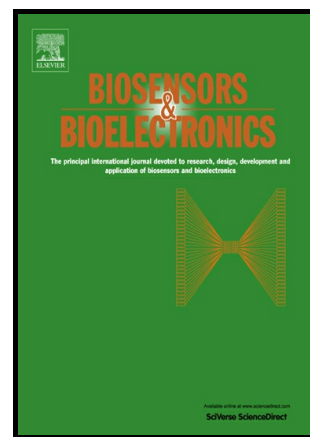


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www.elsevier.com/locate/bios

PII: S0956-5663(18)30943-6
DOI: <https://doi.org/10.1016/j.bios.2018.11.046>
Reference: BIOS10957

To appear in: *Biosensors and Bioelectronics*

Received date: 24 September 2018

Revised date: 9 November 2018

Accepted date: 22 November 2018

Cite this article as: Sa Xiao, Yanqing Zhang, Panpan Song, Junbo Xie and Guangchang Pang, The investigation of allosteric regulation mechanism of analgesic effect using SD rat taste bud tissue biosensor, *Biosensors and Bioelectronics*, <https://doi.org/10.1016/j.bios.2018.11.046>

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**The investigation of allosteric regulation mechanism of analgesic effect using SD
rat taste bud tissue biosensor**

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Abstract

In this study, a taste bud tissue biosensor was prepared by a starch-sodium alginate cross-linking fixation method. Capsaicin was used as a TRPV1 noxious ion channel activator to investigate the antagonism kinetics of six different substances on capsaicin. The results showed that capsazepine, AMG517, loureirin B, and tetrahydropalmatine were all competitive allosteric regulatory ligands for capsaicin, while aconitine and anandamide were mixed allosteric regulatory ligand that combines non-competition and competition effect. Through analyzing the kinetic parameters of capsaicin and its competitive allosteric regulatory ligands, and comparing the structures between spicy substances and endocannabinoids, the importance of amide groups and similar groups in the allosteric regulation of cannabinoids (CB) receptors and analgesic mechanism was elucidated. This indicates that vanilloid activators turn on the TRPV1 ion channel to transmit only pain and other nociceptive signals, while capsaicin and its competitive ligands are capable of activating intracellular G protein/PI3K/PIP2 signaling pathways by binding to endogenous cannabinoid receptors, and then increase intracellular PIP2 levels (the increasing PIP2 can competitively replace capsaicin and other vanilloid activators), thereby closing the TRPV1 channel and exerting the analgesic effect. The elucidation

of this mechanism of pain and analgesia will lay the theoretical foundation and new ideas for investigating nociceptive signal and screening potential analgesic drugs.

Key words: Capsaicin; TRPV1 channel; CB receptor; competitive ligand; anandamide; analgesic

1. Introduction

The transient receptor potential vanilloid 1 (TRPV1) acts as a noxious stimulus molecular integrator that can be activated by a variety of factors (Conway, 2008; Mennella, 2014). TRPV1 not only participates in the development of spicy taste, but also plays an important role in the development of pain perception and signal transmission. The opening of TRPV1 channel in nociceptors produces Ca^{2+} influx, which results in depolarization of the cells and produces nerve impulses. The nerve impulses are transmitted through the afferent nerves to the pain center of the brain to produce a pain sensation (Caterina et al., 1997). Blocking the TRPV1 channel can inhibit the generation of nerve impulses, thereby avoiding the hyperalgesia and sensitization (Szallasi et al., 2007; Khairatkar-Joshi and Szallasi, 2009). Therefore, in recent years, specific blocking of TRPV1 channels has become an important way for the treatment of pain (Moran et al., 2011). At present, a number of compounds have been found that are capable of blocking Ca^{2+} influx mediated by capsaicin (Cap)-induced TRPV1 channel through high throughput screening (HTS). However, the hyperthermia side effects of these compounds have limited the development of analgesic drugs, eventually leading many pharmaceutical companies to leave the field of new analgesic drug development and switch to drug repurposing screens

(Sisignano et al., 2016). The fundamental reason why it fail to obtain effective results in the screening of analgesic drugs is that the mechanism of activation and antagonism of TRPV1 channels is not yet clear.

Although it is now considered that a large number of ion channels are regulated by phosphatidylinositol, particularly phosphatidylinositol-4,5-bisphosphate (PIP₂), the specific mechanism is not clear yet (Hansen et al., 2011). Multiple studies have shown that PIP₂ interacts directly with the proximal C-terminal region of TRPV1 and may play a key role in the regulation of the TRPV1 channel (Prescott and Julius, 2003; Ufret-Vincenty et al., 2011). With the electron cryomicroscopy and lipid nanodisc technology, Gao et al. demonstrated phosphatidylinositide functions as an endogenous, tightly bound co-factor that stabilize TRPV1 in its resting state by serving as competitive vanilloid antagonists and negative allosteric modulators. At the same time, phosphatidylinositide may function as a positive, obligatory co-factor whose binding to TRPV1 in the closed state primes the channel for subsequent activation by vanilloids or other stimuli (Gao et al., 2016). These findings reveal the mechanism of TRPV1 activation : Capsaicin and other vanilloid ligands can compete with phosphatidylinositol for binding to the same site in the proximal C-terminal region of TRPV1, promoting a conformational change in TRPV1, and subsequently opening the TRPV1 channel, leading to the influx of Ca²⁺ causes the cells to depolarize, thereby transmitting nociceptive signals.

Capsaicin, as the earliest discovered TRPV1 channel activator, causes hyperalgesia at low doses. However, it can inhibit pain when it reaches a certain dose (O'Neill et al.,

2012). It is logically assumed that if capsaicin only binds to TRPV1 and exerts a pungent effect, then a high dose of capsaicin should trigger the opening of a continuous TRPV1 channel, making hyperalgesia sensitization. At least, it will not close this ion channel, and impossibly play analgesic effects. Therefore, the dose-dependent bidirectional regulation of capsaicin suggests that there may be other pathways for regulating or shutting down the TRPV1 channel. At the same time, in the tissues or organs with TRPV1 distribution, the spicy substances should have similar laws of action. However, in addition to being able to produce a spicy sensation in the taste bud tissue, capsaicin also stimulates the anus, and allicin stimulates the stomach (Browning et al., 2013; Macpherson et al., 2005). This shows that different spicy substances also have differences of the action sites in the human body. Although it can be explained by the existence of different TRPV1 subtypes in different tissues, it remains to be confirmed whether these TRPV1 subtypes have different responses to different spicy substances. In addition, PIP2, which plays an important role in the regulating TRPV1 channel, acts as an endogenous regulatory lipid that is regulated by phospholipase C, and participates in multiple intracellular signaling pathways (Ufret-Vincenty et al., 2015; Delage et al., 2013). The above phenomenon suggests that in addition to the pungent effect induced by capsaicin-TRPV1 direct binding, capsaicin and analgesic compounds may also indirectly regulate TRPV1 channel switching through other receptors as well as intracellular signaling pathways.

