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(Article begins on next page)

Expression of functional TRPV1 receptors in primary culture of canine keratinocytes.

Running title: TRPV1 expression and functionality in canine keratinocytes

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## Abstract

The interest for the endovanilloid system and for transient receptor potential Vanilloid 1 (TRPV1) is continuously increasing, due to their involvement in inflammation, nociception and pruritus. Even if TRPV1 enrolment was highlighted in both physiological and pathological conditions, some aspects remain unclear, mostly in veterinary medicine. This study aimed to verify the expression and functionality of TRPV1 in canine keratinocytes to investigate in vitro the role of TRPV1 in these cells that are involved in different cutaneous pathologies. Keratinocytes primary cultures were isolated from bioptical samples and cultivated. Binding assay (using 3[H]-resiniferatoxin), displacement assay (in the presence of 1.2 nM 3[H]-resiniferatoxin) and functional assays (in the presence of 1  $\mu$ Ci/45Ca<sup>2+</sup>) with vanilloid agonists and antagonists, specifically addressed to TRPV1 receptor, were performed. Binding assay demonstrated the presence of measurable concentrations of TRPV1 ( $B_{max} = 1,240 \pm 120$  fmol/mg protein;  $K_d = 0.01 \pm 0.004$  nM). Displacement assay highlighted the highest affinity for resiniferatoxin (RTX) and 5-iodo-resiniferatoxin (5-I-RTX), among agonists and antagonists, respectively. The same compounds results as the most potent in the functional assays. This study demonstrated the identification and the characterization of TRPV1 receptor in primary canine keratinocytes cultures. The results are promising for a clinical use, but further in vivo investigations are required.

**Keywords:** TRPV1, keratinocytes, dog, identification, characterization.

## INTRODUCTION

The transient receptor potential vanilloid type 1 (TRPV1) is a non-selective cation channel that belongs to the Transient Receptor Potential (TRP) family of proteins (Veronesi and Oortgiesen, 2011). TRP receptors are able to mediate the receptor response induced by external “transient” stimuli, such as light, temperature, low pH, electrical charge and xenobiotics (Starowicz et al., 2007). TRPs are encoded by 28 genes and are divided into 6 subfamilies based on the amino acid homology: TRPA (ankyrin), TRPC (canonical), TRPM (melastatin), TRPML (mucolipin), TRPP (polycystin) and TRPV (vanilloid) (Vandewauw et al., 2013). TRPV1 (Fig.1) was the first to be cloned among TRP ion channels (Caterina et al., 1997), and became one of the main characters in scientific investigations, and its presence was demonstrated in several organs and tissues, in physiological and pathological conditions, both for humans and animals (Starowicz et al., 2007; Patapoutian et al., 2005).

The skin is the largest organ of the body and it is an interface with the environment. It protects water-rich internal organs from harmful external factors, i.e. dryness, chemicals, temperature and UV irradiation (Radner et al., 2014). It has been demonstrated that  $\text{Ca}^{2+}$  dynamics may play an important role in the homeostasis of epidermis, i.e., the outermost part of the skin (Denda et al., 2000; Graham et al., 2013; Permatasari et al., 2013). An increase in the intracellular  $\text{Ca}^{2+}$  concentration in response to external stimuli may result in epidermal cell differentiation (Graham et al., 2013). These findings raise the possibility that epidermal keratinocytes have functional  $\text{Ca}^{2+}$ -permeable ion-channel receptors, similar to neuronal TRPV1. Indeed, data concerning the expression of functional TRPV1 in human skin are available, and the expression of TRPV1 in human keratinocytes suggests its involvement in the maturation and function of epithelial cells (Ständer et al., 2005), and skin disorders (Inoue et al., 2002; Barbas et al., 2013). In particular, it was demonstrated that the activation of epidermal TRPV1 plays a key role in nociception (Salaat et al., 2013), pruritus (Paus et al., 2006; Shim et al., 2007), and skin inflammation (Southall et al., 2003; Ständer et al., 2004). Noteworthy, certain endocannabinoids (i.e., anandamide) and cannabimimetic endocannabinoid-like compounds (i.e., palmitoylethanolamide) are able to directly or indirectly desensitize TRPV1 (Veronesi and Oortgiesen, 2006), and may thus mitigate pruritus, inflammation and pain (Re et al., 2007). Indeed, TRPV1 is now often referred to as part of the endocannabinoid system (De Petrocellis et al., 2010). To date, mammalian TRPV1 has been cloned and characterized in humans (Hayes et al., 2000), rats (Caterina et al., 1997), guinea-pigs (Savidge et al., 2002), rabbits (Gavva et al., 2004), mice (Correll et al., 2004), and dogs (Phelps et al., 2005). From these and from other studies, the presence of specie-specific differences in the functional profile of TRPV1 arose (Chou et al., 2004; Vercelli et al., 2015a).

