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Transient receptor potential vanilloid 1 (TRPV1) channels in cultured rat Sertoli cells regulate an acid sensing chloride channel

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ABSTRACT

Sertoli cells provide a controlled microenvironment for regulation and maintenance of spermatogenesis for which an acidic milieu is crucial for male fertility. Sertoli cells also contribute to protection of spermatogenetic cells. Here, we showed that TRPV1 is expressed in rat Sertoli cells and regulates an acid sensing Cl^- channel (ASCC). The expression of TRPV1 in rat Sertoli cells was demonstrated by RT-PCR, immunostaining and calcium measurement experiments. ASCC activity was inhibited by capsaicin (IC_{50} = 214.3 ± 1.6 nM), nM), olvanil (IC_{50} = 400 ± 1.7 pM) and resiniferatoxin (IC_{50} = 9.3 ± 1.5 nM) but potentiated by capsazepine (EC_{50} = 5.3 ± 1.3 μM) and ruthenium red (EC_{50} = 2.3 ± 1.5 μM). In the human airway epithelial cell line Calu-3 in which ASCC can be detected but not TRPV1, capsaicin and capsazepine were without any effect. Finally the application of the non-steroidal anti-inflammatory drug ibuprofen prevented the control of ASCC by TRPV1. Our study provides the first evidence for a regulation by TRPV1 of an acid sensing chloride channel in rat Sertoli cells. TRPV1 and ASCC may thus be considered as new potential physiological regulators of spermatogenesis and targets for pharmacological treatments of reproductive disorders as cryptorchidism, Sertoli cell tumors or torsion of the spermatic cord.

1. Introduction

The Sertoli cell comprises the main structural component of the seminiferous epithelium and has a cytoplasmic arrangement surrounding germinal cells providing a controlled microenvironment that creates an immunologically protected space [1]. Sertoli cells are the site of action of all hormonal influences governing spermatogenesis, supporting and nourishing the germ cells [2,3]. Their structure and the specialized junctions between them and the neighbouring germ cells create a sophisticated microenvironment providing all the nutrients and growth factors required for the full development of spermatogenetic cells [2]. An acidic microenvironment is crucial for the male fertility since the sperm cannot fertilize an egg without proper acidification of the acrosome [4]. Sertoli cells are also key cells involved in KCl secretion into the lumen of seminiferous tubules [5]. They express a variety of ionic channels, including calcium-dependent Cl^- channels [6], cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels [7], voltage-dependent Cl^- channels [8],

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Abbreviations: ASCC, acid sensing Cl^- channel; CAP, capsaicin; CPZ, capsazepine; RR, ruthenium red; TRPV1, transient receptor potential vanilloid 1 channels.

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voltage-dependent Ca^{2+} channels [9,10] and transient receptor vanilloid 1 (TRPV1) channels [11].

The transient receptor potential (TRP) channels are universal biological sensors that detect changes in the environment [12] and are characterized by permeability to several cations, including Ca^{2+} [13–15]. One of these TRP channels, TRPV1 or capsaicin receptor, is predominantly expressed on the peripheral and central terminals of primary sensory neurons located in the dorsal root where it plays a key role in the detection of noxious stimuli that may result in tissue damage [16]. Activation of the channel leads to an influx of cations (such as Na^+ and Ca^{2+}) into the cell, resulting in depolarization, neuronal hyperexcitability, and ultimately the sensation of pain. A wide range of stimuli are responsible for activation of TRPV1 including noxious heat ($T > 42\ ^\circ C$), low extracellular pH, and a variety of chemical mediators [16–18]). Over recent years, evidence has been accumulated showing distribution of TRPV1 in other neuronal and non-neuronal tissues. In particular, TRPV1 mRNA has been detected in rat prostate, testis, penis and bladder tissues and in all human genito-urinary tract tissues [19].

Some studies have provided evidence that TRPV1 is required for the development of certain pathological conditions, such as inflammatory heat hyperalgesia, visceral hyperreflexia or airway inflammation [20–22]. Conditions which may adversely affect Sertoli cells are testicular conditions (for example cryptorchidism or Sertoli cell tumors), or extratesticular conditions that damage the spermatic cord or duct system causing testicular damage as torsion of the spermatic cord [3]. Testicular torsion is an urologic syndrome mainly caused by torsion of the spermatic cord that constitutes a surgical emergency affecting newborns, children and adolescent boys. The primary pathophysiologic event in testicular torsion is ischemia following by reperfusion; thus, testicular torsion–detorsion is an ischemia-reperfusion injury to the testis. This syndrome often leads to male infertility [23]. Recently, ibuprofen, a non-steroidal anti-inflammatory drug (NSAID), was successfully used to decrease testicular ischemia-reperfusion injury in rats [24].