In taste mucosal epithelial tissue detached from the taste buds, taste receptor cells (TRCs) can still retain their integrity and normal function, and can be effectively

activated by taste substances (El-Ali et al., 2006). In this study, we used taste bud tissue of SD rats expressing TRPV1 as a sensing component to prepare a taste bud tissue sensor. Using capsaicin as an activator, we studied the antagonistic action of capsazepine (Walpole et al., 1994), AMG517 (Doherty et al., 2007), anandamide and three known analgesic compounds (Wei et al., 2013; Ameri, 1998; Guo et al., 2014) on the activated TRPV1 channel.

2. Materials and Methods

2.1. Materials, Equipment and Pretreatment of glassy carbon electrode

The list of reagents, materials and equipment used in this work and pretreatment of glassy carbon electrode are provided in the Supplementary material.

2.2. Preparation of taste bud tissue sensor

After the SD rats were sacrificed by decapitation, the tongues (from the tip to foliate papillae) were removed. According to the reported method, the rat taste bud tissue was collected (Qiao et al., 2015). Using the sodium alginate-starch gel as a fixing agent, taste-bud tissues of SD rats were fixed between two nuclear microporous membranes. The prepared sandwich-type membrane was immersed into 5.0% CaCl_2 solution for 10s, thereby causing the gelatinization of sodium alginate solution to form the sensing membrane (Qiao et al., 2015). It was then fixed on the pretreated glassy carbon electrode so that the taste buds were coincident with the electrode core. The prepared biosensor is capable of maintaining the activity of taste bud cells and providing the desired microenvironment for ligand-receptor binding (El-Ali et al.,

2006). By replacing the afferent nerves with the glassy carbon electrode, the action potential generated by the stimulated taste bud cells can be transmitted as electrical signals to the signal acquisition system. The collected electrical signals mimic the neural signals transmitted to the rat central nervous system after stimulation induced by capsaicin and analgesic compounds. The schematic diagram of the working principle of the prepared biosensor is drawn (**Fig. 1**).

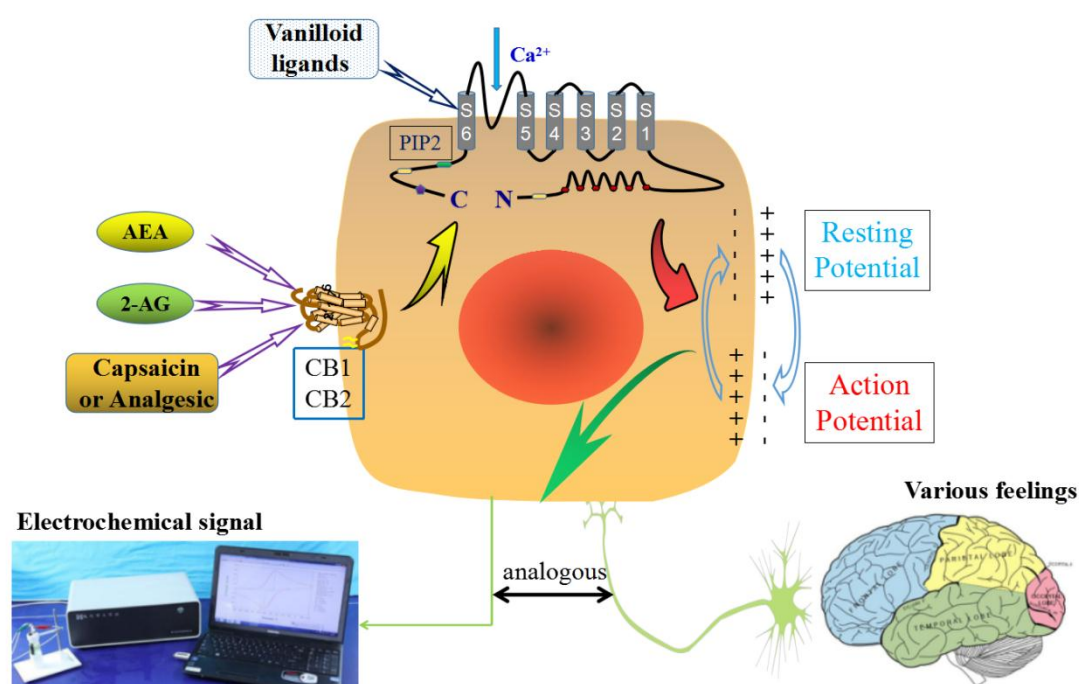


Fig. 1. Schematic diagram of the working principle of the taste bud tissue biosensor.

2.3. Electrochemical measurement of capsaicin and analgesic compounds

A three-electrode system was used for the measurement: a glassy carbon electrode fixed with a taste-measuring membrane as a working electrode, an Ag/AgCl electrode as a reference electrode, and a platinum-wire electrode as a counter electrode.

Ultrapure water is used as the test base fluid. At a voltage of 0.4V, the response currents of capsaicin and analgesic compounds are measured by the amperometric i-t curve method. As shown in the inset in **Fig. S1**. The steady response current at 90 seconds was taken as the measured value. The response current change rate is used as a detection index and the calculation formula is shown in Equation (1).

$$\Delta I / \% = \frac{I_1 - I_2}{I_1} \times 100 \quad (1)$$

Among them, I_1 and I_2 refer to the steady state current values at the same time point before and after the determination, respectively.

