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Temperature-dependent activation of recombinant rat vanilloid VR1 receptors expressed in HEK293 cells by capsaicin and anandamide

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Abstract

Capsaicin activates vanilloid (VR1) receptors found on sensory neurons. These ligand-gated ion channels are also sensitive to low pH, elevated temperature and the endocannabinoid, anandamide. In this study, we have measured capsaicin- and anandamide-induced elevations in intracellular calcium concentrations ($[Ca^{2+}]_i$) in fura-2 loaded HEK293 cells stably expressing the rat VR1 receptor at 22, 37 and 50 °C. Both capsaicin and anandamide produced a concentration-dependent elevation in $[Ca^{2+}]_i$ at all temperatures. pEC_{50} values were 7.74 and 5.69 at 22 °C and 6.90 and 5.15 at 37 °C for capsaicin and anandamide, respectively. At 50 °C, the pEC_{50} value for capsaicin was 6.36 but the response to anandamide did not saturate. Responses to both agonists were sensitive to ruthenium red and capsazepine at all temperatures. This temperature-dependent reduction in potency may result from desensitization. © 2001 Published by Elsevier Science B.V.

Keywords: Vanilloid VR1 receptor, rat; Ca^{2+}, intracellular; Capsaicin; Anandamide; Heat

1. Introduction

One characteristic of nociceptors, the sensory neurones responsible for the first order transmission of pain, is that they are activated by capsaicin, the pungent chemical present in hot peppers (Szallasi and Blumberg, 1999). Recently, a ligand-gated ion channel, termed vanilloid receptor-1 (VR1), has been cloned (Caterina et al., 1997), which displays the appropriate pharmacology (Tominaga et al., 1998; Jerman et al., 2000) and distribution (Mezey et al., 2000) to account for the actions of capsaicin in sensory neurones (Szallasi and Blumberg, 1999). Moreover, VR1 knockout mice demonstrate impaired nociception (Caterina et al., 2000; Davis et al., 2000).

It has been proposed (Smart and Jerman, 2000) that anandamide, an arachidonic acid derivative originally isolated from porcine brain (Devane et al., 1992), is an endogenous mammalian ligand for VR1, as it activates VR1 in both recombinant (Zygmunt et al., 1999; Smart et al., 2000) and endogenous (Szoke et al., 2000) systems. Interestingly, anandamide is antinociceptive even in cannabinoid CB_1 receptor knockout mice (DiMarzo et al., 2000).

Low pH enhances the response to capsaicin (Tominaga et al., 1998; Jerman et al., 2000; Baumann and Martenson, 2000) but not to anandamide (Smart et al., 2000). Heat has also been reported to enhance VR1-mediated responses (Caterina et al., 1997; Tominaga et al., 1998) but the effect of increased temperature on the VR1-mediated anandamide-induced response has not been reported. Therefore, in the present study, concentration–response curves to capsaicin and anandamide were generated at different temperatures in cells expressing rat VR1. Heat decreased the potency of both capsaicin and anandamide in a temperature-dependent fashion, although there was an increase in the maximum response with both ligands at 37 °C compared to 22 °C.

2. Materials and methods

2.1. Cell culture

Rat VR1-HEK293 cells (Jerman et al., 2000) were maintained in minimum essential media (MEM) supplemented with 10% foetal calf serum, 0.2 mM glutamine, 2.5

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μg/ml fungizone, 50 IU/ml and penicillin, 50 μg/ml streptomycin, at 37 °C in 5% CO_2/air. Cells were passaged every 5–6 days and used up to passage 24.

2.2. Measurement of intracellular calcium concentrations ($[Ca^{2+}]_i$)

Monolayer cultures were detached with harvest buffer (EDTA 1.7 mM, NaCl 154 mM and HEPES 10 mM), then washed twice with and resuspended into Krebs–HEPES buffer (NaCl 143.4 mM, KCl 4.8 mM, HEPES 10 mM, $CaCl_2.2H_2O$ 2.6 mM, KH_2PO_4 1.2 mM, $MgSO_4.7H_2O$ 1.2 mM, and glucose 11.7 mM). Suspensions of cells were then incubated at 37 °C in Krebs–HEPES buffer containing 5 μM Fura-2AM for 30 min, followed by a further 20 min in fresh buffer at room temperature to allow for de-esterification.