The aim of this study was to search for physiological regulators of the recently identified acid sensing Cl^- channels (hereafter noted ASCC) [8,25]. ASCC is activated only in the presence of an extracellular acidic pH, with estimated half-maximal activation at pH 5.5. The anionic current is strongly outwardly rectifying. It was not detected at physiological or basic pH and is not sensitive to intracellular or extracellular Ca^{2+} variation. Its anionic selectively sequence was $Cl^- > Br^- > I^- >$ gluconate [8]. The order of potency for pharmacological inhibition of ASCC is DIDS $\approx$ glibenclamiglibenclamide $>$ DPC $\gg$ calix[4]arene, 9-AC [25]. We report here the first evidence for a regulation of ASCC by endogenous TRPV1 in rat Sertoli cells.

2. Material and methods

2.1. Cell cultures

Preparation of rat Sertoli cells: Experiments were performed on cultured Sertoli cells isolated in sterile conditions from 13- to 15-day-old Wistar rats as described previously [8,25] and purified by an hypotonic shock after 48 h of culture [26]. The purity of our cultures was confirmed by vimentine staining (a specific marker of Sertoli cells) and revealed purity above 86%. No 3-β-hydroxysteroid dehydrogenase activity (a specific marker of Leydig cells) was detected in our primary cultures indicating the absence of Leydig cells [27]. All the experiments were performed at minimum on two different cell cultures from the parenchyma of 6–10 testis.

Calu-3 cell line: Calu-3, a cell line of human pulmonary origin (purchased from American Type Culture Collection) was cultured at 37 °C in 5% CO_2 and maintained in DMEM-Ham's F12 (1:1) nutritive mix supplemented by 10% FCS and 1% antibiotics (50 IU/ml penicillin and 50 μg/ml streptomycin) [28].

2.2. RT-PCR

cDNAs were used as PCR template using AmpliTaq Gold® DNA polymerase (Applied Biosystems), with PCR primers specific for rat TRPV1 gene (accession number NM_031982): forward, 5′-TGGCAGATAACACAGTTGACA-3′, and reverse, 5′-ACAGGCCGATAGTAGGCAG-3′, spanning the region between nucleotides 961 and 1450, or with PCR primers specific for human TRPV1 gene (accession number NM_080706.2): forward, 5′-CGGTCAAGCAGAAGTTTCAGC-3′ and reverse, 5′-AGTGTTGACAGTGCTGTCTG-3′, spanning the region between nucleotides 14 and 210. The housekeeping β-actin gene (accession number NM_031144) was used as control, with primers forward, 5′-CTACCTCATGAAGATCCT GA-3′, and reverse, 5′-TTTCATGGATGCCACAGGAT-3′, spanning the region between nucleotides 561 and 829. Others details [8,25].

2.3. Recording calcium signals

We used the Fluo-4 probe to record calcium signals as previously described [29]. Raw fluorescence values were converted into Ca^{2+} concentrations by applying the self-ratio method with assuming a Kd of Fluo-4 of 396 nM and a resting $[Ca^{2+}]$ for Sertoli cells of 89 nM [30].

2.4. Immunofluorescence study

Cells were first incubated with a primary specific antibody (goat anti-TRPV1 antibody, 1:200, Santa Cruz, USA) and then, incubated with the corresponding secondary conjugated antibody Fluo488 (1:400, FluoProbes). In the control, the primary antibody was omitted. The nuclei were labelled with TOPRO-3 (1:1000, Interchim).

2.5. Radiotracer efflux experiments

Chloride channel activity was assayed by measuring the rate of iodide (^{125}I) efflux from a population of Sertoli cells as described [25].

2.6. Chemicals and statistics

All chemicals were from Sigma Chemicals (St. Louis, MO, USA). Results were expressed as mean ± S.E.M. of n independent

experiments and compared with Student's *t*-test. ns: non significant difference, $^{***}P < 0.001$.

