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Life Sciences 82 (2008) 644–651
Life Sciences
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Actions of 3-methyl-*N*-oleoyldopamine, 4-methyl-*N*-oleoyldopamine and *N*-oleoylethanolamide on the rat TRPV1 receptor in vitro and in vivo

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Received 21 September 2007; accepted 16 December 2007

Abstract

N-oleoyldopamine (OLDA) has been identified as an agonist of the transient receptor potential vanilloid type 1 (TRPV1) receptor. A related fatty acid amide, *N*-oleoylethanolamide (OEA), was found to excite sensory neurons and produce visceral hyperalgesia via activation of the TRPV1 receptor, however, a recent study described this agent as an antinociceptive one. The aim of the present paper was to characterize two newly synthesized derivatives of *N*-oleoyldopamine, 3-methyl-*N*-oleoyldopamine (3-MOLDA) and 4-methyl-*N*-oleoyldopamine (4-MOLDA) as well as OEA with regard to their effects on the TRPV1 receptor. Radioactive $^{45}Ca^{2+}$ uptake was measured in HT5-1 cells transfected with the rat TRPV1 receptor and intracellular Ca^{2+} concentration was monitored by fura-2 microfluorimetry in cultured trigeminal sensory neurons. Thermonociception was assessed by determining the behavioral noxious heat threshold in rats. 3-MOLDA induced $^{45}Ca^{2+}$ uptake in a concentration-dependent manner, whereas 4-MOLDA and OEA were without effect. 4-MOLDA and OEA, however, concentration-dependently reduced the $^{45}Ca^{2+}$ uptake-inducing effect of capsaicin. In trigeminal sensory neurons, 3-MOLDA caused an increase in intracellular Ca^{2+} concentration and this effect exhibited tachyphylaxis upon repeated application. Again, 4-MOLDA and OEA failed to alter intracellular Ca^{2+} levels. Upon intraplantar injection, 3-MOLDA caused an 8–10 °C drop of the noxious heat threshold in rats which was inhibited by the TRPV1 receptor antagonist iodo-resiniferatoxin. 4-MOLDA and OEA failed to alter the heat threshold but inhibited the threshold drop induced by the TRPV1 receptor agonist resiniferatoxin. These data show that 3-MOLDA behaves as an agonist, whereas 4-MOLDA and OEA appear to be antagonists, at the rat TRPV1 receptor.

Keywords: TRPV1 receptor; 3-methyl-*N*-oleoyldopamine; 4-methyl-*N*-oleoyldopamine; *N*-oleoylethanolamide; anandamide; $^{45}Ca^{2+}$ uptake; Ca^{2+} imaging; Increasing-temperature (incremental) hot plate

Introduction

The transient receptor potential (TRP) family of ion channels includes the vanilloid (V) subfamily in which only one member, the TRPV1 receptor, is activated by capsaicin, the pungent agent of the red pepper (for a review see Dhaka et al., 2006). Since cloning of this receptor (Caterina et al., 1997) it has been shown that this ligand-gated non-selective cation channel expressed by nociceptive primary afferent neurons is activated by not only capsaicin but also other substances including resiniferatoxin (RTX), low pH (protons), lipoxygenase products and arachidonoylacylamides as well as by noxious heat (for a review see Pingle et al., 2007). Furthermore, since the TRPV1 receptor is indirectly activated also from other receptors like the bradykinin B_2 receptor, it is often referred to as an integrator molecule of polymodal nociceptors.

The fatty acid amide arachidonylethanolamide, also known as anandamide, the endogenous ligand for the cannabinoid receptors (for a review see Pertwee, 2001), was also shown to activate the TRPV1 receptor (Zygmunt et al., 1999; Smart et al., 2000). There has been a long debate about its possible role as the natural endogenous agonist for the TRPV1 receptor (Szolcsányi, 2000; Di Marzo et al., 2001). Recently other

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0024-3205/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.lfs.2007.12.022

related fatty acid amides, *N*-arachidonoyl dopamine and *N*-oleoyldopamine (OLDA, Fig. 1), were identified as TRPV1 receptor agonists (Huang et al., 2002; Chu et al., 2003; Szolcsányi et al., 2004) whereas *N*-arachidonoyl serotonin was described as an antagonist at this receptor (Maione et al., 2007). Parallel to this, a further fatty acid amide, *N*-oleoylethanolamide (OEA, Fig. 1), was also reported to activate sensory neurons and produce hyperalgesia via the TRPV1 receptor (Ahern, 2003; Wang et al., 2005; LoVerme et al., 2006). In contrast, OEA was found antinociceptive in two models of visceral and inflammatory pain both in the mouse and rat (Suardíaz et al., 2007).

