamide and Vanilloid TRPV1 Receptors. Br J Pharmacol **140**: 790-801.

Ross RA, Gibson T M, Brockie H C, Leslie M, Pashmi G, Craib S J, Di Marzo V and Pertwee R G (2001) Structure-Activity Relationship for the Endogenous

Cannabinoid, Anandamide, and Certain of Its Analogues at Vanilloid Receptors in Transfected Cells and Vas Deferens. Br J Pharmacol **132**: 631-640.

Scotland RS, Chauhan S, Davis C, De Felipe C, Hunt S, Kabir J, Kotsonis P, Oh U and Ahluwalia A (2004) Vanilloid Receptor TRPV1, Sensory C-Fibers, and Vascular Autoregulation: a Novel Mechanism Involved in Myogenic Constriction. Circ Res **95**: 1027-1034.

Smart D and Jerman J C (2000) Anandamide: an Endogenous Activator of the Vanilloid Receptor. Trends Pharmacol Sci **21**: 134.

Smart D, Jerman J C, Gunthorpe M J, Brough S J, Ranson J, Cairns W, Hayes P D, Randall A D and Davis J B (2001) Characterisation Using FLIPR of Human Vanilloid VR1 Receptor Pharmacology. Eur J Pharmacol **417**: 51-58.

Szallasi A and Blumberg P M (1999) Vanilloid (Capsaicin) Receptors and Mechanisms. Pharmacol Rev **51**: 159-211.

Szallasi A, Blumberg P M, Annicelli L L, Krause J E and Cortright D N (1999) The Cloned Rat Vanilloid Receptor VR1 Mediates Both R-Type Binding and C-Type Calcium Response in Dorsal Root Ganglion Neurons. Mol Pharmacol **56**: 581-587.

Szolcsanyi J (2000) Anandamide and the Question of Its Functional Role for Activation of Capsaicin Receptors. Trends Pharmacol Sci **21**: 203-204.

Szolcsanyi J, Oroszi G, Nemeth J, Szilvassy Z, Blasig I E and Tosaki A (2001) Functional and Biochemical Evidence for Capsaicin-Induced Neural Endothelin Release in Isolated Working Rat Heart. Eur J Pharmacol **419**: 215-221.

Szolcsanyi J, Sandor Z, Petho G, Varga A, Bolcskei K, Almasi R, Riedl Z, Hajos G and Czeh G (2004) Direct Evidence for Activation and Desensitization of the Capsaicin Receptor by N-Oleoyldopamine on TRPV1-Transfected Cell, Line in Gene Deleted Mice and in the Rat. Neurosci Lett **361**: 155-158.

Tognetto M, Amadesi S, Harrison S, Creminon C, Trevisani M, Carreras M, Matera M, Geppetti P and Bianchi A (2001) Anandamide Excites Central Terminals of Dorsal Root Ganglion Neurons Via Vanilloid Receptor-1 Activation. J Neurosci **21**: 1104-1109.

Toth A, Blumberg P M, Chen Z and Kozikowski A P (2004) Design of a High-Affinity Competitive Antagonist of the Vanilloid Receptor Selective for the Calcium Entry-Linked Receptor Population. Mol Pharmacol **65**: 282-291.

Toth A, Kedei N, Wang Y and Blumberg P M (2003) Arachidonyl Dopamine As a Ligand for the Vanilloid Receptor VR1 of the Rat. Life Sci **73**: 487-498.

Toth A, Wang Y, Kedei N, Tran R, Pearce L V, Kang S U, Jin M K, Choi H K, Lee J and Blumberg P M (2005) Different Vanilloid Agonists Cause Different Patterns of Calcium Response in CHO Cells Heterologously Expressing Rat TRPV1. Life Sci **76**: 2921-2932.

Vellani V, Mapplebeck S, Moriondo A, Davis J B and McNaughton P A (2001) Protein Kinase C Activation Potentiates Gating of the Vanilloid Receptor VR1 by Capsaicin, Protons, Heat and Anandamide. J Physiol **534**: 813-825.

Wang Y, Toth A, Tran R, Szabo T, Welter J D, Blumberg P M, Lee J, Kang S U, Lim J O and Lee J (2003) High-Affinity Partial Agonists of the Vanilloid Receptor. Mol Pharmacol **64**: 325-333.

Zygmunt PM, Julius D, Di Marzo V and Hogestatt E D (2000) Anandamide - the Other Side of the Coin. Trends Pharmacol Sci **21**: 43-44.