It should be considered of interest in veterinary medicine to identify and characterize TRPV1 in canine species, with particular respect to the skin and oral mucosa, two of the most important barriers between the body and the external environment, and often affected by inflammatory, allergic and/or neoplastic processes.

Based on these premises, the aim of the present study was to confirm the presence of TRPV1 receptor in *in vitro* canine keratinocytes cell culture model using binding assays and to verify the expression and to characterize the receptor using displacement and functional studies.

## MATERIALS AND METHODS

## Cell cultures

Keratinocyte primary cultures were prepared from bioptic samples obtained from ear skin, ventral abdominal skin, oral mucosa and soft palate of dogs operated in the surgical rooms of the Veterinary Teaching Hospital of the Department of Veterinary Sciences of Turin (Italy).

Tissues were aseptically cleaned and rinsed twice with povidone iodine scrub followed by 70% alcohol (Gupta et al., 2007), then 1.5 cm<sup>2</sup> full-thickness skin/mucosal biopsies were obtained, and used as a source of keratinocytes. Dermal connective tissue was trimmed off, and the tissue samples were washed in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, Milan, Italy) containing a concentrated antibiotic-antimycotic solution (composed by penicillin G 1000 U/mL, streptomycin 50 µg/mL, amphotericin B 2,5 µg/mL and gentamicin 100 µg/mL; Sigma-Aldrich, Milan, Italy) (Gupta et al., 2007), rinsed and then incubated for 2 h in the same medium in a 5% CO<sub>2</sub> controlled atmosphere at 37 °C. Finally, subcutaneous fat and dermis were accurately removed without damaging the superficial layer of epidermis. The skin was then minced finely with a scalpel to small pieces (approximately 1 mm diameter) and plated for 1-2 weeks (this phase was named "early keratinocyte attachment") in irradiated plastic culture dishes in complete William's Medium E (WME) (Cambrex Bio Science, Petit-rechain, Belgium) added with: cholera toxin 10<sup>-10</sup> M (to control fibroblast overgrowth), epidermal growth factor 10 ng/mL, fetal bovine serum 20%, penicillin G 100 U/mL, streptomycin 100 µg/mL, amphotericin B 0.25 µg/mL, gentamicin 100 µg/mL (Sigma-Aldrich, Milan, Italy).

Isolated keratinocytes were then incubated using the same culture medium in 5% CO<sub>2</sub> controlled atmosphere at 37°C and re-seeded when reaching 80 % confluence (Sun et al., 2014; Poumay et al., 2004; Watson et al., 2004).

Morphological changes, growth and proliferation were observed every 2-3 days under inverted microscope (Nikon Corporation, Tokio, Japan). Keratinocytes were seeded and expanded to obtain a sufficient number of cells for the following experiments, and cells were stored at -80 °C until use, when necessary.

## Binding assays

TRPV1 radioligand binding assays were performed in canine keratinocytes in triplicate, using four pools of membranes containing approximately 180 x 10<sup>6</sup> cells, from which ten Scatchard's analyses (n=10) were carried out.

Binding assays for identification of the TRPV1 receptor in canine keratinocyte membranes were performed according to the methods described by Ross et al. (2001), and Puntambekar et al. (2004), but introducing some minor modifications. Briefly, thawed cells, were suspended in binding buffer (KCl 5 mM, NaCl 5,8 mM, CaCl<sub>2</sub> 0,75 mM, MgCl<sub>2</sub> 2 mM, sucrose 320 mM and HEPES 10 mM; pH 7.4), disrupted using a sonicator (XL 2020, Misonix Inc, NY, USA) and centrifuged at 3000 g for 10 min at 4 °C. The resulting supernatants were ultra-centrifuged at 105 000 g for 45 min at 4 °C, in order to separate cytosolic fraction from cell membrane fraction. The final pellets were suspended in the same buffer, and protein concentration was measured according to the method described by Lowry et al. (1951). Keratinocyte membrane suspensions were diluted to a final concentration of 1 mg protein/mL. Aliquots of membrane suspension (100 µL) were incubated for 1 h at 37 °C in a shaking water bath with increasing concentrations (0.025-3.2 nM) of labelled resiniferatoxin ([<sup>3</sup>H]RTX, 43 Ci/mmol, PerkinElmer, Boston, USA), a high affinity selective