The major aim of the present paper was to characterize two newly synthesized methylated derivatives of OLDA, 3-methyl-*N*-oleoyldopamine (3-MOLDA) and 4-methyl-*N*-oleoyldopamine (4-MOLDA) (Fig. 1), with regard to their effect on the rat TRPV1 receptor. In vitro and in vivo methods were employed: measurement of $^{45}Ca^{2+}$ uptake in TRPV1 receptor-transfected non-neuronal cells, monitoring intracellular Ca^{2+} concentration in cultured rat trigeminal sensory neurons and assessment of thermonociception by determining the behavioral noxious heat threshold in unrestrained rats. Owing to the above-mentioned contradictory data obtained with OEA, its effects on the TRPV1 receptor were also investigated. Finally, the effect of anandamide with regard to TRPV1 was also examined but only in the in vivo behavioral model as it had previously been studied extensively in vitro.

Materials and methods

Measurement of radioactive $^{45}Ca^{2+}$ uptake in HT5-1 cells transfected with the rat TRPV1 receptor

The experiments were performed essentially as has been described previously (Szolcsányi et al., 2004). HT5-1 cells transfected with the rat TRPV1 receptor (Sándor et al., 2005) were plated in 15 µl cell culture medium onto Microwell Minitrays (Sigma Inc). The next day, the cells were washed 5 times with HEPES (10 mM, pH 7.4) buffered Hank's balanced salt solution containing 2 mM $CaCl_2$. When testing compounds for TRPV1 receptor agonistic activity, the cells were incubated in 10 µl of the same buffer containing the desired amount of drug and 200 µCi/ml $^{45}Ca^{2+}$ isotope (1.3 Ci/mmol, Amersham) for 5 min at room temperature.

When drugs with presumed antagonistic activity were tested, the cells were preincubated with the desired amount of the drug for 15 min at room temperature. Afterwards the drug was replaced with a mixture of the drug, 100 nM capsaicin and 200 µCi/ml $^{45}Ca^{2+}$ isotope and the cells were incubated for 5 min at room temperature.

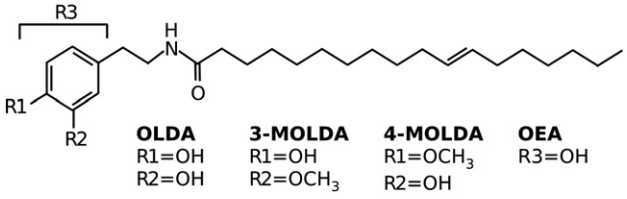

Fig. 1. Chemical structures of *N*-oleoyldopamine (OLDA), 3-methyl-*N*-oleoyldopamine (3-MOLDA), 4-methyl-*N*-oleoyldopamine (4-MOLDA) and *N*-oleoylethanolamide (OEA).

After drug treatment the cells were washed 5 times with assay buffer (5 mM KCl, 2 mM $MgCl_2$, 12 mM glucose, 10 mM HEPES [pH 7.4], 137 mM sucrose, 5.8 mM NaCl, and 0.75 mM $CaCl_2$), the residual buffer was evaporated, the retained isotope was collected in 10 µl 0.1% sodium dodecyl sulfate and the radioactivity was measured in 2 ml scintillation liquid in a Tri-Carb 2800 TR scintillation counter (Packard Inc.).

Measurement by fura-2 microfluorimetry of the intracellular Ca^{2+} concentration in cultured rat trigeminal sensory neurons

Primary cultures of trigeminal neurons were made from trigeminal ganglia of 1–7 day old Wistar rat pups with collagenase (type XI, 0.8 mg/ml) and deoxyribonuclease I (type IV, 600 U/ml) treatment as has been described previously (Szőke et al., 2000). Neurons were grown on 100 µg/ml poly-D-lysine-coated glass cover slips in cell culture medium composed of 85 ml Dulbecco's Modified Eagle Medium, 5 ml horse serum, 5 ml fetal bovine albumin, 5 ml newborn calf serum, 2×10^5 NE/ml penicillin, 100 µg/ml streptomycin and 7 ng/ml nerve growth factor (from mouse, 2.5S). Nerve growth factor (5 ng/ml) was added to the cultured cells every second day.

The trigeminal ganglion neurons on the glass cover slips were incubated for 30 min in incubation buffer (122 mM NaCl, 3.3 mM KCl, 0.4 mM $MgSO_4$, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 10 mM glucose, 25 mM HEPES, pH 7.4) containing 1 µM fura-2 AM (Molecular Probes Inc. Eugene, OR, USA). Dye-loaded cells were examined at room temperature in extracellular solution buffer (160 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.3). The different drugs were dissolved in extracellular solution and were applied with a SF-77B Perfusion Fast-Step system (Warner Inc.). Analysis was performed on the stage of an upright fluorescent microscope (Olympus BX50WI, Tokyo, Japan) alternately at 340 and 380 nm light generated by a monochromator (Polychrome II., Till Photonics Inc., Gräfelfing, Germany) and fluorescence emitted at >510 nm from separate cells was detected with a digital camera (CCD, SensiCam PCO, Kelheim, Germany). Up to 10 dye-loaded small (<25 µm diameter) cells were selected in each field of vision to monitor their fluorescence individually. Fluorescence intensity ratios as a function of time were determined in small areas (regions of interest, ROIs) covering a single neuron and representing approximately 800–1500 image pixels. The ratio of the emitted light (>510 nm) intensities generated by the alternating 340 and 380 nm exciting light (R=F340/F380) was monitored at a rate of 1 Hz. The data were recorded and the 340/380 nm fluorescence ratio was analyzed by the Axon Imaging Workbench 2.1 software (Axon Instruments Inc., Foster City, CA, USA) on a personal computer. The 340/380 nm fluorescence ratio values generated by the Axon Imaging Workbench 2.1 software were then processed by the Origin software version 7.0 (Originlab Corporation, Northampton, MA, USA).