Loaded cells were then resuspended in Krebs–HEPES supplemented with 0.2% bovine serum albumin (BSA) and maintained on ice. Two hundred microliters of cell suspension was added to 1.8 ml BSA supplemented buffer in a cuvette prewarmed to the desired temperature (22, 37 or 50 °C, see below). Cells were allowed to equilibrate for 3 min before the agonist (capsaicin or anandamide) was added. Fluorescence emission measured at 510 nm with excitation at 340 and 380 nm was recorded using a Perkin-Elmer LS50B fluorimeter as described previously (Hirst et al., 1999). Ratio (340:380) was converted to $[Ca^{2+}]_i$ according to Grynkiewicz et al. (1985) where R_{max} and R_{min} were determined using Triton-X100 and EGTA, respectively. K_d values at 22, 37 and 50 °C were 145 (Simpson, 1999), 225 (Simpson, 1999) and 329, respectively. Value at 50 °C was derived based on the relationship $Q_{10} = 2$. In some experiments, the VR1 antagonists capsazepine (competitive) and ruthenium red (pore blocker) were included at least 5 min prior to agonist addition. Cuvette temperature was adjusted using a circulating water bath whose temperature was monitored continuously using a thermocouple thermometer. Preliminary studies indicated that with a stable bath temperature (higher than the desired final temperature) and addition of 0.2 ml ice cold cells to 1.8 ml prewarmed buffer, 2 min were required to reach the desired cuvette temperature.

2.3. Data analysis

Unless stated otherwise, all data are expressed as mean ± S.E.M. and are change (Δ) in $[Ca^{2+}]_i$ (basal subtracted). pEC_{50} values (negative logarithm to $base_{10}$ of the agonist concentration producing 50% of the maximum response, E_{max}) were obtained by computer-assisted curve fitting using GRAPH-PAD PRISM (V2.0). Appropriate data were analysed using ANOVA with Bonferroni correction for multiple comparisons and differences were considered significant when $p < 0.05$.

2.4. Materials

All tissue culture media and supplements were from Life Technologies (Paisley, Scotland). Capsaicin, anandamide, capsazepine and ruthenium red were from Sigma