Zygmunt PM, Petersson J, Andersson D A, Chuang H, Sorgard M, Di Marzo V, Julius D and Hogestatt E D (1999) Vanilloid Receptors on Sensory Nerves Mediate the Vasodilator Action of Anandamide. Nature **400**: 452-457.

Footnotes

Support: This research was supported in part by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research. In addition, A.T. was supported by the János Bolyai Research Fellowship and by the Hungarian Research Fund (OTKA F/15 048873).

Person to receive reprint requests: Attila Tóth, PhD

Division of Clinical Physiology, Institute of Cardiology, University of Debrecen

P. O. BOX 1, 4004, Debrecen, Hungary

E-mail: atitoth@jaguar.unideb.hu

Molecular Pharmacology Fast Forward. Published on December 7, 2005 as DOI: 10.1124/mol.105.015644
This article has not been copyedited and formatted. The final version may differ from this version.

Legends for figures

Fig. 1 Specificity of capsaicin mediated vasoconstriction

Skeletal muscle arteries were isolated and tested with capsaicin. Response to 1 μM capsaicin was measured in the absence (panel A, first bar, n=5) or in the presence of the TRPV1 specific antagonist capsazepine (10 μM, second bar, n=5). To measure the capsaicin mediated desensitization of TRPV1 in a separate series of experiments vessels were treated with capsaicin (1 μM, 20 min, maximal response is shown on panel B, first bar) and the responsiveness of the same vessels were determined after 40 min regeneration (1 μM capsaicin, second bar, n=5). Finally, the expression of TRPV1 was detected in the skeletal muscle arteries by immunohistochemistry (panel C). Consecutive slides of m. gracilis were stained with a polyclonal anti TRPV1 antibody (Calbiochem, dilution 1:100) and TRPV1 immunostaining was visualized with Vector VIP substrate. Slides with the omission of the primary antibody served as controls for background staining. The lumen (V) and the smooth muscle layer (arrows) of the vessels are shown.

Fig. 2 Effects of anandamide on the diameter of isolated skeletal muscle arterioles

Skeletal muscle arteries were isolated and tested with anandamide and capsaicin. Response to anandamide (100 μM, n = 5) is shown on the first bar. In case of the second bar, vessels were preincubated with 100 nM CY-A for 5 min before anandamide stimulation (n=4). Capsaicin response (1 μM, n = 5) is shown on the third bar. Fourth bar

represent the capsaicin response after anandamide treatment (30 μM, 15 min, n=5). The fifth bar shows the response to capsaicin after incubation with anandamide (30 μM for 15 min) and CY-A (100 nM, for 5 min at the end of anandamide treatment, n=5). Bars denote mean ± SEM. Significant differences ($p<0.05$) are labelled by asterisks. Abbreviations are: anandamide (Ana), cyclosporin-A (CY-A) and capsaicin (Caps).

Fig. 3 Characterization of the agonist effect of anandamide on CHO-TRPV1 cells

CHO-TRPV1 cells were treated with different concentrations of anandamide (from 1 to 200 μM) for 5 min and the $^{45}Ca^{2+}$ uptake was measured by scintillation counting. Dose-response relations for anandamide were determined in the absence of effectors (Control, n=7) or in the presence of the fatty acid amide hydrolase inhibitor PMSF (0.5 mM, n=4), the calcineurin inhibitor cyclosporin-A (100 nM CY-A, n=5), the PKC activator PMA (100 nM PMA, n=5), or in the simultaneous presence of CY-A and PMA (100 nM CY-A + 100 nM PMA, n=3). Four parallel determinations were done in each separate experiment and a representative experiment is shown in Panel A. The data from the replicates are summarized in Panel B (maximal effects (Bmax) compared to the effect of 300 nM capsaicin on the same plate, bars are mean ± SEM) and Panel C (apparent Kd values, bars are mean ± SEM). Significant differences versus control ($p<0.05$) are labelled by asterisks.

Fig. 4 Antagonist effect of anandamide on CHO-TRPV1 cells

Molecular Pharmacology Fast Forward. Published on December 7, 2005 as DOI: 10.1124/mol.105.015644
This article has not been copyedited and formatted. The final version may differ from this version.

CHO-TRPV1 cells were treated with anandamide (0-100 μM) for 15 min, then $^{45}Ca^{2+}$ and 50 nM capsaicin (elicits half maximal effects in this system) was added for an additional 5 min. The $^{45}Ca^{2+}$ uptake was detected by scintillation counting. Four parallel determinations were performed at each point in each experiment. A representative result is shown of the three independent separate experiments.