Assessment of thermonociception by measurement of the behavioral noxious heat threshold in unrestrained rats

Thermonociception was investigated in female Wistar rats (140–180 g) by measurement of the behavioral noxious heat threshold with an increasing-temperature (incremental) hot plate (IITC Life Sciences Inc, Woodland Hills, CA, USA) as described previously (Almási et al., 2003). The animal was placed onto the plate, the temperature of which was linearly increased at a rate of 12 °C/min from 30 °C until the animal showed nocifensive behavior confined to either hindpaw (licking or lifting). The corresponding plate temperature was regarded as the behavioral noxious heat threshold.

To reveal an agonistic effect at TRPV1 receptors the compounds were applied by intraplantar (i.pl.) injection at a volume of 100 µl following measurement of the control heat threshold. Subsequently threshold measurements were performed at 5 min intervals. Previous experiments showed that the noxious heat threshold is reproducible even at such short intervals (Almási et al., 2003).

For revealing an antagonistic effect of compounds at TRPV1 receptors, the previously validated resiniferatoxin (RTX) heat allodynia/hyperalgesia model was used (Almási et al., 2003). Briefly, a drop of the heat threshold (heat allodynia or hyperalgesia) was evoked by i.pl. injection of RTX (0.05 nmol in 100 µl), a potent agonist of the capsaicin TRPV1 receptor (Szolcsányi et al., 1990). In order to test for possible TRPV1 receptor antagonistic (antihyperalgesic) effects, one group of animals was pretreated with the given dose of drug investigated and the other with its solvent i.pl. 5 min prior to RTX application. The heat threshold of the treated hindpaw was determined 5, 10, 15 and 20 min after RTX injection. Each animal was included in one group only. For a statistical comparison of the threshold drops (defined as the difference between the control and the drug-induced threshold value) in the drug- versus solvent-pretreated animals at a given time point, the Student's *t*-test for unpaired samples was used ($p<0.05$ values were considered statistically significant). The overall inhibitory effect of drugs on the RTX-induced threshold drop was expressed as percentage inhibition according to the following formula: $(Drop_{solv} - Drop_{drug})/Drop_{solv} \times 100$, where $Drop_{solv}$ and $Drop_{drug}$ refer to the average of the sum of threshold drops measured at 5, 10, 15 and 20 min in the solvent- and drug-treated animals, respectively. The sums of threshold drops obtained in the solvent- and drug-treated animals were also compared by the Student's *t*-test for unpaired samples.

The experiments were performed according to the ethical guidelines of the International Association for the Study of Pain (Zimmermann, 1983) and approved by the ethical committee of the University of Pécs. The number of animals used was kept to the minimum necessary for the purpose of the investigation and care was taken to avoid unnecessary suffering of the animals.

Drugs

3-MOLDA and 4-MOLDA were synthesized in the Chemical Research Center of the Hungarian Academy of Sciences, Budapest, Hungary while all other chemicals used in the experiments were obtained from Sigma-Aldrich Inc. unless stated otherwise. Stock solutions of capsaicin, 3-MOLDA, 4-MOLDA and OEA (10 mM) and RTX (1 mM) were prepared in DMSO and further diluted in the appropriate buffers, as required. OEA solutions were made fresh before every experiment.

Results

Effects of 3-MOLDA, 4-MOLDA and OEA in the $^{45}Ca^{2+}$ uptake test and the inhibitory effects of 4-MOLDA and OEA on the capsaicin-induced $^{45}Ca^{2+}$ uptake

In the first series of experiments 3-MOLDA, 4-MOLDA and OEA were tested for agonistic activity in HT5-1 cells expressing the rat TRPV1 receptor in the concentration range from 0.1 to 100 µM. The reference TRPV1 receptor agonist capsaicin was also tested and drug-induced $^{45}Ca^{2+}$ uptake values are presented relative to the maximum response obtained with 330 nM capsaicin. The concentration–response curves obtained are shown in Fig. 2. 3-MOLDA even at the highest applied concentration (100 µM) evoked an effect corresponding to only 70% of the maximum capsaicin response, however, higher concentrations were not tested due to the concern of precipitation and non-receptor-related effects. The EC_{50} value (defined as the concentration of the drug needed to evoke the response corresponding to 50% of the maximum produced by that drug) of 3-MOLDA was 19.7±6.7 µM indicating that it is about 500-fold less potent than capsaicin (EC_{50}=36 nM). Neither 4-MOLDA nor OEA evoked any $^{45}Ca^{2+}$ uptake in these experiments.