It has been demonstrated that the interaction between taste substances and its receptors is hyperbolic (similar to the kinetics of enzymes and substrates), and has substrate saturation effects (Wei et al., 2017). Therefore, in this study, the linear concentration range was first determined by measuring different concentrations of capsaicin solution, and the kinetic curve of capsaicin action was determined by subdividing the linear concentration range. Then, the capsaicin activation constant, K_a value, was calculated (Wei et al., 2017). The concentration of each analgesic compound used (capsazepine, AMG 517, loureirin B, aconitine, anandamide and tetrahydropalmatine) was determined based on the K_a value of capsaicin. The capsaicin-analgesic compound mixed solution was prepared by adding the same content of analgesic compound in different concentrations of capsaicin solution. Finally, the response current of the mixed solution was measured under the same conditions. The antagonistic effects of analgesic compound was analyzed by comparing the changes in kinetic parameters (K_a and V_{max}) before and after the

analgesic compound was added.

2.4. Hyperthermia effects of analgesic compounds

The analgesic drugs screened by capsaicin inhibition often cause adverse reactions, in which the elevated body temperature was the most common symptom. As a result, the effect on body temperature is the key index for developing safe analgesics through TRPV1 channels (Gavva, 2008). A typical example is: TRPV1 pyrimidine antagonist AMG517 was forced to be suspended in clinical trials due to significant and prolonged hyperthermia in sensitive populations (Doherty et al., 2007). Therefore, this study further tested whether these substances could cause the increase of body temperature in experimental animals with AMG517 as a positive control drug. The half effective concentration of analgesic effect of AMG517 (200 µg/kg) (Doherty et al., 2007) and other analgesic compounds (capsazepine 188.45 µg/kg (Walpole et al., 1994), loureirin B 41.3 µg/kg (Wei et al., 2013), tetrahydropalmatine 2 mg/kg (Ameri, 1998), and aconitine 28.45 µg/kg (Guo et al., 2014)) were injected into SD rats by tail vein injection, respectively. The rectal temperature of the rat was measured at 0, 30, 60, 90, 120, 150, 180 min after the injection. During the experiment, 25 SD rats (200g $\pm$ 20g) were randomly divided into 5 groups (n=5), and the body temperature difference of the rats in each group does not exceed 1°C. The rats were fasted for 2 hours before the experiment, and the temperature in the laboratory was kept at 25 °C~28 °C. The increase in body temperature exceeding 0.6 °C means that analgesic compounds have hyperthermia side-effect in rats (Watabiki et al., 2011).

2.5. Data analysis

The sensor data were analyzed and processed using Origin 8.0 software (OriginLab Northampton, MA). The experimental detection limit (LOD) was defined as the target concentration giving a response current signal at least three times higher than the one from the standard deviation of the blank control signal.

3. Results

3.1. Electrochemical Characterization of Pretreatment Electrodes

After being activated by 1mol/L H_2SO_4 , the glassy carbon electrode can generate negatively charged carboxyl, hydroxyl and other oxygen-containing groups on the surface (Ye et al., 2014). At the same time, a porous structure is formed on the surface of electrode, increasing its effective surface area (Thiagarajan et al., 2009). The cyclic voltammogram has a peak potential difference of less than 80 mV (**Fig. 2A**), indicating that the surface of the electrode is clean and the reproducibility of the electrode is good after the pretreatment. The AC impedance spectrum ($10^{-2}\sim 10^6$ Hz) before and after electrode treatment was measured (**Fig. 2B**). Before the pretreatment, the curve consists of a semicircle in the high frequency region and a straight line in the low region, indicating that the electrode process is jointly controlled by both charge transfer and diffusion processes; after pretreatment, the curve approximates to a straight line, indicating that electron transfer to the electrode surface is only controlled by diffusion process (Gurudatt et al., 2016). Cyclic voltammetric measurements of pretreated electrodes were carried out at different scanning rates (1→8 in order of 25, 50, 75, 100, 125, 150, 200, 250 mV/s) (**Fig. 2C**). The results

show that the redox peak current of the pretreated electrode has a good linear relationship with the square root of the scan rate (**Fig. 2D**). This means that the glassy carbon electrode redox peak current is only controlled by diffusion process (Sheppard et al., 2017). It can be seen that the pretreatment effect of glassy carbon electrode in this study is good and can be used for subsequent studies.

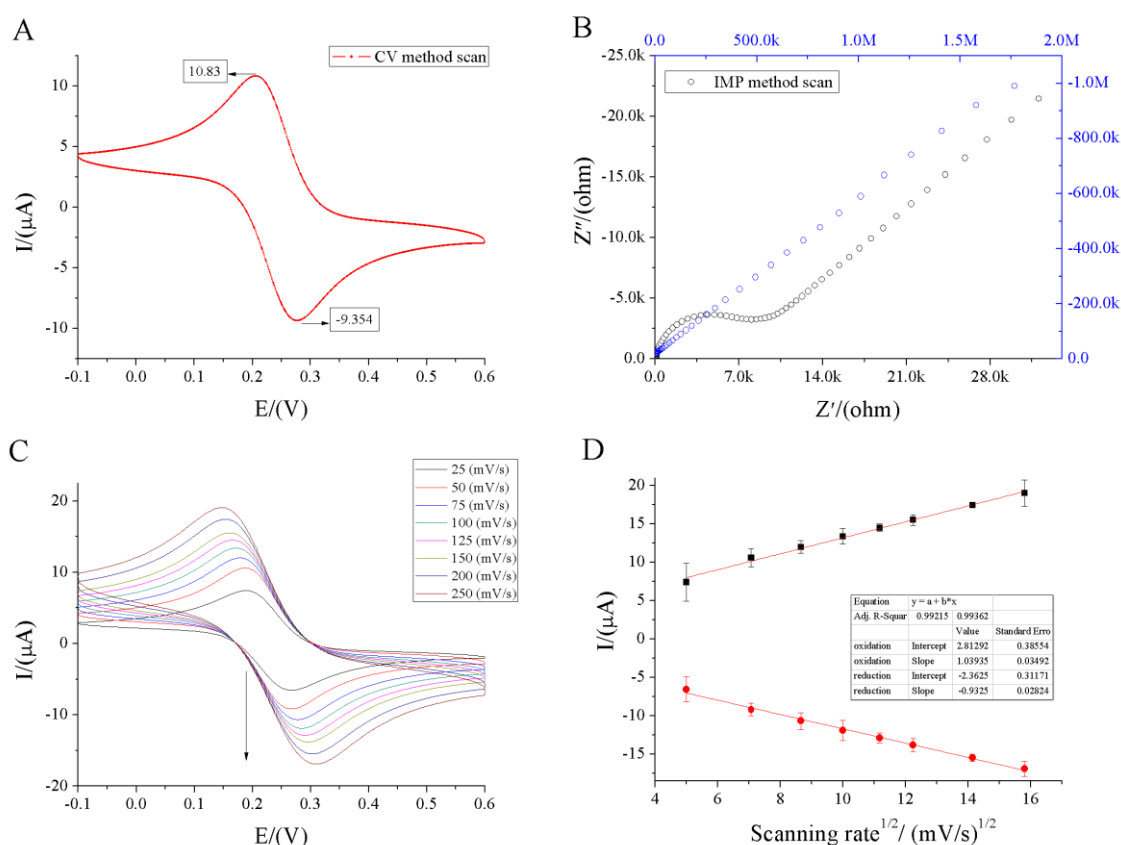


Fig. 2. Electrochemical characterization of pretreated glassy carbon electrodes.