TRPV1 agonist (Szallasi et al., 1999), in absence (total binding) and in presence (non-specific binding) of 1  $\mu$ M unlabelled RTX (Vercelli et al., 2015a; Barbero et al., 2006) to a final volume of incubation of 300  $\mu$ L. The binding reaction was stopped by chilling incubation tubes in an ice-cold bath for 20 minutes. The incubation mixture was immediately filtered under vacuum using pre-soaked glass fibre filters (Whatman GF/B, Whatman International, Maidstone, UK). Filters were then carefully washed with  $3 \times 3$  mL of buffered saline (NaCl 154 mM, Tris-HCl 50 mM, pH 7.4) and solubilized with 4 mL of scintillation fluid (Filter Count, Canberra Packard, Meridien, CT, USA). The radioactivity retained on wet filters was measured for 2 minutes using a Tri-Carb 1600 TR scintillator spectrometer (Canberra Packard, Meridien, CT, USA), with an efficiency of 60%.

The maximum number of binding sites ( $B_{max}$ ) and the equilibrium dissociation constant ( $K_d$ ) were estimated by use the Scatchard (Scatchard, 1949) analysis using a computer program (GraphPad Prism Software, San Diego, CA, USA).

### Displacement assays

For competition experiments, aliquots of membrane suspension (100  $\mu$ L), diluted to a final concentration of 1 mg protein/mL, and containing 1.2 nM [ $^3$ H] RTX (minimal concentration that saturates the receptor system), were incubated and processed as previously described for binding assays, with increasing concentrations (10<sup>-11</sup>M–10<sup>-3</sup>M) of three TRPV1 unlabelled agonists (capsaicin, anandamide and RTX) and three TRPV1 unlabelled antagonists (capsazepine, 5'-iodoresiniferatoxin - 5-I- RTX - and SB-366791) (Barbero et al., 2006). Unlabelled receptor ligand concentrations that inhibited 50% (IC<sub>50</sub>) of [ $^3$ H]-RTX specific binding were determined from the competition curves obtained by nonlinear regression analysis of the data (GraphPad Prism Software, San Diego, CA, USA). The fitting for one-site model was tested by R<sup>2</sup> from nonlinear regression and by the run test. The affinity constants ( $K_i$ ) for the competitors were calculated using the Cheng–Prusoff equation (Cheng and Prusoff, 1973):

where C is the concentration of radioligand used in the assay, and  $K_d$  the dissociation constant of the radioligand as obtained from the Scatchard plots (Scatchard, 1949).

### Functional studies

The functionality of TRPV1 was assessed by measuring  $^{45}\text{Ca}^{2+}$  cell uptake. The potencies of the different vanilloid analogues to stimulate the uptake of  $\text{Ca}^{2+}$  and the capacity for antagonists to inhibit  $\text{Ca}^{2+}$  influx, were measured in the presence of 1  $\mu$ Ci  $^{45}\text{Ca}^{2+}$ /mL.

Briefly, for  $^{45}\text{Ca}^{2+}$  uptake assay, 250  $\mu$ L of cell suspension ( $5 \times 10^3$ /100  $\mu$ L) were incubated for 20 min at 37 °C in a shaking water bath in the presence of 1  $\mu$ Ci  $^{45}\text{Ca}^{2+}$ /mL ( $^{45}\text{Ca}$  - PerkinElmer, Boston USA; batch of 27.63 mCi/mL) in two parallel set of tubes with increasing concentrations (10<sup>-11</sup> - 10<sup>-3</sup> M) of the selective ligands: capsaicin, anandamide and RTX, as receptor agonists, and 5-I-RTX and capsazepine, as receptor antagonists (Acs et al., 1996; Acs et al., 1997; Biro et al., 1998). The antagonists have been tested in the presence of RTX 2 nM for the measurement of the percentage of inhibition of  $^{45}\text{Ca}^{2+}$  uptake (IC<sub>50</sub>). Immediately after the incubation, extracellular  $^{45}\text{Ca}^{2+}$  was removed by washing cells three times with ice-cold PBS (NaCl 140 mM, KCl 27 mM, KH<sub>2</sub>PO<sub>4</sub> 15 mM, Na<sub>2</sub>HPO<sub>4</sub> 90 mM, pH 7.4), then, PBS cell suspensions were transferred into

vials containing 3 mL of scintillation fluid (Ultima Gold<sup>TM</sup>, Canberra Packard; Meridien, CT, USA) and radioactivity (counts per minute – cpm - /well) was measured by a Tri-Carb 1600 TR Canberra Packard scintillator spectrometer, with an efficiency of 60%. For each data point, four wells were assayed.