Since neither 4-MOLDA nor OEA showed any measurable $^{45}Ca^{2+}$ uptake, they were tested for possible TRPV1 receptor antagonistic activity against 100 nM capsaicin (Fig. 3). Again, $^{45}Ca^{2+}$ uptake values are presented relative to that obtained with 100 nM capsaicin alone. Both 4-MOLDA and OEA exerted a concentration-dependent inhibitory effect on the $^{45}Ca^{2+}$

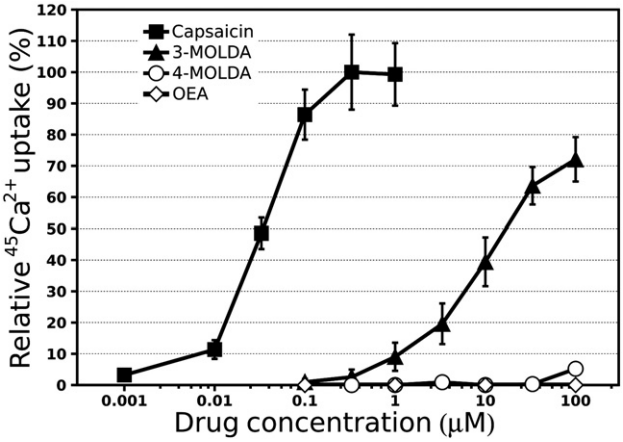

Fig. 2. Concentration–response curves for capsaicin, 3-methyl-*N*-oleoyldopamine (3-MOLDA), 4-methyl-*N*-oleoyldopamine (4-MOLDA) and *N*-oleoylethanolamide (OEA) for uptake of radioactive $^{45}Ca^{2+}$ in HT5-1 cells transfected with the rat TRPV1 receptor. The results are presented as relative values compared to $^{45}Ca^{2+}$ uptake induced by 330 nM capsaicin (100%). Four experiments were done with each drug (n=4) and values are presented as means±SEM.

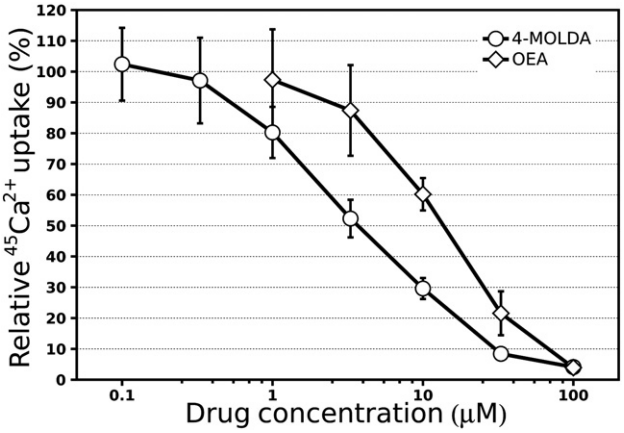

Fig. 3. Inhibition by 4-methyl-*N*-oleoyldopamine (4-MOLDA) and *N*-oleoylethanolamide (OEA) of the capsaicin (100 nM)-induced uptake of radioactive $^{45}Ca^{2+}$ in HT5-1 cells transfected with the rat TRPV1 receptor. In four experiments ($n=4$) cells were treated with the mixture of 100 nM capsaicin and increasing concentrations of 4-MOLDA or OEA. Activity values are presented as mean±SEM, relative to $^{45}Ca^{2+}$ uptake induced by 100 nM capsaicin alone (100%).

uptake induced by capsaicin. The IC_{50} values (corresponding to drug concentrations reducing the capsaicin response by half) were 3.8±1.0 and 16.3±3.5 μM for 4-MOLDA and OEA, respectively.

Effects of 3-MOLDA, 4-MOLDA and OEA on the intracellular Ca^{2+} concentration in cultured rat trigeminal sensory neurons

3-MOLDA, 4-MOLDA and OEA were tested for capacity to elevate intracellular Ca^{2+} concentration in trigeminal sensory neurons by employing fura-2 microfluorimetry in neurons isolated from the trigeminal ganglia of newborn rats. A representative recording of a single cell is presented for each compound in Fig. 4. 3-MOLDA at a concentration of 10 μM (10 s pulse duration) evoked a transient elevation of the intracellular Ca^{2+} concentration as shown by a increase in fura-2 ratio (Fig. 4, panel A). Upon repeated application remarkably diminished responses were observed indicating a pronounced tachyphylaxis. Neither 4-MOLDA nor OEA (10 μM, 10 s pulse duration) induced a measurable increase in fura-2 ratio (Fig. 4, panel B and C). In the same cells, however, capsaicin (330 nM, 10 s pulse duration) evoked an elevation of the intracellular Ca^{2+} concentration indicating the expression of functional TRPV1 receptors in the neurons.