(A) Cyclic voltammogram obtained at a scan rate of 50 mV/s over a voltage range of -0.1-0.6 V. (B) Comparison of electrochemical impedance spectroscopy of glassy carbon electrodes before and after pretreatment. (C) Cyclic voltammetry curves at different scan rates (25, 50, 75, 100, 125, 150, 200, 250 mV/s). (D) A linear fit between the square root of the scan rate and the redox peak current of the cyclic voltammetry curve at different scan rates. All data show mean $\pm$ SD of triplicate

measurements.

3.2. The role of capsaicin and TRPV1 channel

In the detection of gradient concentrations of capsaicin, the minimum detection limit was reached when the concentration was 1×10^{-18} mol/L. For the consideration of gradient concentration range mapping, the concentration of capsaicin was redefined as follows:

$$pCa = 19 + \log(C) \quad (2)$$

Among them, pCa refers to the redefining concentration of capsaicin, and C refers to the initial concentration of capsaicin.

The curve is plotted with pCa as the abscissa and the rate of response current change as the ordinate. The response current change rate has a good linear relationship with pCa (**Fig. 3A**) when the concentration of capsaicin is in the range of $1 \times 10^{-18} \sim 1 \times 10^{-15}$ mol/L. Therefore, the concentration of capsaicin in the range of 1×10^{-18} to 1×10^{-15} mol/L was further subdivided and measured (**Fig. 3B**). Curve fitting showed that the rate of response current change exhibits a good hyperbolic relationship with capsaicin concentration (**Fig. 3C**). The fitting equation is:

$$\Delta I = \frac{0.72161 \times C \times 10^{-18}}{2.22759 + C \times 10^{-18}} (R^2 = 0.976)$$

This shows that the interaction between capsaicin and TRPV1 channel is similar to the kinetics of enzymatic reaction. It is composed of a first order reaction at low concentration, a mixed order reaction at medium concentration and a zero order reaction at high concentration, and has ligand saturation effect (Warshel, 1984). The

double reciprocal curve was obtained by the Lineweaver-Burk plot method using the reciprocal of the capsaicin concentration as the abscissa and the reciprocal of the rate of response current change as the ordinate (**Fig. 3D**). The fitting regression equation is:

$$\frac{1}{\Delta I} = 1.40337 + 2.8373 \times \frac{1}{C} \times 10^{-18} (R^2 = 0.994)$$

From this, it can be calculated that the K_a value of capsaicin concentration reaching half of the maximum cell output signal is 2.0218×10^{-18} mol/L. This means that when this concentration is applied to the prepared biosensor, the velocity of response current change rate is close to infinity. The K_a value is at the transition point of the capsaicin kinetic curve, and the change in the rate of response current change is the most significant. Therefore, in order to study the antagonism mechanism of different analgesic compounds, the concentration of the selected analgesic compound is $5K_a$, i.e. about 1×10^{-17} mol/L.