## RESULTS

### Radioligand binding assay

The results obtained by radiobinding assay are shown in Table 1, and data are expressed as mean values  $\pm$  SEM. A linear fit to the non-specific binding was used to calculate the non-specific component of the total binding at each free [3H]-RTX concentration, and the remaining part was defined as specific binding. The specific binding was fitted to a three-parameters function in the formula:

$$\text{bound} = B_{\text{max}} / (1 + (K_d/[L])^n)$$

where [L] is the free [3H]-RTX concentration,  $B_{\text{max}}$  is the maximum specific binding,  $K_d$  is the dissociation constant, and  $n$  is the Hill coefficient (Barbero et al., 2006).

Scatchard plots were linear, having a  $r$  value  $>0.9$ , and the receptor system was saturated by the increasing concentrations of [3H]-RTX used in the experiment. Scatchard analysis demonstrated the presence of measurable and saturable concentrations ( $B_{\text{max}} = 1240 \pm 120$  fmol/mg protein;  $K_d = 0.01 \pm 0.004$  nM) of a single class of specific binding sites for [3H]-RTX.

### Displacement studies

Figure 2 shows the results obtained by competition studies on canine keratinocyte membranes using three selective agonists (capsaicin, anandamide, and RTX) and by three selective antagonists (capsazepine, 5-I-RTX and SB-366791).

The tables reported in the insets of Fig. 2 show the affinity values ( $K_i$ ) for the different selective TRPV1 agonists and antagonists derived from competition assays. All the experiments were carried out in the presence of saturating concentration of [3H]-RTX (1.2 nM) performed using canine keratinocyte membranes and performed 8 times ( $n=8$ ). Among the agonists, RTX was the strongest analogue, having a  $K_i$  value of  $8.7 \times 10^{-10}$  M, while capsaicin and anandamine had  $4.5 \times 10^{-10}$  and  $1.5 \times 10^{-10}$  M, respectively. Considering antagonists, 5-I-RTX had the highest  $K_i$  value ( $7.3 \times 10^{-10}$  M), while capsazepine and SB-366791  $K_i$  values resulted  $1.4 \times 10^{-8}$  and  $1.09 \times 10^{-8}$  M, respectively. It was also possible to calculate the maximum effect ( $E_{\text{max}}$ ) corresponding to the maximal response induced by the system: among agonists, capsaicin was able to induce an effect of 100.90% when bound to the receptor, while among antagonists, SB-366791 had an  $E_{\text{max}}$  value of 94.90%. All the  $K_i$  and  $E_{\text{max}}$  values are summarized in the tables in the inset of Figure 2.

### Functional studies

The results obtained by the functional evaluation of TRPV1 in canine keratinocytes are indicated by the potencies of the different vanilloid analogues to stimulate the uptake of  $\text{Ca}^{2+}$  and the ability of the antagonists to inhibit  $\text{Ca}^{2+}$  influx determined in the presence of  $1\ \mu\text{Ci/mL}\ 45\text{Ca}^{2+}$ . Figure 3 shows the curves representing  $\text{Ca}^{2+}$  uptake induced in by the increasing concentrations of the three selective agonists (capsaicin, anandamide and RTX), and the corresponding table shows the 50% Efficacy Concentrations (EC50) of each compound. Similarly it was represented the inhibition of  $\text{Ca}^{2+}$  influx produced in canine keratinocytes by the increasing concentrations of the selective antagonists (5-I-RTX and capsazepine). Among agonists, RTX was the most efficient compound, having an EC50 value of  $3.09 \times 10^{-8}\text{M}$ , while among antagonists, the highest efficiency was demonstrated by 5-I-RTX, having a IC50 value of  $4.2 \times 10^{-9}\text{M}$ . The maximum effect (Emax) was induced by capsaicin and capsazepine, among agonists and antagonists respectively. All the EC50/IC50 and Emax values of the functional studies are summarized in the tables in the inset of Figure 3.

## DISCUSSION

In authors opinion, TRPV1 receptor is one of the most investigated targets of the last two decades, considering the huge amount of papers published on this topic.. It is known to be involved in nociception, pruritus (Paus et al., 2014; Shim et al., 2007), and skin inflammation in humans (Southall et al., 2003; Ständer et al., 2004).

Functional TRPV1 receptors have been identified in human epidermal keratinocytes (Inoue et al., 2002; Southall et al., 2003), fibroblasts (Kim et al., 2006), and skin mast cells (Ständer et al., 2004). Concerning the canine species, Phelps and co-workers (2005) cloned and functionally characterized TRPV1 in dog's neuronal cells, and Vercelli et al. (2015a) demonstrated the expression of TRPV1 in canine mammary cancer cells, no other cell being investigated so far in dogs.

The aim of the present study was to identify and characterize TRPV1 receptor in canine primary keratinocytes, that are currently considered one of the main cell populations involved in skin allergies and atopic dermatitis both in humans and dogs (Albanesi, 2010; Asahina and Maeda, 2017; Santoro et al., 2015).