3. Results

3.1. New pharmacology of the acid-sensing chloride channel

We recently characterized a novel Cl^- transport activated by extracellular acidic pH in Sertoli cells that we provisionally named acid-sensing Cl^- channel (ASCC) [8,25]. In the present study we hypothesized that ASCC in Sertoli cells, like TRPV1 channels, share similar pharmacology (for review see [31]). We thus tested drugs known to modulate acid sensors. Capsaicin (CAP) is an agonist and capsazepine (CPZ) is an antagonist of TRPV1, a pH-sensitive protein. Using iodide efflux, we studied the effect of CAP and CPZ on ASCC stimulated by pH 5 (Fig. 1A). We observed with 1 μM CAP a complete inhibition of ASCC activity and, in contrast, in presence of 10 μM CPZ, we observed a potentiation of this activity (Fig. 1A). In separate experiments, both CAP and CPZ applied alone did not produce any stimulation of iodide efflux (not shown). In Sertoli cells we previously identified, beside the acid-sensing Cl^- channel, at least two other Cl^- channels activated by hypo-osmotic challenge or by calcium [8,25]. Similar experiments were then performed to study the effect of TRPV1 modulators on these Cl^- transports. Iodide efflux activated by hypo-osmotic challenge (Fig. 1B) or by the calcium ionophore A23187 (Fig. 1C) were affected neither by CPZ nor by CAP. These results suggested that the regulation by CAP and CPZ is selective of the ASCC.

To begin to understand the mechanism of action of these molecules on ASCC we first determined the half-maximal effective concentration for inhibition (IC_{50}) of ASCC by CAP as shown Fig. 2A. We found an IC_{50} of 214.3 ± 1.6 nM (Fig. 2A) with 80% inhibition at 1 μM. We also tested two other TRPV1 agonists: olvanil and resiniferatoxin. Olvanil (Fig. 2B) and resiniferatoxin (Fig. 2C) inhibited ASCC activity with IC_{50} of 400 ± 1.7 pM and 9.3 ± 1.5 nM, respectively. Importantly, in the presence of the TRPV1 antagonists capsazepine (CPZ) and ruthenium red (RR), we observed the opposite effect on ASCC, i.e. a potentiation of ASCC activity with a half-maximal effective concentration for activation (EC_{50}) for CPZ of 5.3 ± 1.3 μM (Fig. 2D) and for RR an EC_{50} of 2.3 ± 1.5 μM (Fig. 2E). Again in the absence of extracellular acidic milieu, these pharmacological agents have no effect on basal iodide efflux (not shown).

3.2. Endogenous expression of TRPV1 in Sertoli cells

As these TRPV1 modulators are very specific [32], the above observations suggested that TRPV1 by itself regulates ASCC in Sertoli cells. However, the expression of TRPV1 has never been shown in Sertoli cells. For this reason we conducted a series of new experiments to search for the functional expression of TRPV1 proteins in Sertoli cell cultures. First we identified TRPV1 transcripts by RT-PCR analysis (Fig. 3A) and we used hyppocampal cDNA as a control since TRPV1 has previously been detected in this tissue [33]. Then we localized TRPV1 in the plasma membrane and cytosol of rat Sertoli cells by immunostaining (Fig. 3B). We finally measured the intracellular Ca^{2+} mobilization using specific TRPV1 modulators. In the presence of 1 μM capsaicin and 1 nM olvanil we recorded