Fig. 5 Sensitization of TRPV1 to anandamide by enhanced phosphorylation

CHO-TRPV1 cells were loaded with the Ca^{2+} indicator fura-2. Cells were pretreated with buffer alone (Panel A, control), with 100 nM PMA (Panel B) or with 100 nM CY-A (Panel C) for 15 min. Then, intracellular Ca^{2+} elevations were evoked by addition of anandamide (30 μM) at 1 min. Responses were recorded for an additional 9 min. Responses of 27-49 individual cells were recorded and averaged in each separate experiment. A representative experiment is shown of the three independent experiments performed.

Fig. 6 Resensitization of TRPV1 to anandamide by enhanced phosphorylation

CHO-TRPV1 cells were loaded with the Ca^{2+} indicator fura-2. Intracellular Ca^{2+} elevations were evoked by anandamide (30 μM) at 1 min. When the cells desensitized and responses reached a plateau (usually in 3-5 min), buffer alone (Panel A, control), 100 nM PMA (Panel B) or 100 nM CY-A (Panel C) was added in the continued presence of

Molecular Pharmacology Fast Forward. Published on December 7, 2005 as DOI: 10.1124/mol.105.015644
This article has not been copyedited and formatted. The final version may differ from this version.

anandamide and responses were recorded for an additional 5 min. Responses of 32-49 individual cells were detected and averaged in each separate experiment. A representative experiment is shown from the three experiments performed.

Fig. 7 Proposed mechanism of TRPV1 responsiveness to anandamide in CHO-TRPV1 cells

Under resting conditions (bottom left) the phosphorylation state of TRPV1 (phoshorylated receptor is represented by –P on the top of the cells, while dephosphorylated is without P on the bottom) is regulated by kinases (like protein kinase C, PKC) and protein phosphatase 2B (calcineurin, PP2B). This determines its sensitivity to ligands, like anandamide (●). The Ca^{2+} concentration rises upon anandamide binding to TRPV1 (receptor is activated) as a result of the Ca^{2+} influx through the activated channel. It results in the higher activity of PP2B compared to PKC and the consequent dephosphorylation of the receptor. The dephosphorylated receptor remains in its desensitized state as far as anandamide is present. However, if the level of receptor phosphorylation increased (as a result of kinase activation or phosphatase inhibition) the receptor could be re-activated (resensitized). Finally, when anandamide dissociates, the receptor returns to its resting (inactivated) state irrespectively to its phosphorylation level.

Molecular Pharmacology Fast Forward. Published on December 7, 2005 as DOI: 10.1124/mol.105.015644
This article has not been copyedited and formatted. The final version may differ from this version.

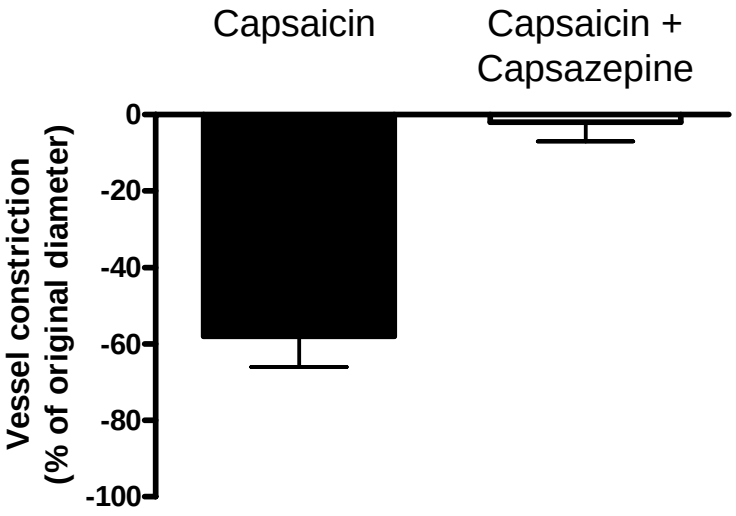
	Vessel diameter (μm) or Change in vessel diameter (% of maximum)
With Ca^{2+} at 80 Hgmm	179 ± 33 μm
Without Ca^{2+} at 80 Hgmm	234 ± 20 μm
Myogenic constriction	25 ± 4 %
Acetylcholine (10^{-7} M)	95 ± 13 %
Norepinephrine (10^{-7} M)	31 ± 14 %
Anandamide (10^{-4} M)	3 ± 5 %
Capsaicin (10^{-6} M)	51 ± 12 %