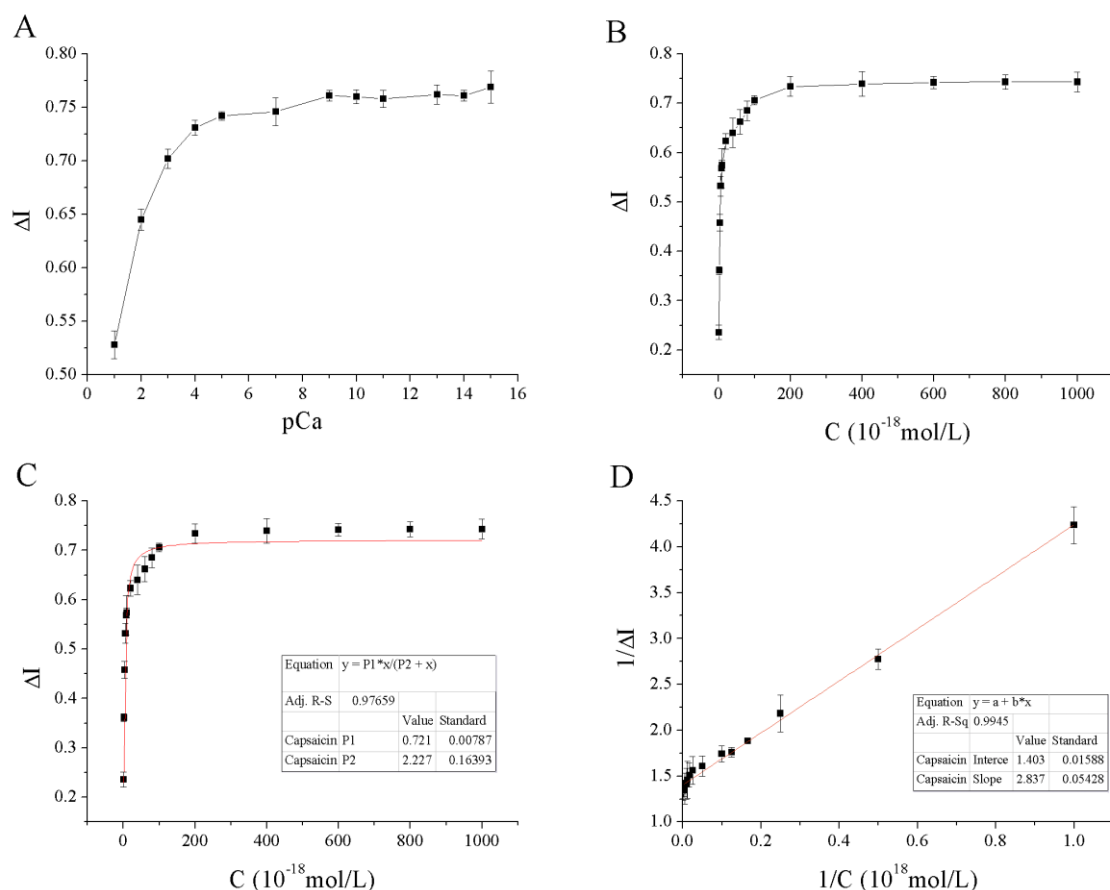


Fig. 3. Kinetics of the action of capsaicin with the biosensor. All data show mean $\pm$ SD of triplicate measurements. **(A)** The action law of response current change rate and pCa in the concentration range of capsaicin of $1 \times 10^{-18} \sim 1 \times 10^{-4}$ mol/L. **(B)** The action law of response current change rate and capsaicin concentration in the concentration range of capsaicin of $1 \times 10^{-18} \sim 1 \times 10^{-15}$ mol/L. **(C)** A fitting curve of the action law between the response current change rate and capsaicin concentration in the concentration range of capsaicin of $1 \times 10^{-18} \sim 1 \times 10^{-15}$ mol/L. **(D)** A fitting curve of the action law between the reciprocal of the response current change rate and the reciprocal of the capsaicin concentration in the concentration range of capsaicin of $1 \times 10^{-18} \sim 1 \times 10^{-15}$ mol/L.

3.3. Antagonistic kinetics of different analgesic compounds on capsaicin

Ligand-receptor interactions have similar kinetic characteristics to substrate-enzyme interactions. When inhibitors are present, the biological effects of the receptor or enzyme are reduced or even eliminated. The inhibition of the enzyme is due to inhibition of the regulatory or active site of the enzyme, resulting in decreased or inactive enzyme activity (Robin et al., 2018). Similarly, the ligand recognition center of the extracellular domain of a receptor is affected by a modulator to produce activation or inhibition of the receptor signaling pathway. For enzyme inhibitors, the inhibition constant (K_i) can be determined by measuring the change in the kinetic characteristics of the enzymatic reaction before and after the addition of the inhibitor (Cornish-Bowden, 1974; Yang et al., 2013). Therefore, in this study, different analgesic compounds with a fixed concentration of 1×10^{-17} mol/L were added to the capsaicin solution (in a concentration range of 1×10^{-18} to 1×10^{-15} mol/L) to prepare a mixed test liquid for detection. The reciprocal of the capsaicin concentration was plotted on the abscissa, and the reciprocal of the response current change rate was plotted on the ordinate to obtain a double reciprocal curve (**Fig. 4A-F**). The resulting fitted linear regression equations are:

$$\frac{1}{\Delta I_{(Cap-capsazepine)}} = 1.39223 + 9.96698 \times \frac{1}{C} \times 10^{-18} (R^2 = 0.996)$$

$$\frac{1}{\Delta I_{(Cap-loureirin B)}} = 1.39959 + 20.35593 \times \frac{1}{C} \times 10^{-18} (R^2 = 0.994)$$

$$\frac{1}{\Delta I_{(Cap-aconitine)}} = 1.69616 + 4.10783 \times \frac{1}{C} \times 10^{-18} (R^2 = 0.992)$$

$$\frac{1}{\Delta I_{(Cap-tetrahydropalmatine)}} = 1.4078 + 3.47576 \times \frac{1}{C} \times 10^{-18} (R^2 = 0.988)$$

$$\frac{1}{\Delta I_{(Cap-AMG517)}} = 1.40869 + 16.51559 \times \frac{1}{C} \times 10^{-18} (R^2 = 0.993)$$

$$\frac{1}{\Delta I_{(Cap-Anandamide)}} = 1.55557 + 4.55483 \times \frac{1}{C} \times 10^{-18} (R^2 = 0.991)$$