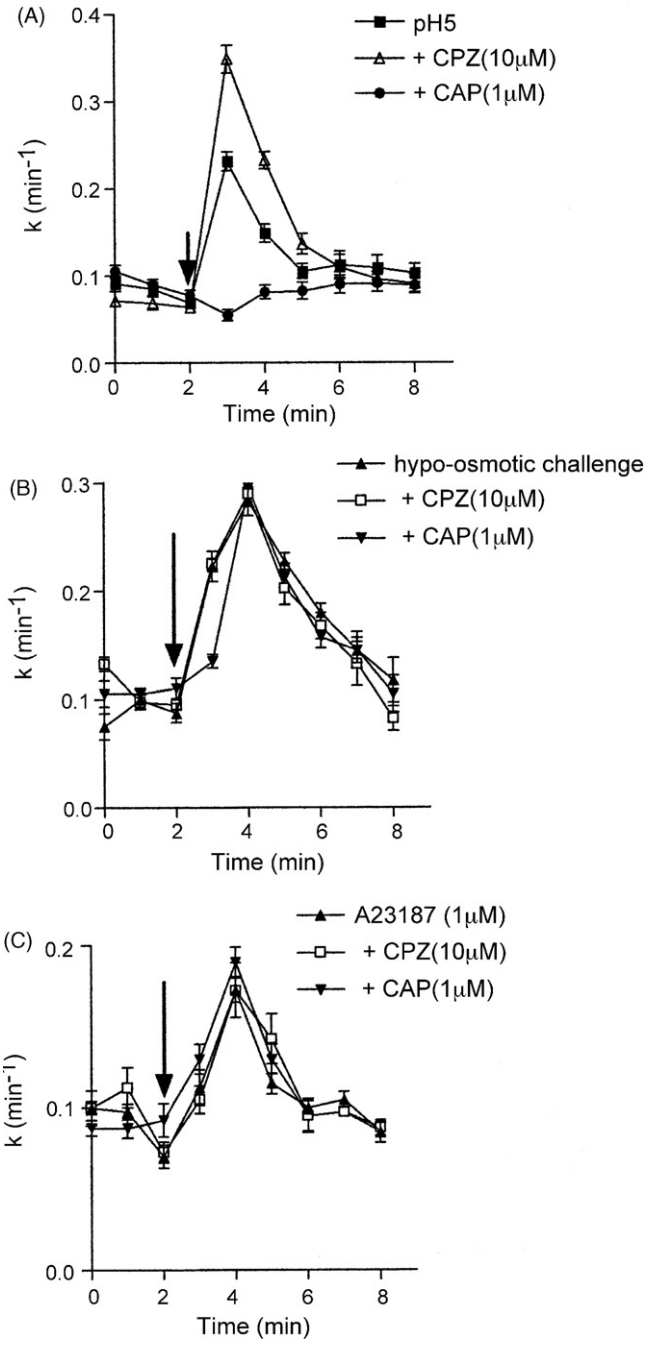

Fig. 1 – Specificity of the modulation of the pH 5-activated iodide efflux by acid sensor modulators. (A) Time-dependent iodide efflux on rat Sertoli cells bathed with a NaCl-rich solution at pH 5 with HCl in presence or absence of 10 μM CZP or 1 μM CAP. (B) Iodide efflux performed on rat Sertoli cells bathed with a hypo-osmotic challenge and in the presence of 10 μM CPZ or 1 μM CAP. (C) Iodide efflux performed on rat Sertoli cells bathed with A23187 solution and in the presence of 10 μM CPZ or 1 μM CAP.