Table 1 General characteristics of isolated skeletal muscle arteries

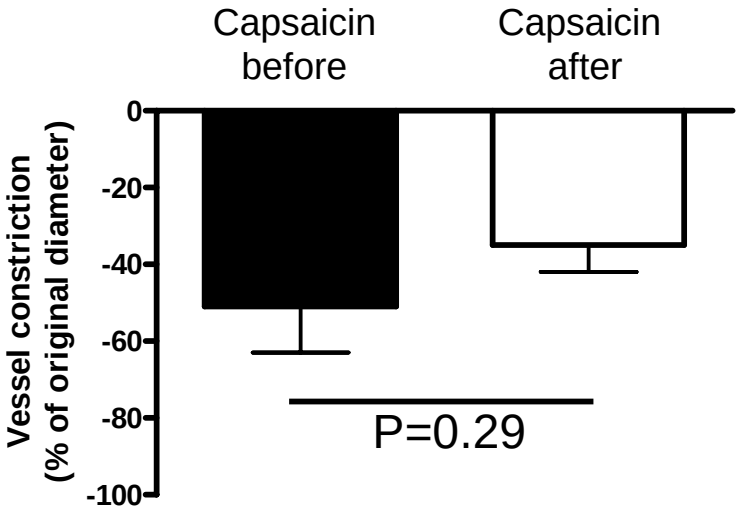
Molecular Pharmacology Fast Forward. Published on December 7, 2005 as DOI: 10.1124/mol.105.015644
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 1