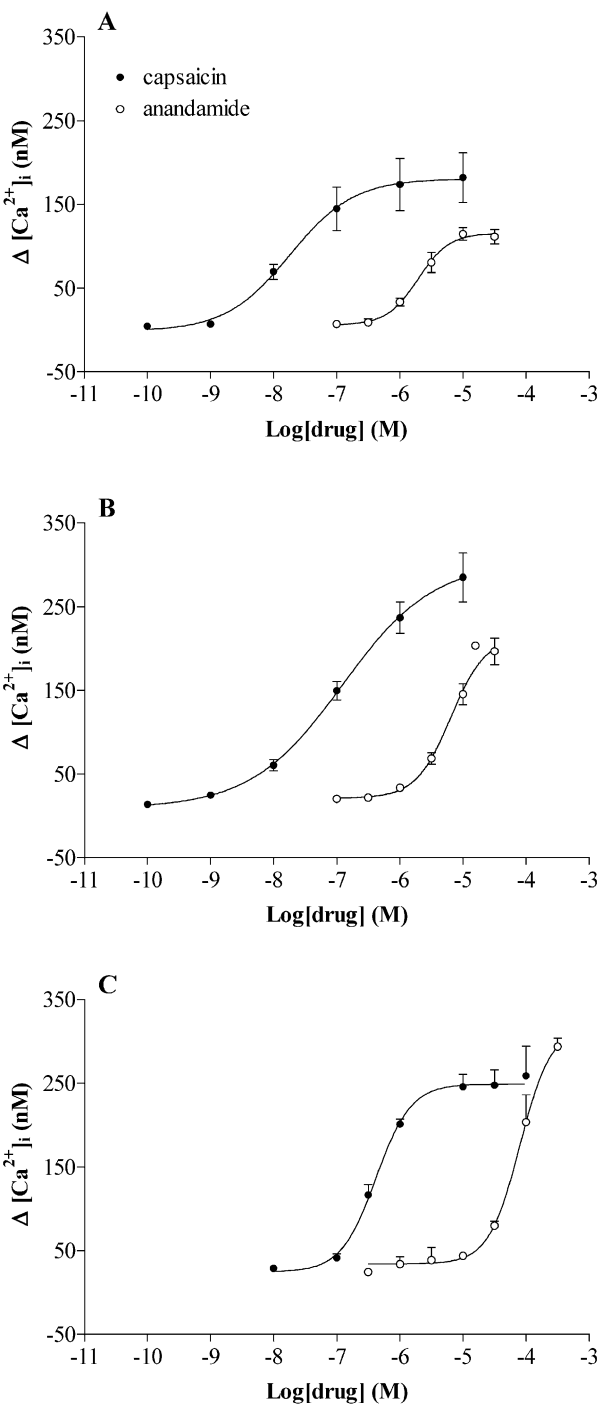

Fig. 1. Capsaicin and anandamide increase $[Ca^{2+}]_i$ in a concentration-dependent manner at (A) 22, (B) 37 and (C) 50 °C in fura-2 loaded rat VR1-HEK293 cells. Data are mean ± S.E.M. ($n = 3–5$).

Table 1
Effects of capsaicin and anandamide on $[Ca^{2+}]_i$ in fura-2 loaded rat VR1-HEK293 cells at 22, 37 and 50 °C
Data are mean ± S.E.M. ($n = 3–5$).

Temperature	Capsaicin			Anandamide		
	22 °C	37 °C	50 °C	22 °C	37 °C	50 °C
pEC_{50} (nM)	7.74 ± 0.07 (18)	6.9 ± 0.07[a] (126)	6.36 ± 0.09[a] (437)	5.69 ± 0.12 (2042)	5.15 ± 0.04[a] (7079)	crc incomplete
Relative 22 °C	1.0	7.0	24.3	1.0	3.5	
E_{max} $\Delta[Ca^{2+}]_i$ (nM)	182 ± 28	304 ± 34[b]	244 ± 14	115 ± 8	220 ± 14[b]	294 ± 10[c]
Slope	0.85 ± 0.1	0.61 ± 0.04	2.4 ± 0.5	2.35 ± 0.26	1.49 ± 0.27	

[a] pEC_{50} significantly lower than at 22 °C.
[b] E_{max} significantly higher than at 22 °C.
[c] Concentration response curve (crc) did not saturate, value for 300 μM of anandamide is quoted.

(Dorset, UK). All other reagents were of the highest purity available.

3. Results

At 22, 37 and 50 °C, both capsaicin and anandamide increased $[Ca^{2+}]_i$ as reported previously (Smart et al., 2000). The increase seen with both capsaicin and anandamide was concentration-dependent, although at 50 °C, the anandamide-induced concentration–response curve did not plateau even at the highest concentration used, 300 μM. At all temperatures, anandamide was significantly less potent (56–112 fold) than capsaicin (Fig. 1, Table 1). There was a temperature-dependent decrease in potency for both capsaicin and anandamide. Moreover, the efficacy of capsaicin and anandamide increased when the temperature was elevated from 22 to 37 °C with no further increase on elevation to 50 °C for capsaicin. Basal $[Ca^{2+}]_i$ rose in a temperature-dependent manner (48 ± 3 nM at 22 °C, 105 ± 15 nM at 37 °C and 212 ± 14 nM at 50 °C).