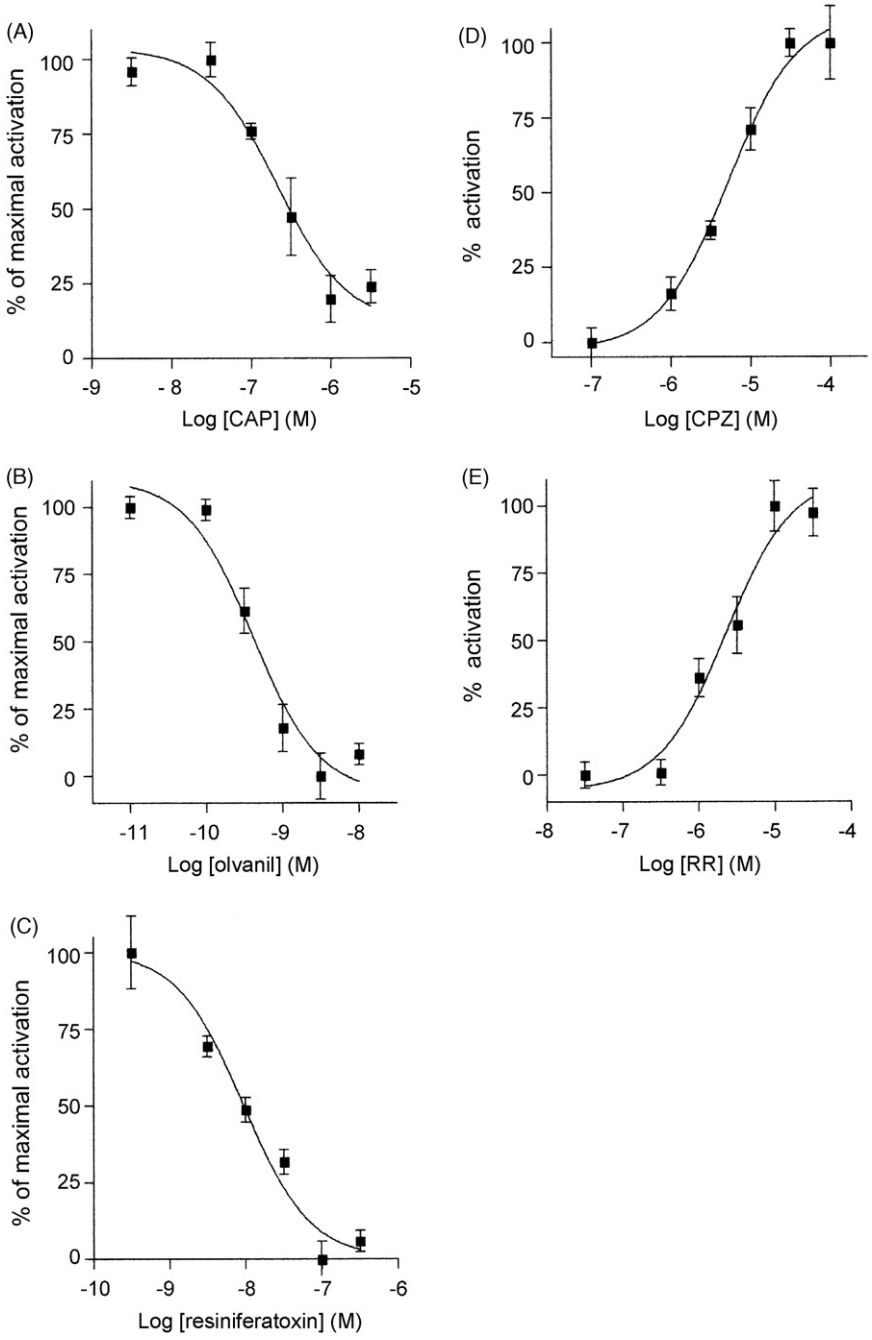

Fig. 2 – Modulation of ASCC by agonists and antagonists of TRPV1 channel. Concentration–response relationships for CAP (A), olvanil (B), resiniferatoxin (C), CZP (D) and RR (E). The data are expressed as a percentage of maximal activation of the iodide efflux in the presence of different concentrations of the TRPV1 modulator. *n* = 4–8 for each concentration.

an increase of intracellular Ca^{2+} (Fig. 3C), which was rapidly inhibited (Fig. 3C) by 10 μM ruthenium red ($P < 0.001$). The corresponding averaged Ca^{2+} mobilization expressed in nM/min is presented (Fig. 3D). These results demonstrate that TRPV1 is endogenously expressed and functional in rat Sertoli cells.

To better define the regulation of ASCC by TRPV1 we reasoned that if ASCC is detected in the absence of TRPV1 in another cell model, then we should not observed pharmacological regulation of ASCC by TRPV1 modulators. We have been able to detect ASCC in the human airway epithelial cell line Calu-3, like in Sertoli cells (Fig. 4A). However, on the contrary to Sertoli cells, no TRPV1 transcripts (Fig. 4B) or positive immunostaining of TRPV1 proteins (Fig. 4C) were detected. In addition, no modulation by CAP or CPZ of the ASCC was observed in Calu-3 cells (Fig. 4A). These results clearly indicated that the presence of TRPV1 is necessary to observe a regulation of ASCC by TRPV1 modulators.