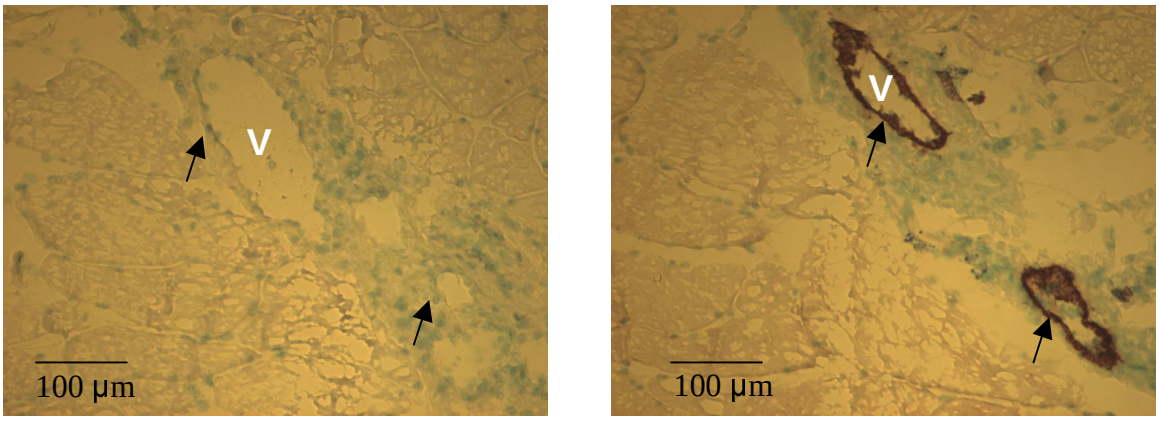
A



B



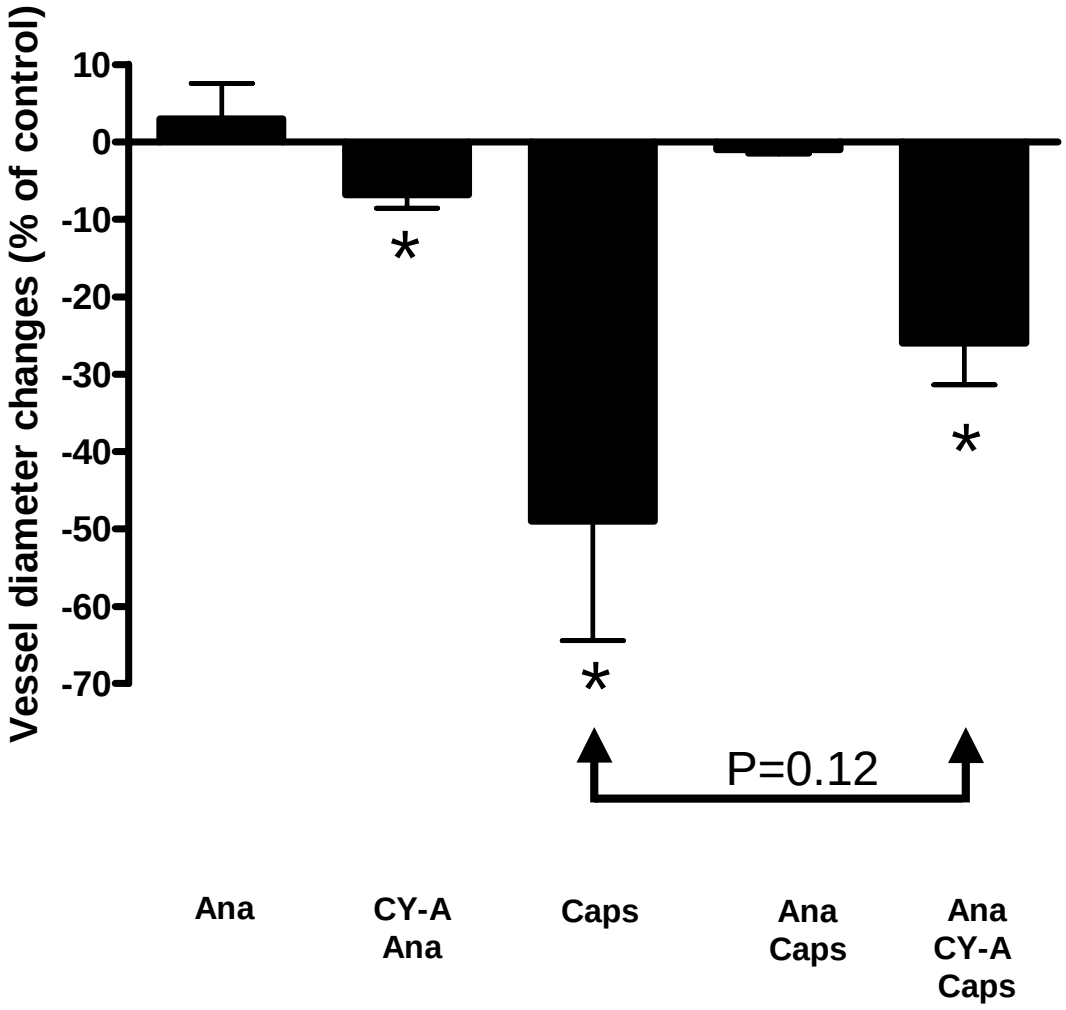
C



Background TRPV1

Molecular Pharmacology Fast Forward. Published on December 7, 2005 as DOI: 10.1124/mol.105.015644
This article has not been copyedited and formatted. The final version may differ from this version.