As a control for the effects of temperature, we examined the effects of carbachol (presumably acting at a G_q-coupled muscarinic receptor) at 22 and 50 °C. At 22 °C, pEC_{50} and E_{max} values were 4.56 and 219 nM ($n = 2$), respectively. In contrast to the enhancement of the maximum response seen with capsaicin, the response at 50 °C was abolished in two experiments and substantially reduced in the third (pEC_{50} 4.87, E_{max} 76 nM).

At all temperatures, the responses to both capsaicin (0.1 or 1 μM) and anandamide (3, 10 or 100 μM) were sensitive to capsazepine and ruthenium red (Table 2).

Table 2
Effects of casazepine and ruthenium red on the response to capsaicin and anandamide in fura-2 loaded rat VR1-HEK293 cells at 22, 37 and 50 °C
Data are %inhibition ± S.E.M. ($n = 4–7$). At 22 °C, 100 nM capsaicin and 3 μM anandamide were antagonized by 10 μM capsazepine and 1 μM ruthenium red. At 37 °C, 1 μM capsaicin and 10 μM anandamide were antagonized by 100 μM capsazepine and 10 μM ruthenium red. At 50 °C, 1 μM capsaicin and 100 μM anandamide were antagonized by 100 μM capsazepine and 10 μM ruthenium red (all values significantly inhibited compared with control, $p < 0.05$).

Temperature (°C)	Capsaicin		Anandamide	
	Capsazepine	Ruthenium Red	Capsazepine	Ruthenium Red
22	90 ± 5	99 ± 1	88 ± 8	91 ± 4
37	78 ± 6	91 ± 2	84 ± 2	79 ± 5
50	92 ± 3	91 ± 1	100 ± 0	100 ± 0

4. Discussion

The recently cloned ligand-gated ion channel, VR1 (Caterina et al., 1997), plays an important role in nociceptive signalling (Szallasi and Blumberg, 1999; Caterina et al., 2000), particularly thermal hyperalgesia (Davis et al., 2000). VR1 is activated by the plant extract capsaicin (Caterina et al., 1997) and the endogenous mammalian ligand anandamide (Smart and Jerman, 2000; Szoke et al., 2000). Low pH enhances the capsaicin- (Tominaga et al., 1998; Jerman et al., 2000), but not the anandamide- (Smart et al., 2000) induced response in VR1-expressing cells. Noxious heat also enhances some VR1-mediated responses (Caterina et al., 1997; Tominaga et al., 1998). Therefore, in the present study, the effect of temperature on the capsaicin- and anandamide-induced calcium responses in VR1-expressing HEK293 cells was assessed using Fura-2AM. Heat decreased the potency of both capsaicin and anandamide in a temperature-dependent fashion, although there was an increase in the maximum response with both ligands at 37 °C compared to 22 °C.

At room temperature, both capsaicin and anandamide increased $[Ca^{2+}]_i$ in VR1-expressing HEK293 cells in a concentration-dependent manner, as reported previously (Caterina et al., 1997; Jerman et al., 2000; Smart et al., 2000). Interestingly, anandamide appeared to be a partial agonist at 22 °C, contrary to our previous observations (Smart et al., 2000). This may reflect a species difference as the earlier study utilised human VR1 (Smart et al., 2000) while the current investigation utilised rat VR1. Indeed, anandamide has been reported to be a partial agonist at rat VR1 (Zygmunt et al., 1999). Alternatively, this discrepancy might reflect methodological differences, as in the human studies, the apparent efficacy of anan-

damide varied with the type of assay, e.g. whole cell patch clamping vs. calcium imaging (Smart et al., 2000; Smart and Jerman, 2000).