Behavioral experiments: effects of compounds on the unconditioned or sensitized noxious heat threshold in unrestrained rats

The noxious heat threshold of untreated rats was 44.9±0.2 °C ($n=16$) as measured with the increasing-temperature (incremental) hot plate. 3-MOLDA (5 nmol) applied i.pl. evoked an instantaneous nocifensive reaction consisting of paw lickings and liftings which disappeared within 10 min (data not shown). The subsequent threshold measurements revealed that this dose of 3-MOLDA significantly decreased the heat threshold with a maximum drop of threshold observed 15 min after administration (Fig. 5, panel A). The heat threshold-lowering effect of 3-MOLDA was inhibited by pretreatment with the TRPV1 receptor antagonist iodo-resiniferatoxin (I-RTX, 0.05 nmol i.pl., 5 min before) at each time point of measurement as assessed by comparison of threshold drops in the solvent- (of I-RTX) and I-RTX-treated animals (Fig. 5, panel A). In contrast to 3-MOLDA, 4-MOLDA and OEA failed to evoke nocifensive behavior or alter the heat threshold up to i.pl. applied doses of 5 nmol, respectively (data not shown).

Since neither 4-MOLDA nor OEA evoked nocifension or a measurable drop of heat threshold, they were tested for possible antagonistic action against resiniferatoxin (RTX), the reference TRPV1 receptor agonist in this assay (Almási et al., 2003). Pretreatment with the middle and highest dose (1.5 and 5 nmol) of 4-MOLDA (i.pl., 5 min before) diminished the heat threshold-lowering effect of RTX (0.05 nmol i.pl.) at each time point of measurement to a comparable degree (Fig. 5, panel B). The percentage inhibition value for 4-MOLDA calculated on the basis of the sum of threshold drops measured in the four time points was also not higher (in fact smaller) for the highest dose than that for the middle one suggesting that 4-MOLDA's inhibitory effect reached its maximum at about 50% in this model. Higher doses were not tested because of danger of precipitation of the substance. Owing to lack of clear dose–response relationship no ID_{50} value was determined for 4-MOLDA. In contrast, OEA pretreatment (0.5–5 nmol i.pl.,

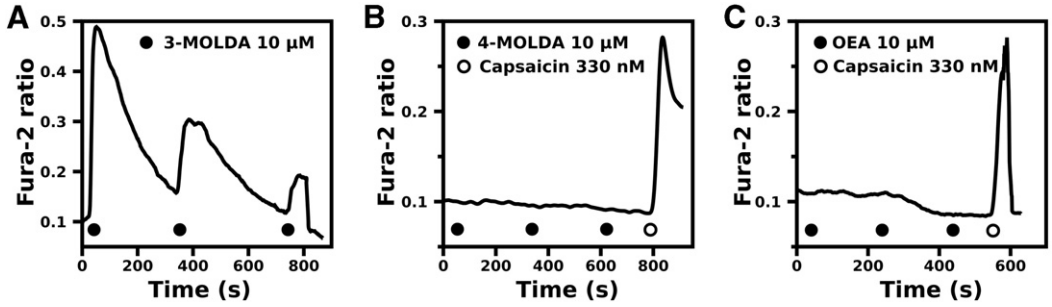

Fig. 4. Effects of capsaicin, 3-methyl-*N*-oleoyldopamine (3-MOLDA), 4-methyl-*N*-oleoyldopamine (4-MOLDA) and *N*-oleoylethanolamide (OEA) on the intracellular Ca^{2+} concentration in cultured rat trigeminal sensory neurons as measured by fura-2 microfluorimetry. The 340/380 nm fluorescence ratio measured over a single representative cell is presented in each graph. Dark circles indicate three consecutive, 10 s long applications of 10 mM 3-MOLDA, 4-MOLDA and OEA, respectively. White circles represent 10 s long applications of 330 nM capsaicin as a positive control.