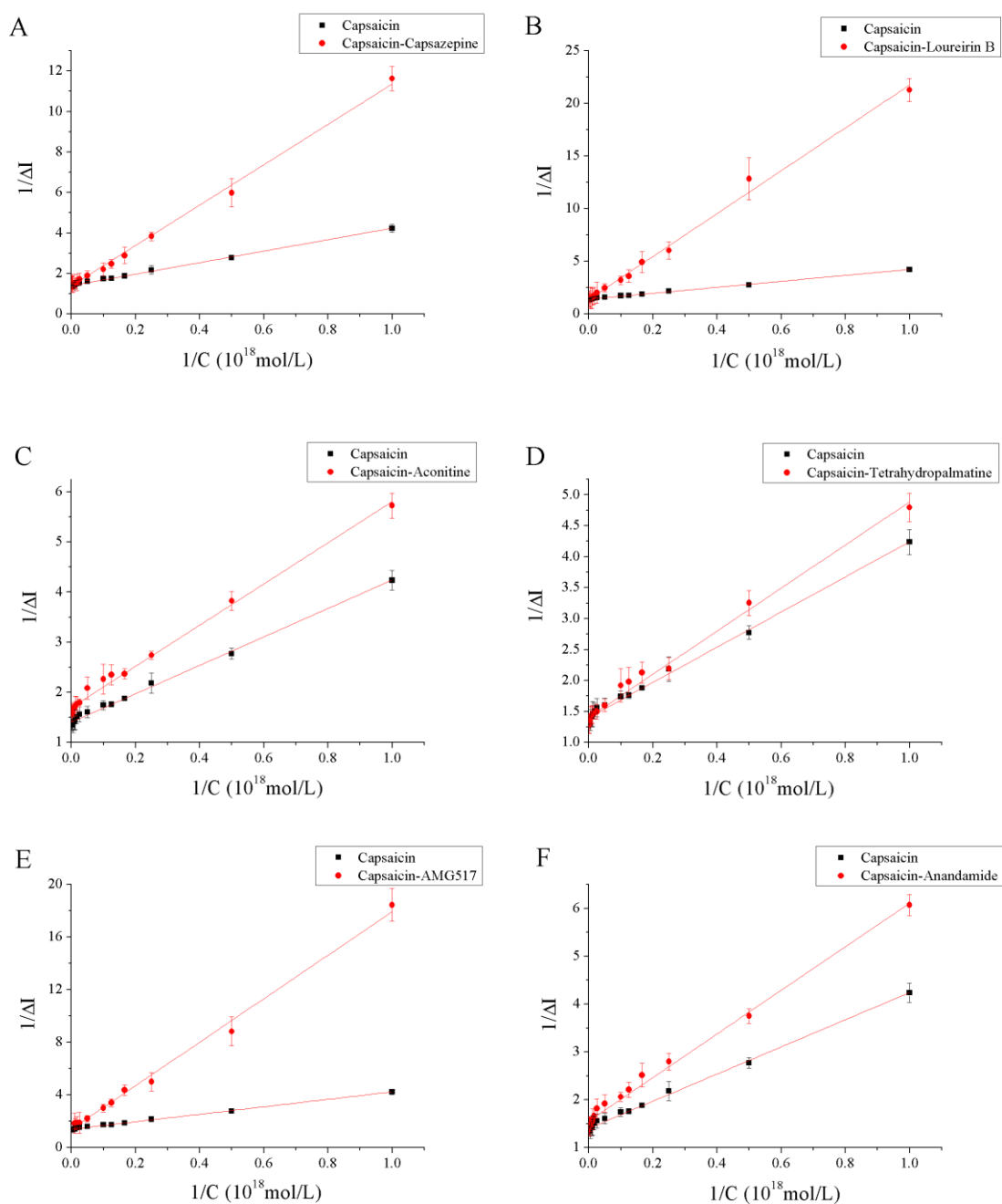


Fig. 4. Comparison of antagonism of Ca^{2+} influx mediated by capsaicin-induced TRPV1 channel by different analgesics. All data show mean $\pm$ SD of triplicate measurements. **(A)** The interaction between capsaicin and capsazepine. **(B)** The interaction law of capsaicin-loureirin B. **(C)** The interaction of capsaicin-aconitine. **(D)** The interaction between capsaicin and tetrahydropalmatine. **(E)** The interaction law of capsaicin-AMG517. **(F)** The interaction law of capsaicin-anandamide.

Its kinetic parameters are shown in **Table 1**. After adding analgesic compounds, the main manifestations were the changes of K_a and $V_{\max}$. This is very similar to the reversible inhibition of the enzyme. The calculation formulas for K_a and $V_{\max}$ are shown in equations (3) and (4).

$$\text{Slope} = \frac{K_a}{V_{\max}} \quad (3)$$

$$\text{Intercept} = \frac{1}{V_{\max}} \quad (4)$$

The maximum response value ($V_{\max}$) of the output signal produced by capsaicin stimulation did not change significantly after the addition of capsazepine, AMG517, loureirin B, and tetrahydropalmatine, whereas the K_a value increased significantly. This is consistent with the competitive inhibition of the enzyme. After the competitive compound is added, the compound can competitively bind to the ligand binding site of the receptor. At this time, the ligand is repelled outside the binding center, resulting in the ligand cannot bind to the receptor and thereby inhibiting the activation of the receptor (Benini et al., 2004). However, as the ligand concentration gradually

increases, the binding center of the receptor is gradually occupied by the ligand. Therefore, the generated maximum output signal, stimulated by a mixed solution with high concentration of capsaicin, did not significantly decrease. Therefore, capsazepine, AMG517, loureirin B, and tetrahydropalmatine are competitive ligands for capsaicin, and the inhibition constants are: $K_i \text{ capsazepine} = 3.9796 \times 10^{-18} \text{ mol/L}$, $K_i \text{ AMG517} = 2.0743 \times 10^{-18} \text{ mol/L}$, $K_i \text{ loureirin B} = 1.6196 \times 10^{-18} \text{ mol/L}$, $K_i \text{ tetrahydropalmatine} = 4.4440 \times 10^{-17} \text{ mol/L}$ respectively. The inhibitory intensity of the four substances is: loureirin B > AMG517 > capsazepine > tetrahydropalmatine.

When stimulated with aconitine (or anandamide) at the same concentration, $V_{\max}$ decreased and K_a value increased significantly. This is inconsistent with the three basic inhibitory types of the enzyme, indicating that the inhibitory type of aconitine (or anandamide) to the TRPV1 channel is a mixed type. In the mixed inhibition of the enzyme, the slope and intercept of the two straight lines, resulting from the double reciprocal mapping with the addition of the inhibitor before and after, have a certain relationship with the inhibitory constant (K_i) (Cornish-Bowden, 1974; Yang et al., 2013):

$$\frac{\text{Slope}_{(\text{capsaicin and antagonist})}}{\text{Slope}_{(\text{capsaicin})}} = 1 + \frac{[I]}{K_i} \quad (5)$$

$$\frac{\text{Intercept}_{(\text{capsaicin and antagonist})}}{\text{Intercept}_{(\text{capsaicin})}} = 1 + \frac{[I]}{K_i'} \quad (6)$$

Among them, $[I]$ indicates the concentration of the inhibitor used, K_i is the dissociation constant of the inhibitor, and K_i' is the apparent inhibition constant.

Therefore, the ratio of slope and longitudinal intercept of the kinetic curve after and

before aconitine (or anandamide) addition were used to calculate the K_i $K_{i\text{aconitine}}=2.2332\times 10^{-17}$ mol/L, $K_{i'\text{aconitine}}=4.7931\times 10^{-17}$ mol /L, $K_{i\text{anandamide}}=1.6520\times 10^{-17}$ mol/L, $K_{i'\text{anandamide}}=9.2206\times 10^{-17}$ mol/L. Since $K_{i'}>K_i$, it can be judged that the inhibition of TRPV1 by aconitine (or anandamide) is a combination of non-competition and competition inhibition. This indicates that when aconitine (or anandamide) and capsaicin act simultaneously on the biosensor, aconitine (or anandamide) binds to both the receptor and the capsaicin-receptor complex. At this time, the binding between capsaicin, aconitine (or anandamide) and receptors has some interference with each other (Kaustov et al., 2003).

3.4. Hyperthermia of analgesic compounds

Most of the analgesic compounds developed with the TRPV1 target have hyperthermia side effects. In this study, the effects of five analgesic compounds on body temperature in rats were examined (**Fig. S2**). AMG517 caused a significant increase in body temperature in rats (with the maximum increase of 0.79 °C), and the body temperature returned to normal after 150 min. Similarly, loureirin B also caused a significant increase in body temperature with the maximum increase of 0.95 °C. However, capsazepine, aconitine, and tetrahydropalmatine did not cause the body temperature of rats to reach standard levels (>0.6 °C) within the measured time range.