Increasing the temperature (22–50 °C) caused a small, non-specific, temperature-related increase in basal $[Ca^{2+}]_i$, in VR1-expressing cells, as reported previously (Nagy and Rang, 1999; Savidge and Rang, 2000). Increasing the temperature from 22 to 37 °C also increased the maximum response to both capsaicin and anandamide by 67% and 91%, respectively. This is consistent with previous reports that similar increases in temperature enhance the proton-induced response in VR1-expressing cells (Tominaga et al., 1998; Cesare et al., 1999), even though 37 °C is insufficient to activate VR1 directly (Caterina et al., 1997; Savidge and Rang, 2000). However, raising the temperature higher to 50 °C had no further effect on the maximum capsaicin-induced response. In sensory neurones, raising the temperature from 37 to 50 °C also failed to enhance the maximum capsaicin-induced calcium response (Nagy and Rang, 1999). Nevertheless, the efficacy of anandamide was increased further by raising the temperature to 50 °C, although it should be noted that the response did not plateau at this temperature. Low pH has also been shown to differentially regulate the anandamide- and capsaicin-induced responses in VR1-expressing cells (Smart et al., 2000). The effects of temperature on efficacy were specific for VR1, as the efficacy of carbachol at the endogenous muscarinic receptor was decreased by increasing temperature.

The potency of both anandamide and capsaicin was reduced in a temperature-dependent manner over the range 22–50 °C, while the potency of carbachol at the endogenous muscarinic receptor was unaffected. This is most probably due to desensitisation of VR1 at higher temperatures, as heat and capsaicin have been shown to cross-desensitise in sensory neurones (Cesare et al., 1999) and recombinant systems (Tominaga et al., 1998), although it should be noted that at 37 °C, heat does not directly activate VR1 (Caterina et al., 1997). This is the opposite effect to low pH which enhances the potency of capsaicin in VR1-expressing cells (Baumann and Martenson, 2000; Jerman et al., 2000). One possible explanation for this is that protons and heat desensitise VR1 by different mechanisms (Cesare et al., 1999).

Collectively, these data provide evidence for a decreased agonist potency but increased efficacy at elevated temperatures. However, the interpretation of these observations is complicated due to the nature of the assay system employed. Outwardly, the observed behavior is suggestive of a reduced agonist affinity coupled with a higher maximum probability of channel opening at 100% agonist occupancy. The latter is likely to arise from temperature-dependent changes in the lifetimes of different channel states (i.e. closed, open and desensitised). Indeed, the probability of VR1 receptors occupying an open state is strikingly increased as temperature is raised, thus allowing it to act as a molecular thermometer. The effects of temperature on ligand binding are also complex, particularly due to the relative contributions of temperature-independent enthalpic and temperature-dependent entropic components to the free energy of the receptor ligand (Barlow, 1980).

Both capsaicin and anandamide may need to cross the plasma membrane to reach their site of action. This process has been suggested to be transporter-mediated for the latter agonist (Piomelli et al., 1999). The effects of temperature on aqueous/lipid partition and/or active transport, as well as on the enzymatic breakdown of anandamide, may all contribute to the efficacy and apparent affinity observed. Additional problems to the interpretation of the observed changes lie in the basis of the assay. These predominantly arise from the fact that the assay is performed on a population of non-equivalent cells. In such assays, raising the maximum probability of channel opening (i.e. increased efficacy) causes an apparent decrease in EC_{50} and increase in Hill coefficient. In contrast, decreases in Ca^{2+}-reporter dye affinity increase EC_{50} and lower Hill coefficient.

At 22 °C, both the capsaicin- and anandamide-induced responses were abolished by capsazepine and ruthenium red, as reported previously (Caterina et al., 1997; Zygmunt et al., 1999; Smart et al., 2000). Capsazepine and ruthenium red also blocked these responses at 37 and 50 °C, confirming that the effects of temperature on the capsaicin- and anandamide-induced responses were specific to VR1. Interestingly, the effects of temperature on VR1 have been reported to be relatively insensitive to these antagonists (Nagy and Rang, 1999; Savidge and Rang, 2000), although this has been disputed (Tominaga et al., 1998; Kirschstein et al., 1999).

In conclusion, increasing temperature decreased the potency of both capsaicin and anandamide at VR1 in a temperature-dependent fashion, although there was an increase in the maximum response with both ligands at 37 °C compared to 22 °C. Further studies to specifically address desensitization at different temperatures are warranted.

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