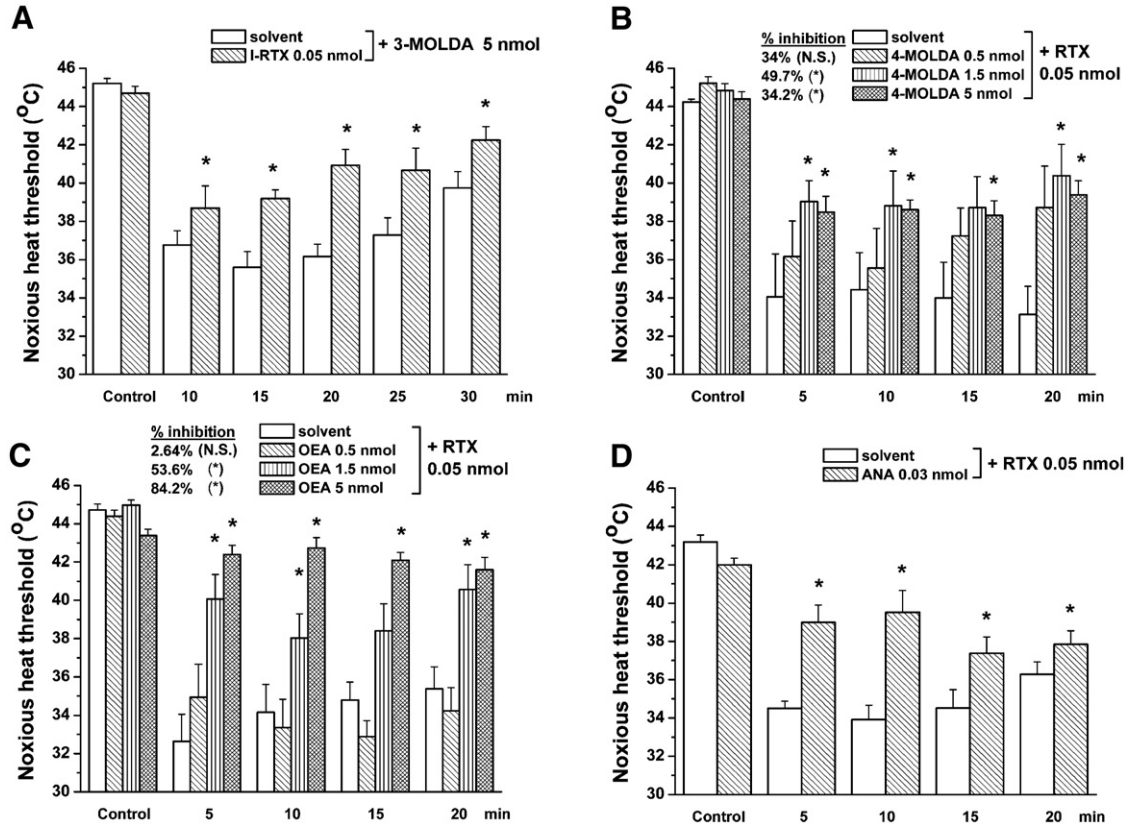

Fig. 5. Effects of drugs applied by intraplantar injection (i.pl., at a volume of 50 μl) on the behavioral noxious heat threshold measured by an increasing-temperature (incremental) hot plate in unrestrained rats (n=6–8 animals in each group). Heat threshold values are presented as mean±SEM. Effect of 3-methyl-*N*-oleoyldopamine (3-MOLDA, 5 nmol i.pl.) alone and following pretreatment with the TRPV1 receptor antagonist iodo-resiniferatoxin (I-RTX, 0.05 nmol i.pl.) (panel A). Effects of 4-methyl-*N*-oleoyldopamine (4-MOLDA, 0.5–5 nmol i.pl., panel B), *N*-oleoylethanolamide (OEA, 0.5–5 nmol, i.pl. panel C) and anandamide (ANA, 0.03 nmol i.pl. panel D) on the heat threshold drop induced by the TRPV1 receptor agonist resiniferatoxin (RTX, 0.05 nmol i.pl.). In panel B and C solvent refers to vehicle of the middle dose of drugs. The % inhibition values indicated were calculated with the formula: $(Drop_{solv} - Drop_{drug})/Drop_{solv} \times 100$, where $Drop_{solv}$ and $Drop_{drug}$ refer to the average of the sum of threshold drops measured at 5, 10, 15 and 20 min in the corresponding solvent- and drug-treated animals, respectively. Asterisks indicate statistically significant ($p<0.05$) threshold drops at a given time point or sum of threshold drops induced by drug compared to that observed in the actual solvent-treated group (Student's *t*-test for unpaired samples).

5 min before) reduced the RTX-induced heat threshold drop in a dose-dependent manner with percentage inhibition values (calculated with the sum of threshold drops) ranging from 2.6 to 84.2% and an ID_{50} value of 1.4 nmol (Fig. 5, panel C). Anandamide, a drug considered as a dual cannabinoid–vanilloid (TRPV1) receptor agonist (Németh et al., 2003; Ahluwalia et al., 2003), at a dose of 0.03 nmol i.pl. also diminished the heat threshold-lowering effect of RTX (0.05 nmol i.pl.) at each time point of measurement (Fig. 5, panel D) but failed to evoke nocifensive behavior or alteration of the unconditioned heat threshold. The inhibitory effect of anandamide was abolished by co-administration with the CB_1 cannabinoid receptor antagonist SR141716A (0.18 nmol i.pl., data not shown).