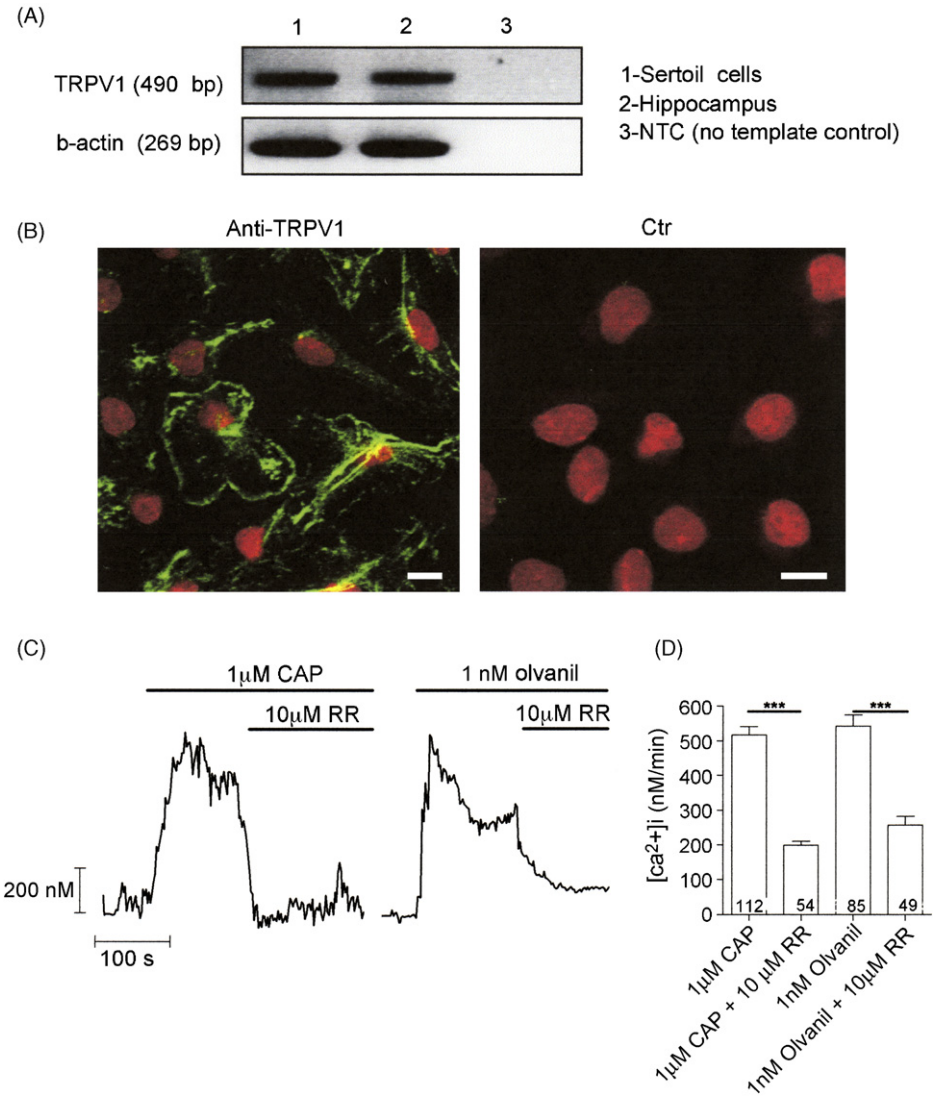

Fig. 3 – Expression and function of endogenous TRPV1 in rat Sertoli cells. (A) RT-PCR analysis of TRPV1 mRNA expression in rat Sertoli cells. Positive control for expression of TRPV1 mRNA was rat hippocampus. The housekeeping β-actin gene was also used as control. Transcript size is indicated. (B) Immunofluorescence study of TRPV1 protein in Sertoli cells in presence (in green left image) or in absence of anti-TRPV1 antibody (right image). Sertoli cells nucleus are stained in red. The scale bar is 10 μm. (C) Example of Ca^{2+} response induced by 1 μM capsaicin and 1 nM olvanil, and inhibited by 10 μM ruthenium red. (D) Histogram showing the Ca^{2+} cytoplasmic mobilization induced by various stimulations expressed in nM/min. Results are presented as mean ± S.E.M. and the number of experiments is noted on each bar graph. ***P < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

3.3. *Ibuprofen prevents the effect of TRPV1 on ASCC*

Because Sertoli cells are important to protect spermatogenetic cells and since TRPV1 is a therapeutic target for analgesic drugs [34] we tested ibuprofen, a non-steroidal anti-inflammatory drug (NSAID), on the acid sensor. By itself ibuprofen did not affect ASCC as shown in the experiments presented Fig. 5A and the histograms Fig. 5B (compare pH 5 versus pH 5 + ibuprofen). However, 100 μM ibuprofen completely prevented the inhibition of ASCC by 1 μM CAP (Fig. 5) ($P < 0.001$). We conclude that ibuprofen has no direct effect on ASCC but prevented its regulation by TRPV1, and therefore that this agent may have therapeutic implication in the immunologically protected seminiferous epithelium.