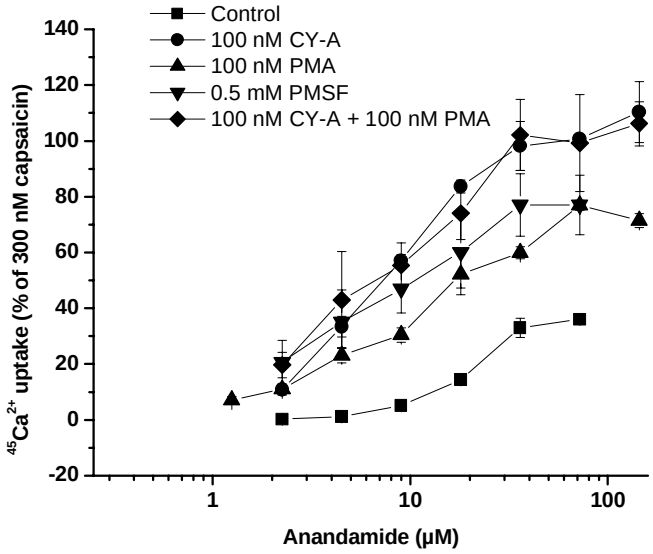
Fig. 2



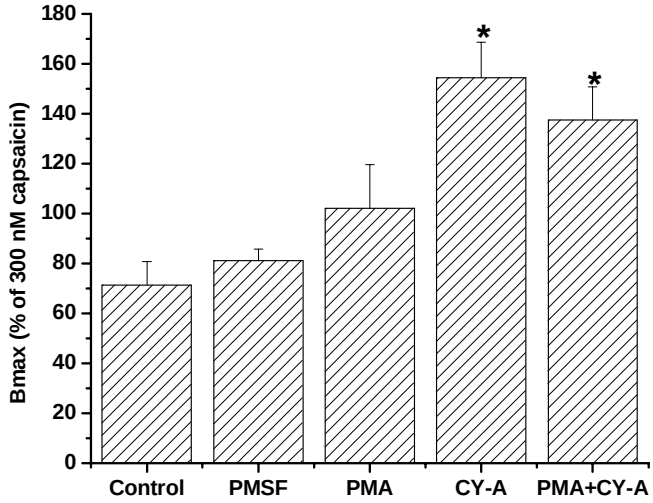
Molecular Pharmacology Fast Forward. Published on December 7, 2005 as DOI: 10.1124/mol.105.015644
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 3