The specific binding assays used in the present study provide the first unequivocal evidence for the existence of TRPV1 receptor in membranes derived from cultured canine primary keratinocytes. [3H]-RTX binding affinity for TRPV1 receptor (average Kd) in canine primary keratinocytes (0.01 nM) was similar to those previously reported in rat sensory neurons (0.04 nM) (Szallasi and Blumberg, 1999) and rat transfected cell line (0.034 nM) and sensibly higher compared to human transfected cell line (0.44 nM) (Chou et al., 2004) and organs, such as airways (0.25 nM, rat; 2 nM, human; and 7nM, guinea pig) and colon (3.0 nM) (Preti et al., 2012; Szallasi and Blumberg, 1999).

The receptor characterization was completed by displacement studies performed using [3H]-RTX in presence of selective agonists and antagonists. According to the affinity values (Ki), the relative affinity of different compounds to TRPV1 receptor in membranes of canine keratinocytes was assessed. On the basis of the Ki values, the range of TRPV1 affinity for agonists was RTX >> capsaicin >> anandamide. Among antagonists, 5-I-RTX demonstrated the highest affinity to the receptor, i.e., more than capsazepine, and SB-366791.

The affinity of capsaicin and capsazepine to TRPV1 expressed by canine keratinocyte membranes was much higher compared to finding by Acs and colleagues (1996) in rat neuronal membranes,

i.e.,  $K_i = 4.93 \times 10^{-6} \text{ M}$  and  $3.89 \times 10^{-6} \text{ M}$  for capsaicin and capsazepine respectively. The functionality of TRPV1 expressed in canine keratinocytes in term of stimulation of  $45\text{Ca}^{2+}$  uptake indicated the following order of potency for the tested agonists: RTX >> capsaicin >> anandamide. Based on  $\text{IC}_{50}$  values, 5-I-RTX was considered more potent than capsazepine to inhibit  $45\text{Ca}^{2+}$ .

Displacement and functional data of TRPV1 agonists and antagonists are both scarce and difficult to compare, mainly because of differences in assay employed (e.g., calcium uptake, patch-clamp electrophysiology) and species-related heterogeneity in sensitivity to TRPV1 activation. Nonetheless, some comparison might be attempted. In the present study, RTX showed an agonistic activity ( $\text{EC}_{50}$  of  $3.09 \times 10^{-8} \text{ M}$ ) weaker than those previously reported in human TRPV1-transfected cell line (Appendino et al., 2007), rat neuronal membranes (Acs et al., 1996), mouse TRPV1-transfected cell line (Correll et al., 2004), and dog TRPV1-transfected cell line (Phelps et al., 2005) i.e.,  $\text{EC}_{50}$  values of  $1.9 \times 10^{-11} \text{ M}$ ,  $9.4 \times 10^{-10} \text{ M}$ ,  $1.5 \times 10^{-10} \text{ M}$ , and  $2.27 \times 10^{-9} \text{ M}$ , respectively. On the contrary, the agonistic effect of capsaicin in canine keratinocytes ( $\text{EC}_{50} = 9.4 \times 10^{-8} \text{ M}$ ) was stronger than rat neuronal membranes ( $\text{EC}_{50} = 2.7 \times 10^{-7} \text{ M}$ ) (Acs et al., 1996) and similar to human embryonic kidney cells (Appendino et al., 2007), dog TRPV1-transfected cell line (Phelps et al., 2005) and rat vagal afferent neurons (Fenwick et al., 2017), the  $\text{EC}_{50}$  values ranging from  $4.0 \times 10^{-8} \text{ M}$  to  $4.6 \times 10^{-8} \text{ M}$ . Similarly, anandamide showed stronger agonistic activity in canine keratinocytes compared to rat neuronal cultures (Fenwick et al., 2017), i.e.,  $\text{EC}_{50}$  of  $1.1 \times 10^{-7}$  vs  $1.95 \times 10^{-6}$ .

As far as antagonism concerns, the highest efficacy shown by 5-I-RTX in the present study agrees with previously published data, all reporting 5-I-RTX to be the most potent inhibitor (Appendino et al., 2010; Appendino et al., 2007; Phelps et al., 2005; Correll et al., 2004). An intriguing hypothesis is that the introduction of the iodine atom might not only increase the binding, but also induce better membrane penetration and thus higher potency given the intracellular localization of the vanilloid binding site on TRPV1 (Appendino et al., 2010). The comparison of  $\text{IC}_{50}$  value found in the present study for I-5-RTX points to a lower efficacy on canine keratinocyte TRPV1 ( $4.2 \times 10^{-9} \text{ M}$ ) as compared to dog, mouse and rat TRPV1-transfected cell lines ( $\text{IC}_{50} = 2.64 \times 10^{-10} \text{ M}$ ,  $3.5 \times 10^{-10} \text{ M}$ ,  $1.5 \times 10^{-10}$ , respectively) (Phelps et al., 2005; Correll et al., 2004) but in the same order of magnitude to what shown in human TRPV1 transfected cell line, i.e.,  $\text{IC}_{50} = 1.06 \times 10^{-9} \text{ M}$  (Correll et al., 2004).