4. Discussion

Nowadays, the research on the TRPV1 channel as a novel target for analgesic screening has mainly focused on the affinity relationship between the ligand and the

TRPV1 channel (Elokely et al., 2016). However, there is less study on the intrinsic connections between the biological effects produced by binding of ligands to receptors and the structure of ligands. In present study, the taste bud tissue sensor was prepared, and the electrical signals output induced by capsaicin and analgesic compounds interacting with receptors were then measured. Thus the relationship between ligand structure and biological function was evaluated. The results showed that capsazepine, AMG517, analgesic loureirin B, and tetrahydropalmatine were competitive allosteric regulatory ligands for capsaicin, while aconitine and anandamide were mixed allosteric regulatory ligand that combined both competition and non-competition. All of these allosteric regulatory ligands were able to significantly inhibit capsaicin-induced Ca^{2+} influx through the TRPV1 channel. Molecular structure analysis shows that capsazepine, AMG517, loureirin B and tetrahydropalmatine all have similar H receptor/donor parts (B) and substituted benzene ring moiety (C), while the hydrophobic aliphatic side chain portion (A) is greatly different (**Fig. S3**). This indicates that the H receptor/donor parts (B) and the substituted benzene ring moiety (C) are the key pharmacophore sites for the competitive allosteric modulatory ligands. Similar to enzyme-substrate interactions, competitive interactions mean that there is a common structure or group between the antagonist and the ligand (Gohlke et al., 2014).

Both the competitive allosteric regulation and bidirectional regulation of capsaicin suggest that the blockade of TRPV1 channels may be mediated through other pathways distinct from the activation mechanisms. It has been clearly revealed the

“switching” role of the PIP2 locus during the ON/OFF state adjustment of the TRPV1 channel¹¹. TRPV1 is a Ca^{2+} -infiltrating channel that can be activated by multiple stimuli and inhibited by intracellular PIP2 (Clapham, 2003). In addition, Minke et al. found that drosophila TRP channels can be regulated by GPCR-mediated signal transduction pathways (Minke and Cook, 2002). Therefore, the blockade of the TRPV1 channel is most likely through the GPCR-mediated phosphatidylinositol-4,5-bisphosphate (PIP2) regulatory pathway.

Up to now, it is not yet clear what type of specific G protein-coupled receptors are responsible for the antagonism of capsaicin and analgesic compounds on TRPV1 channels. Through the structural analysis of the four spicy substances (**Fig. S4**) and the five analgesic compounds (**Fig. S3**), it was found that the amide groups and analogous groups are common to them. These peculiar groups may play a key role in capsaicin and analgesic compounds combining with their specific GPCRs. It is known that selective activation of the cannabinoid CB2 receptor inhibits hyperalgesia caused by intradermal capsaicin, and cannabinoid-like substances have a significant analgesic effect (Hohmann et al., 2004; Agarwal et al., 2007). By analyzing the structural features of the endocannabinoid Anandamide (AEA) and 2-Arachidonyl Glycerol (2-AG) (**Fig. S4**), we can easily see that they have similar amide groups and analogous groups. Studies of Hua et al. on the crystal structure of cannabinoid receptors and the mechanism of ligand regulation have revealed that amide groups and analogous groups play a vital role in the binding mechanism of endocannabinoids and CB receptors (Hua et al., 2016). Therefore, we infer that capsaicin and analgesic

compounds may activate the receptor by binding to endocannabinoid receptors, triggering its intracellular signal transduction, thereby exerting analgesic effects. It has been demonstrated that endocannabinoids can activate intracellular G protein by binding to CB receptor, leading to the dissociation of $G\alpha$ subunit and $G\beta\gamma$ subunits, soon afterwards $G\beta\gamma$ subunits increase PIP2 levels by inhibiting PI3K and reducing the process of PI3K-mediated phosphorylation of PIP2 to form PIP3 (Pisanti et al., 2013; Carracedo and Pandolfi, 2008). Moreover, intracellular PIP2 synthesis pathways also exist, which can promote PI to form PIP2 through activation of the corresponding enzymes (Berridge, 2014). Therefore, based on the experimental results, we suggest that capsaicin and analgesic compounds may activate intracellular signaling pathways by binding to endocannabinoid receptors, resulting in an increase in PIP2 levels. Then, the increased PIP2 binds the specific phosphatidylinositol site in the proximal C-terminal region of the TRPV1 channel by competing with the vanilloid activator, thereby closing the opened TRPV1 channel and stabilizing it in a resting closed state, resulting in an analgesic effect.

In short, there may be two ways in the TRPV1 channel state adjustment process (**Fig. 5**): First, vanilloid activators and other stimuli open the TRPV1 channel by binding to the PIP2 site of the TRPV1 channel, resulting in Ca^{2+} influx, which induces depolarization and thus produces a spicy and painful feel. Second, capsaicin and analgesic compounds increase intracellular PIP2 levels via CB receptor/G protein/PI3K/PIP2 signaling pathways, thereby shutting down TRPV1 channels and producing analgesic effects.