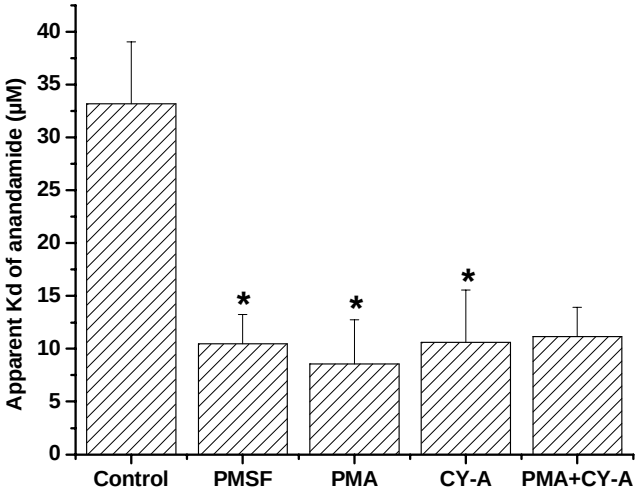
A



B

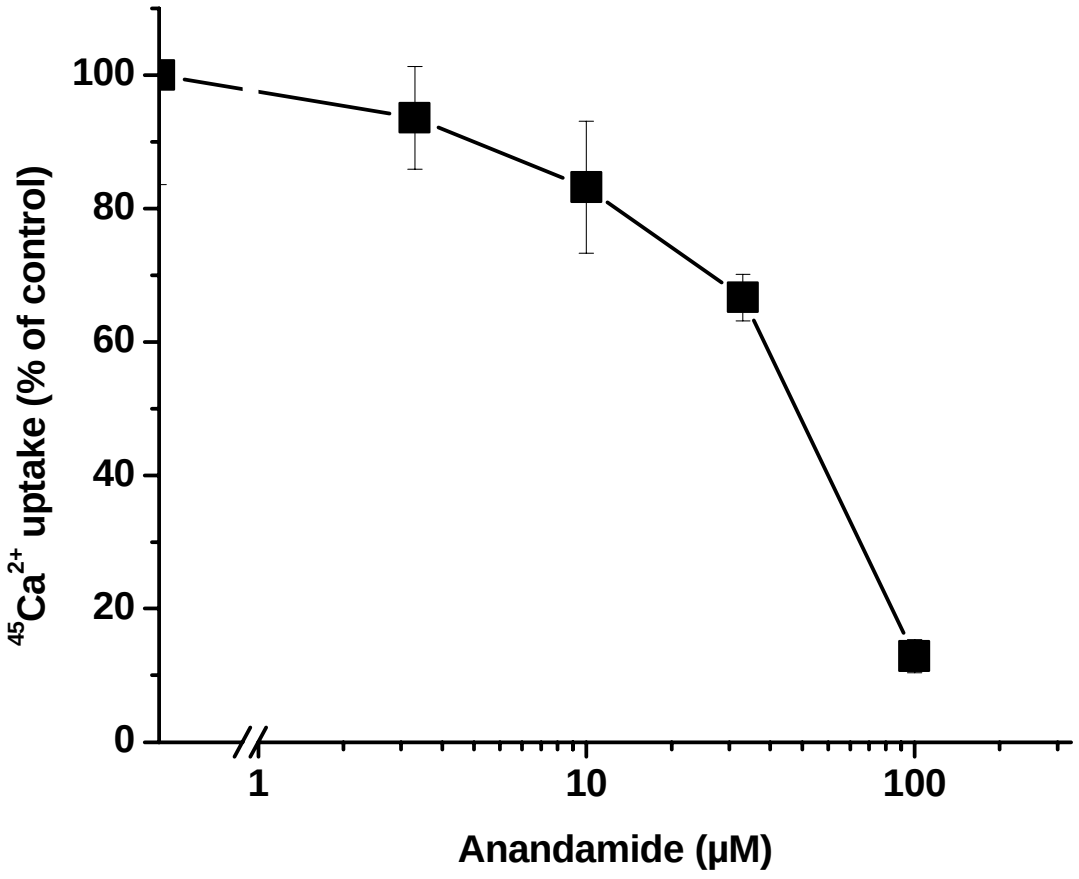


C



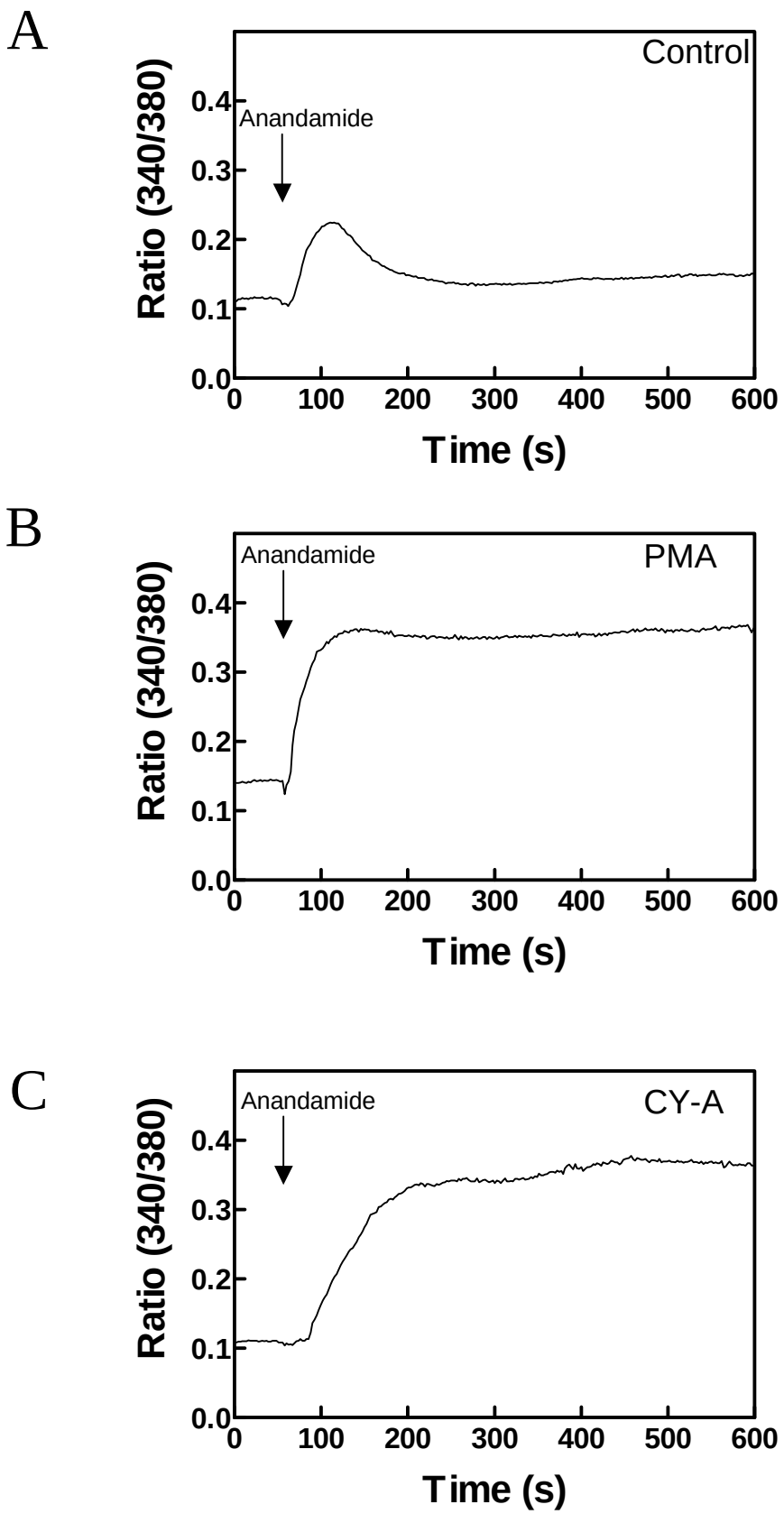
Molecular Pharmacology Fast Forward. Published on December 7, 2005 as DOI: 10.1124/mol.105.015644
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 4



Molecular Pharmacology Fast Forward. Published on December 7, 2005 as DOI: 10.1124/mol.105.015644
This article has not been copyedited and formatted. The final version may differ from this version.