Inhibition by capsazepine shown in the present study ( $\text{IC}_{50} = 2.06 \times 10^{-8} \text{ M}$ ) resulted to be superior to those found in mouse, rat, human TRPV1 ( $\text{IC}_{50} = 1.43 \times 10^{-6} \text{ M}$ ,  $1.18 \times 10^{-6} \text{ M}$ ,  $5.35 \times 10^{-7} \text{ M}$ , respectively) (Correll et al., 2004) and even dog TRPV1-transfected cell line ( $\text{IC}_{50} = 4.84 \times 10^{-7} \text{ M}$ ) (Phelps et al., 2005).

Given all this, the species-related heterogeneity in TRPV1 response to different ligands should always be kept in mind. Although the high grade of homology between human and rat (85.7%), guinea pig (84.4%) rabbit (87.6%), and canine (89.1%) TRPV1s (Phelps et al., 2005), the pharmacologic modulation of TRPV1 among species is thus fairly heterogeneous. Further to what discussed above, also different sensitivity to phorbol 12-phenylacetate 13- acetate 20-homovanillate has been shown between human, guinea pig and rat TRPV1s (Vercelli et al., 2015b). In this light, the results obtained in human keratinocytes should not be simply translated into canine species, and competitive/non-competitive TRPV1 agonists and antagonists continue to be investigated in a species-specific way with the final aim to obtain a targeted therapy (Garcia-Martinez et al., 2002; Pomonis et al., 2003; Gunthorpe et al., 2009; Palazzo et al., 2012).. The complex functional roles of TRPV1 signaling in human skin in physiological and pathological conditions has been studied (Lee



et al., 2015; Radtke et al., 2011), i.e. in pruritus (Paus et al., 2006; Shim et al., 2007), and skin inflammation (Southall et al., 2003; Ständer et al., 2004), but needs further analysis and clarification to hypothesize new therapeutic strategies. One straightforward possibility is that the activation of TRPV1, functioning as a calcium-permeable channel (Szallasi et al., 1999), leads to an increase in intracellular  $\text{Ca}^{2+}$  concentration and hence may initiate calcium-mediated processes (Vercelli et al., 2015a). Such calcium-coupled mechanisms have been described for human mast cells and epidermal keratinocytes (pro-inflammatory mediator release), urinary epithelial cells (nitric oxide release), and glial cells (proliferation, differentiation, apoptosis) (Birder et al., 2001; Southall et al., 2003; Biro et al., 1998). In addition, since most skin cell functions are strongly affected by  $\text{Ca}^{2+}$  concentrations, TRPV1 may possess a pivotal role, for example, in regulating keratinocyte differentiation and proliferation, as demonstrated for other TRP receptors (Santoni et al., 2011). TRPV1 is expressed not only on epidermal keratinocytes of normal skin and hair follicle (Bodó et al., 2005), but also by on neuroectodermal and mesenchymal cell types such as Langerhans cells, sebocytes, sweat gland epithelium, endothelial and smooth muscle cells of skin blood vessels, and mast cells (Ständer et al., 2004; Nilius, 2007). This widespread pattern of TRPV1 protein expression suggests multiple, previously unappreciated, additional functions for TRPV1-mediated signaling, well beyond nociception (Ambrosino et al., 2013).

Importantly, cannabinoid receptors 1 and 2 are also expressed in the canine skin (Esposito et al., 2013; Abramo et al., 2014), and as previously cited, TRPV1 is now often referred to as a component of the endocannabinoid system (De Petrocellis et al., 2010), due to the fact that certain endocannabinoids (i.e., anandamide) and/ or endocannabinoid-like compounds (i.e., palmitoylethanolamide) are able to directly or indirectly interact with TRPV1 receptor and may thus modulate pruritus, inflammation and pain perceptions (Re et al., 2007). In particular, the endocannabinoid-like compound palmitoylethanolamide (an endogenous fatty acid amide) was shown to (i) potentiate  $\text{Ca}^{2+}$  responses triggered by anandamide in TRPV1-transfected cells (De Petrocellis et al., 2005), (ii) activate and desensitize TRPV1 channels in peripheral sensory neurons (Ambrosino et al., 2013), and (iii) potentiate endocannabinoid actions at TRPV1 receptor in keratinocyte cell line (Petrosino et al., 2016). Moreover, several *in vivo* studies provide evidence supporting TRPV1 to be one of the molecular target for the anti-inflammatory and pain relieving effects of palmitoylethanolamide (Costa et al., 2008; Ho et al., 2008; Petrosino et al., 2010; Starowicz et al., 2013; Capasso et al., 2014). Taking into consideration all the mentioned factors, and considering that canine keratinocytes express functional TRPV1, new therapeutic strategies based on TRPV1 modulation might be investigated in veterinary dermatology.