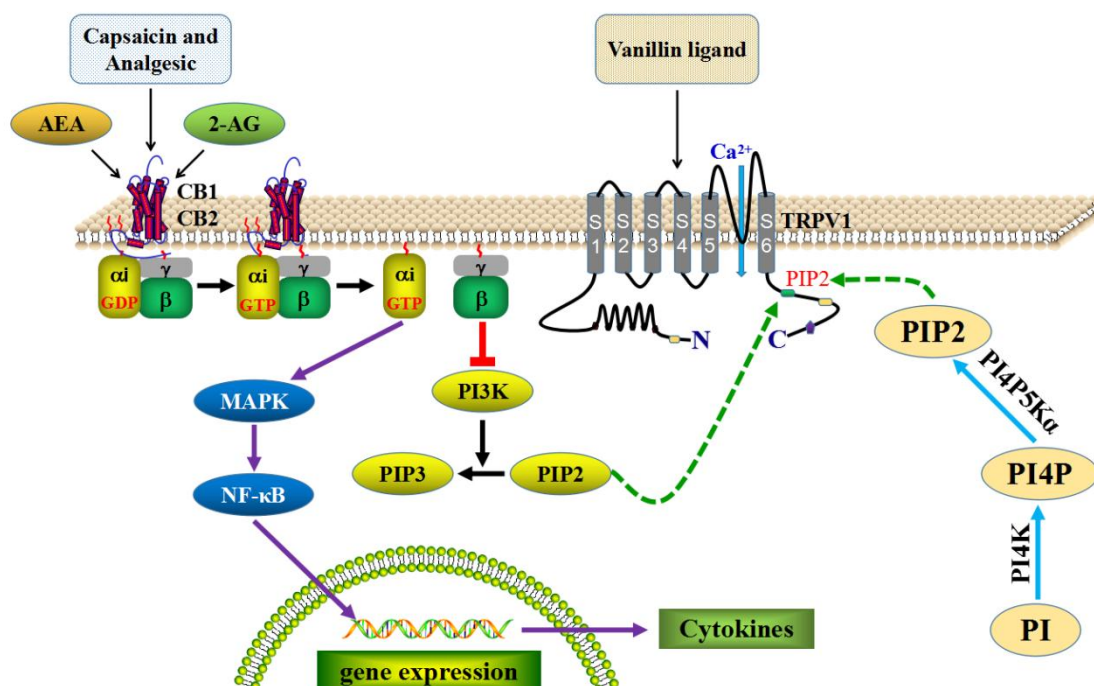


Fig. 5. Signaling network between CB receptor and TRPV1 channel.

In this study, for the first time, the regulation of CB receptors and TRPV1 channels by loureirin B, aconitine, tetrahydropalmatine and anandamide with clear analgesic effects was investigated by prepared taste bud tissue biosensor. The material required for the biosensor is easily available, the preparation method is simple, and the detection is rapid. Compared with the traditional detection method based on Ca²⁺ influx, the activation constant of capsaicin measured by the biosensor can reach 2.0218×10^{-18} mol/L, and the detection level of analgesic compound also reaches the atto-mole grade. Therefore, the biosensor can greatly improve the detection sensitivity of capsaicin and analgesic compounds. Through the analysis of kinetic parameters, the antagonistic types of different TRPV1 channel antagonists can be analyzed using

the biosensor, and the antagonism intensity of antagonists can also be quantified.

In recent years, many different types of TRPV1 channel antagonists have been found, including urea compounds (A-425619 and JYL1421), cinnamoyl amines (SB-366791 and AMG9810), piperazines (BCTC), and pyrimidines (AMG517). These antagonists all have the side effect of elevating body temperature in mice (Caterina, 2008; El-Remessy et al., 2011). The possible reason for this phenomenon is that most of these antagonists were screened with cancer cells as the research object. However, there are significant differences between cancer cells and normal cells in terms of nutrition sensing, environmental monitoring, and material and energy metabolism (Kalyanaraman, 2017). This suggests that the biological information of candidate drugs obtained from drug screening based on cancer cells is questionable. Therefore, it is of great significance to develop an analgesic drug screening method based on normal cells or tissues. The results of a biosensor based on normal taste bud tissue in this study showed that loureirin B and AMG 517 cause hyperthermia, whereas tetrahydropalmatine and capsazepine did not induce hyperthermia in SD rats, so these two compounds can be used as the candidate analgesic drugs. Therefore, this study also provides a new direction for the construction of lead compounds and molecular structure optimization in the development of analgesic drugs for TRPV1 channels.

5. Conclusions

In our present study, capsaicin was first used as a TRPV1 channel activator to study

the antagonistic effects of capsazepine, AMG 517, loureirin B, aconitine, tetrahydropalmatine and anandamide through the prepared taste bud tissue biosensors. By establishing an activation-inhibition model of TRPV1 and analyzing kinetic parameters, the relationship between the allosteric regulatory ligands of the CB receptor and TRPV1 was investigated, and the antagonistic strength and inhibitory constants of different ligands to the activated TRPV1 channel were quantitatively studied. It turned out that capsazepine, AMG 517, loureirin B and tetrahydropalmatine were competitive allosteric regulatory ligands for capsaicin, while aconitine and anandamide were non-competitive and competitive hybrid allosteric regulatory ligand. Meanwhile, the hyperthermia side effects test results showed that aconitine and tetrahydropalmatine could be used as candidate analgesics to be selected for follow-up study. Through kinetic laws and structural analysis, it was first discovered that capsaicin and analgesic compounds bind to endocannabinoid receptors, activate intracellular G protein/PI3K/PIP2 signaling, increase intracellular PIP2 levels, and shut down TRPV1 channels to achieve analgesic effect. It provides a momentous theoretical basis for revealing the mechanisms of pain production and analgesia. Furthermore, the molecular mechanism by which the allosteric regulatory ligands of the CB receptor regulate the state of TRPV1 channel on/off remains to be further determined. The effect of the synthesis/decomposition pathway of intracellular PIP2 on the state of TRPV1 channel should also be studied in more depth.

Acknowledgements

This research was supported by the Natural Science Foundation of China (Grant No. 31671857).

Conflict of interest

The authors declare no conflict of interest.

Accepted manuscript

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Table 1. Kinetic parameters of the interaction between capsaicin and different analgesics and the biosensor.

Detection component	Intercept	Slope	V_{max} (V_{max})	$K_a(K_a')$ / 10^{-18} mol* L^{-1}	Antagonistic type	Inhibition constant $K_i(K_i')$ / 10^{-17} mol* L^{-1}
Capsaicin	1.4034	2.837 3	0.712 6	2.0218	-	-
Cap-capsazepine	1.3922	9.967 0	0.718 3	7.1590	Competitive	0.3980
Cap-AMG517	1.4087	16.51 56	0.709 9	11.7241	Competitive	0.2074
Cap-loureirin B	1.3996	20.35 59	0.714 5	14.5442	Competitive	0.1620
Cap-aconitine	1.6962	4.107 8	0.589 6	2.4218	Non-competitive + competitive	4.7939
Cap-tetrahydropalma	1.4078	3.475 8	0.710 3	2.4689	Competitive	4.4440

tine						
Cap-anandamid		4.554	0.642		Non-competit	
e	1.5556			2.9281	ive +	9.2206
		8	9		competitive	

Highlights

1. First research on TRPV1 related analgesic drugs through biosensors.
2. Mathematical models were used to reveal the antagonistic type and effects on TRPV1.
3. The role of amide groups and their similar groups in analgesic drugs was discovered.
4. First revealed the role of allosteric regulation of CB receptors in analgesia.