Discussion

The present study has shown that of the three fatty acid amides investigated both in vitro and in vivo, 3-MOLDA behaved as a TRPV1 receptor agonist while 4-MOLDA and OEA appeared to be antagonists of the TRPV1 receptor. According to behavioral data, anandamide failed to activate the TRPV1 receptor, rather it exerted an inhibitory effect on the action of the TRPV1 receptor agonist RTX via activation of CB_1 cannabinoid receptors.

In the $^{45}Ca^{2+}$ uptake assay in TRPV1 receptor-transfected non-neuronal cells, 3-MOLDA increased the intracellular Ca^{2+} levels, however, its maximum effect was only 70% as compared to the reference TRPV1 receptor agonist capsaicin. 3-MOLDA was also much less potent than capsaicin as indicated by its 170-fold higher EC_{50} value. In agreement with these results, 3-MOLDA increased the intracellular Ca^{2+} concentration in cultured rat trigeminal sensory neurons as well and again it appeared much less potent than capsaicin. Upon repeated administration, 3-MOLDA exhibited a pronounced tachyphylaxis which is also characteristic for capsaicin in this assay (Varga et al., 2006). In the behavioral model, 3-MOLDA induced a drop of the noxious heat threshold i.e. it evoked a heat hyperalgesia/allodynia. Its threshold-lowering action was pronounced, ranging up to a decrease of 10 °C and being comparable to that of RTX which is the reference TRPV1 receptor agonist in this assay (Almási et al., 2003). In this

model, 3-MOLDA appeared less potent than RTX. The threshold-lowering effect of 3-MOLDA was attenuated by I-RTX (Wahl et al., 2001) whose TRPV1 receptor antagonistic action was previously validated in the presently employed experimental setting (Almási et al., 2003). All these data strongly support the view that 3-MOLDA is an agonist of the rat TRPV1 receptor.

In contrast to 3-MOLDA, 4-MOLDA and OEA exhibited activities consistent with antagonism of the rat TRPV1 receptor in the models studied. Both agents failed to induce $^{45}Ca^{2+}$ uptake in TRPV1 receptor-transfected cells and to alter the intracellular Ca^{2+} concentration in trigeminal sensory neurons. The reference compound capsaicin was highly effective in both models providing evidence for expression of functional TRPV1 receptors. In the in vitro assay designed for studying TRPV1 receptor antagonism in TRPV1 receptor-transfected non-neuronal cells, both 4-MOLDA and OEA reduced the $^{45}Ca^{2+}$ uptake-inducing effect of capsaicin in a concentration-dependent manner with 4-MOLDA being slightly more potent than OEA. The precise mechanisms of the inhibitory actions of 4-MOLDA and OEA cannot be determined on the basis of the present results. However, considering that these inhibitory effects were studied in a reduced model i.e. in non-neuronal cells expressing TRPV1 receptors, two mechanisms are likely to account for the inhibition of capsaicin-induced $^{45}Ca^{2+}$ uptake: a competitive TRPV1 receptor antagonism i.e. inhibition of binding of capsaicin to its receptive site within the TRPV1 protein and/or block of the cation channel part of the TRPV1 receptor i.e. direct inhibition of Ca^{2+} entry. As 4-MOLDA is structurally very closely related to 3-MOLDA and OLDA that proved to be TRPV1 receptor agonists in the present (see above) and previous studies (Chu et al., 2003; Szolcsányi et al., 2004), respectively, the competitive antagonism appears more likely for 4-MOLDA. Nevertheless, further experiments, including binding studies, are required for revealing the mechanism(s) of the TRPV1-receptor inhibiting actions of 4-MOLDA and OEA. The behavioral data obtained with these agents are in accord with a TRPV1 receptor antagonistic behavior as neither 4-MOLDA nor OEA induced nociception and altered the noxious heat threshold in rats but both agents inhibited the heat threshold drop evoked by the TRPV1 receptor agonist RTX. While the in vivo inhibitory action against RTX showed a clear dose-dependence in the case of OEA, no clear-cut dose–response relationship could be revealed for 4-MOLDA. This is in contrast with the $^{45}Ca^{2+}$ uptake assay in which both agents diminished the effect of capsaicin in a concentration-dependent manner. The effect of the highest dose of 4-MOLDA did not exceed that of the middle one (in fact the percentage inhibition value was actually smaller and numerically equal to that of the lowest dose, the effect of which was, however, non-significant). The reason for this apparently smaller effect could reside in the fact that each dose has been compared with its own solvent control that showed variability. We have no clear explanation why 4-MOLDA's inhibitory effect in the RTX hyperalgesia assay reached its maximum at about 50%: theoretically the drug may evoke another, TRPV1-unrelated effect that somehow enhances the action of RTX thereby limiting its RTX antagonistic efficacy.

It is worth emphasizing that 3-MOLDA and 4-MOLDA, two methylated derivatives of the TRPV1 receptor agonist OLDA differing only in the position of the methyl group on the dihydroxylated aromatic ring, behaved as a TRPV1 receptor agonist and antagonist, respectively. These results show that even a slight chemical difference can have a dramatic influence on the effect of the fatty acid amide compounds on the TRPV1 receptor protein.