In human medicine, classical vanilloids, i.e., capsaicin, are nowadays used in creams, patch and subcutaneous injection to induce a local desensitization of cutaneous sensory nerves and control neuropathic pain (Vercelli et al., 2015b; Bley, 2004). The common side effects of these products (e.g. burning, itching, dryness, pain, redness, swelling, or soreness at the application site), prompted new efforts to develop safer and more efficacious approaches targeting TRPV1 for pain, inflammation and pruritus relief. In this light, competitive and non-competitive TRPV1 antagonists have been developed and continued to be currently under investigation (Bley, 2004; Garcia-Martinez et al., 2002; Pomonis et al., 2003; Gunthorpe et al., 2009; Palazzo et al., 2012).

On the other hand, compound like palmitoylethanolamide is currently used in veterinary dermatology and seems to offer three major advantages. First, it is an endogenous compound, being identified in almost all mammalian tissues (Palazzo et al., 2012; Esposito et al., 2013), included canine skin (Abramo et al., 2014; Scarpampella et al., 2001). Second, it modulates an array of promiscuous molecular targets (e.g., cannabinoid receptors 1 and 2, peroxisome proliferator-

activated receptors, G protein-coupled receptor, and TRPV1 of course), all being involved, although differently, in the patho-physiological pathways of inflammation, pruritus and pain in human and canine species (Re et al., 2007; Petrosino et al., 2016). Lastly, it is a safe and well tolerated compound (Nestmann, 2016; Noli et al., 2015)

In conclusion, the present study identified and functionally characterized for the first time TRPV1 in canine keratinocytes. Taking into account the role played by this cation channel in skin pathophysiology, keratinocyte TRPV1 might be a novel therapeutic target for treatment of pruritus, and skin inflammation.

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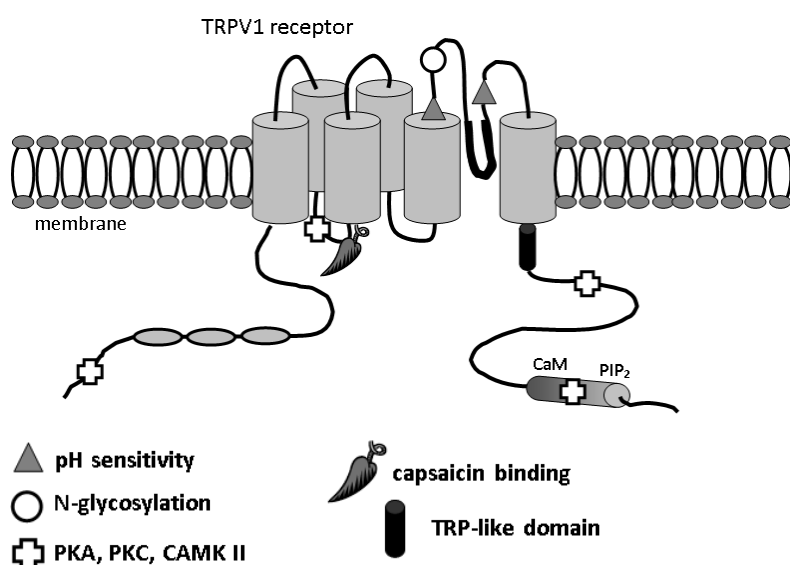
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**FIGURE 1** Molecular architecture of the vanilloid receptor. TRPV channel subunits have a common topology of six transmembrane segments with a pore region between fifth and sixth segment, and cytoplasmic N and C termini. These subunits assemble as tetramers around a central aqueous pore, producing nonselective cation channels. TRPV channels contain three to four ankyrin domains in the N terminus that are thought to interact with cytosolic proteins (protein kinase A, C, calcium/calmodulin-dependent protein kinase II). Cytosolic C-terminal domain-carrying calmodulin (CaM) and phosphatidylinositol-4,5- bispophosphate (PIP<sub>2</sub>) binding sites.