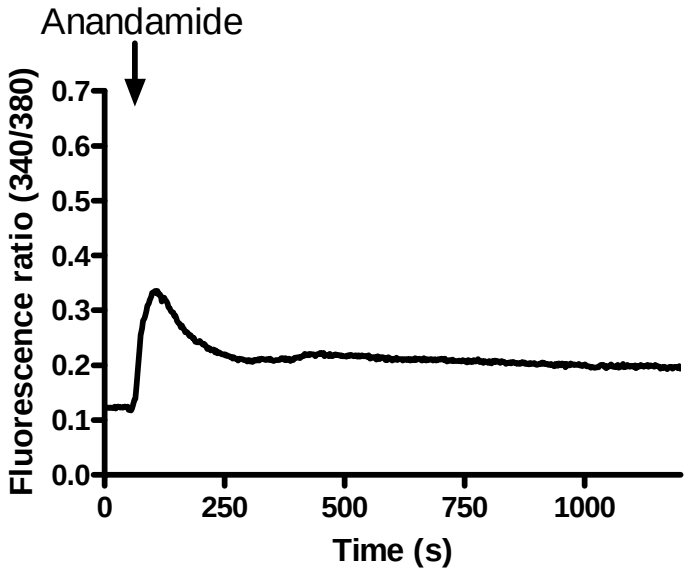
Fig. 5



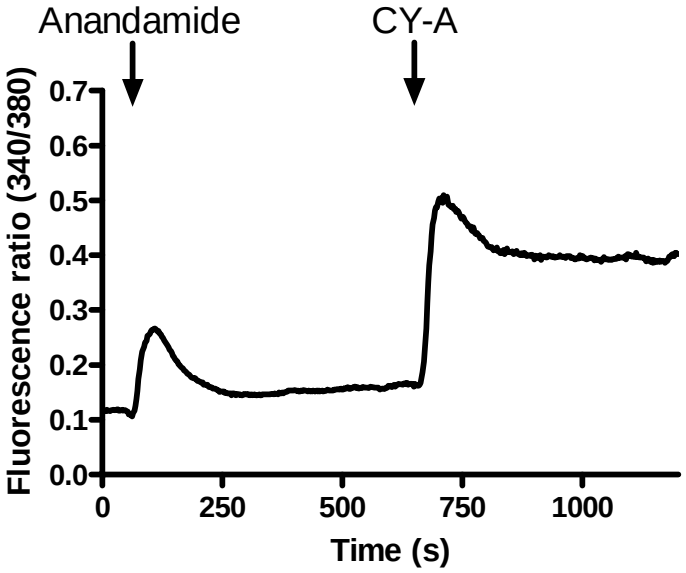
Molecular Pharmacology Fast Forward. Published on December 7, 2005 as DOI: 10.1124/mol.105.015644
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 6

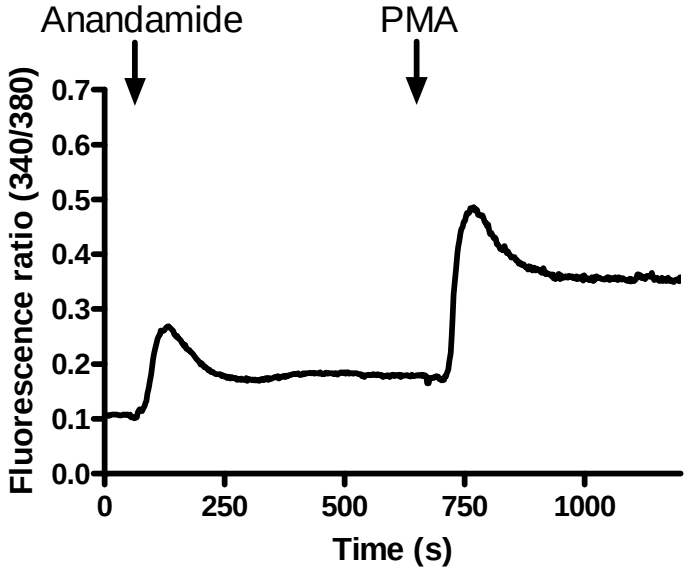
A



B



C



Molecular Pharmacology Fast Forward. Published on December 7, 2005 as DOI: 10.1124/mol.105.015644
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 7