The present results obtained with OEA, pointing to a TRPV1 receptor antagonistic activity both in vitro and in vivo, are in accord with the study in which OEA was found antinociceptive in two models of visceral and inflammatory pain, respectively: acetic acid-induced writhing in the mouse and formalin-evoked chemonociception in the rat and mouse (Suardíaz et al., 2007). It should be remembered, however, that the formalin-evoked chemonociception in the mouse was independent of TRPV1 as shown by our previous work employing TRPV1 receptor knockout mice (Bölcskei et al., 2005). In contrast, in the mouse OEA excited vagal sensory neurons and produced visceral nociception via activation of the TRPV1 receptor (Wang et al., 2005). Also in the mouse, OEA applied by intraplantar injection induced TRPV1-dependent nocifensive behavior, although at 6–30 times higher doses than that which failed to induce nociception in the present study in rats (LoVerme et al., 2006). These discrepancies might be due to the different cellular environment and/or species in which the TRPV1 receptors were investigated; however, a further explanation is also possible. This is based on the assumption that OEA is in fact a partial TRPV1 receptor agonist with low intrinsic efficacy that can competitively inhibit binding and consequently the effect of more efficacious TRPV1 agonists (this might be true for 4-MOLDA as well). This hypothesis is supported by the fact that OEA's in vitro TRPV1 receptor agonistic effect could be detected only after activation of protein kinase C but it inhibited anandamide-evoked TRPV1-mediated currents (Ahern, 2003). Furthermore, OEA proved to be a partial agonist at human TRPV1 receptor in both patch clamp and Ca^{2+} imaging assays (Movahed et al., 2005). Other, chemically related polyunsaturated fatty acids were also shown to activate TRPV1 on the one hand and antagonize the effect of the full TRPV1 agonist capsaicin on the other hand (Matta et al., 2007). In the present study, the OEA concentrations/doses employed might have been too low to evoke a detectable TRPV1 receptor agonistic effect but could partially displace the full agonists capsaicin or RTX from TRPV1 leading to competitive antagonism. The threshold concentration/dose for revealing a TRPV1 agonistic effect might be tissue/model dependent owing to e.g. various levels of TRPV1 receptor expression: in systems with higher TRPV1 receptor densities OEA might appear as a partial agonist whereas in those with lower levels of TRPV1 expression no agonistic effect can be seen. In both cases, however, OEA can competitively antagonize the effects of more efficacious agonists. Further experiments are needed for a better characterization of OEA regarding the TRPV1 receptor and nociception.

The other fatty acid amide investigated, arachidonylethanolamide, better known as anandamide, also inhibited the heat threshold-lowering effect of RTX, i.e. it exerted a thermal

antihyperalgesic action. Anandamide is an endogenous ligand for the cannabinoid receptors, having higher affinity for the CB_1 than for the CB_2 receptors (for a review see Pertwee, 2001). In addition, anandamide is capable of activating the TRPV1 receptor (Zygmunt et al., 1999; Smart et al., 2000), albeit at much higher concentrations than those required for stimulation of the cannabinoid receptors (Németh et al., 2003; Ahluwalia et al., 2003). There has been a debate about the possibility that anandamide can function as an endogenous, physiological activator of TRPV1 receptors (Szolcsányi, 2000; Di Marzo et al., 2001). The present data do not support this hypothesis because anandamide inhibited the effect of the TRPV1 receptor agonist RTX and this inhibitory effect was abolished by a CB_1 receptor antagonist. If anandamide were a partial agonist at TRPV1 receptors, it could still reduce the effect of RTX by a competitive antagonism. However, the abolishment of its effect by CB_1 receptor antagonism clearly shows that this action is exclusively mediated by CB_1 cannabinoid receptors leaving no room for action at TRPV1 receptors.

Conclusion

The present study investigated various fatty acid amides in relation to the rat TRPV1 receptor employing measurement of $^{45}Ca^{2+}$ uptake in TRPV1 receptor-transfected HT5-1 cells and intracellular Ca^{2+} concentration in cultured rat trigeminal sensory neurons as well as assessment of thermonociception by determination of the behavioral noxious heat threshold in rats. While 3-MOLDA proved to be an agonist of the rat TRPV1 receptor, the chemically only slightly different 4-MOLDA exerted effects suggesting an antagonistic activity at TRPV1 receptors. OEA also behaved as a TRPV1 receptor antagonist or weak partial agonist both in vitro and in vivo. Anandamide, tested in the behavioral assay only, failed to activate TRPV1 receptors and its inhibitory effect on TRPV1 receptor agonism-induced heat hyperalgesia was mediated by CB_1 cannabinoid receptors.

Acknowledgements

The authors thank Ms Cecília Disztl and Mrs Katalin Gógl for expert technical assistance. This work was supported by Hungarian Grants ETT 348/2006 and ETT 285/2006.

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