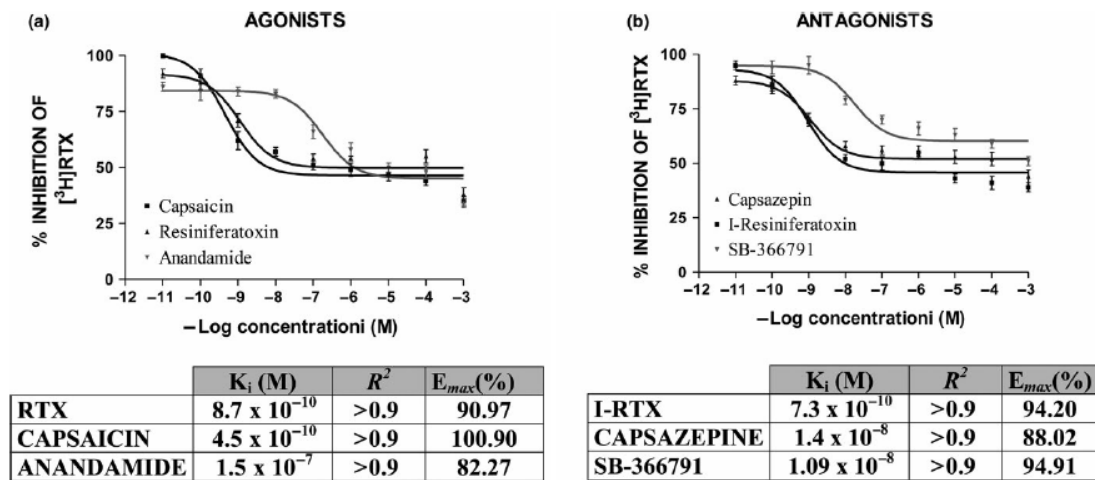


FIGURE 2 Displacement studies of TRPV1 in canine keratinocyte membranes. The displacement studies ( $n = 8$ ) were performed in the presence of saturating concentrations of  $[^3H]$ -RTX (1.2 nM), using increasing concentrations ( $10^{-11}$ – $10^{-3}$  M) of (a) three selective agonists: anandamide, capsaicin and resiniferatoxin, and (b) three selective antagonists: capsazepine, 5-I- RTX and SB-366791. The error bars indicate SEM. The inset tables show the affinities ( $K_i$ ) for the different selective TRPV1 agonists (a) and antagonists (b).

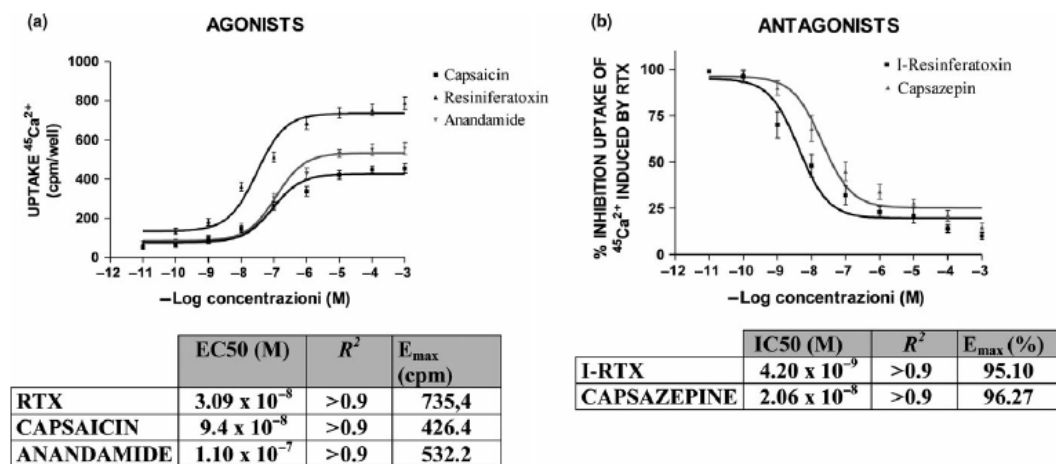


FIGURE 3 Stimulation of  $^{45}Ca$  uptake induced in canine keratinocyte membranes. The functionality studies were performed using canine keratinocytes membranes and increasing concentrations ( $10^{-11}$ – $10^{-3}$  M) of three selective agonists: anandamide, RTX and capsaicin, and two selective TRPV1 antagonists: 5-I-RTX and capsazepine. For each data point in each experiment, four wells were assayed ( $n = 4$ ). The error bars indicate SEM. The tables show the efficacy concentration of 50% ( $EC_{50}$ ) of the used vanilloid agonists on TRPV1 in canine keratinocyte membranes and the inhibitory concentration 50 ( $IC_{50}$ ) of  $Ca^{2+}$  influx of 5-I- RTX and capsazepine.