4. Discussion

In this study, we described an ibuprofen-dependent regulation of ASCC by TRPV1 in rat Sertoli cells.

Of the vanilloid-type (TRPV) subfamily of TRP receptors, TRPV1 responds to stimulation with CAP and resiniferatoxin, two of the best known and most thoroughly studied natural TRPV1 agonists. Usually the rank of order of potency for the agonists proposed for rat cells is resiniferatoxin ≫ olvanil = -

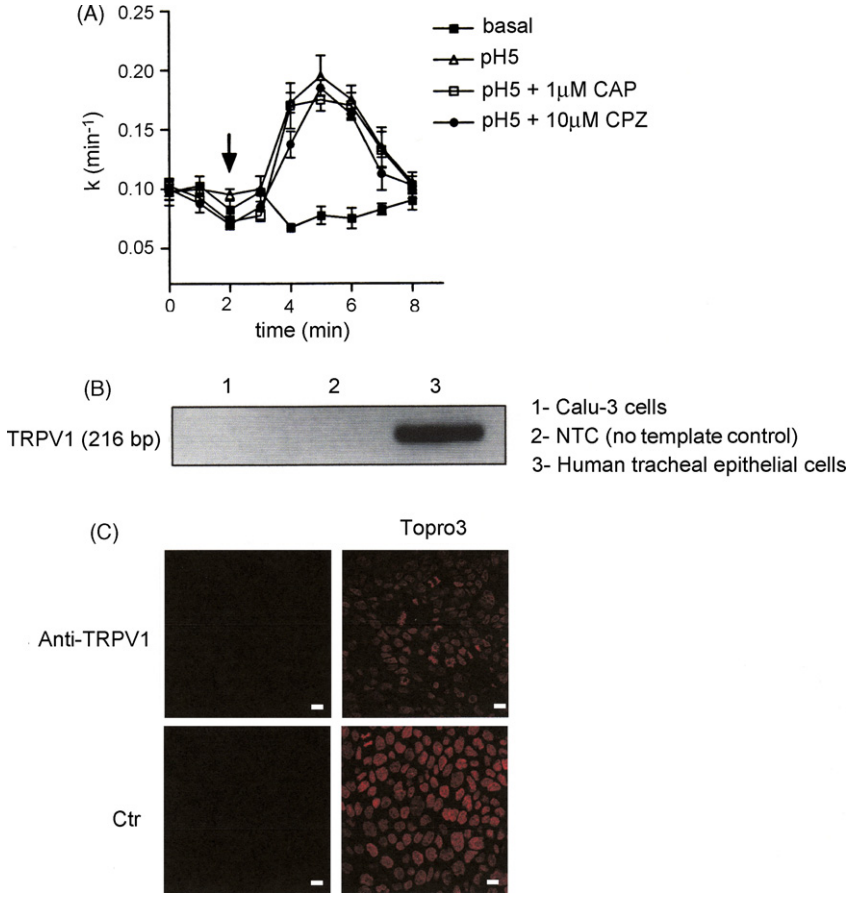

Fig. 4 – ASCC in Calu-3 cells is not regulated by TRPV1 (A) Time-dependent iodide efflux performed on Calu-3 cells bathed with a NaCl-rich solution at pH 5 with HCl in presence of 10 μM CPZ or 1 μM CAP. (B) RT-PCR analysis of TRPV1 mRNA expression in Calu-3 cells. Positive control for expression of TRPV1 mRNA was human tracheal epithelial cells. Transcript size is indicated. (C) Immunofluorescence study of TRPV1 channel in Calu-3 cells in presence (top images) or in absence of anti-TRPV1 antibody (bottom images). Calu-3 cells nucleus are stained in red. The scale bar is 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

CAP [35–37], different from those observed here (olvanil > resiniferatoxin > CAP). However, some difference can be observed depending of the cell origin [37]. For example, CAP and olvanil were described with similar potency in rat mesenteric arterial bed [37] or on heterologous expressed mice TRPV1 [38]; on contrary, on porcine [35] or dog [36] orthologue of TRPV1 the potency of olvanil is higher than CAP. Moreover, some kinetic differences were also observed depending of the cell type. Indeed, a study reported that whilst resiniferatoxin was only 5-fold more potent than CAP on rat vanilloid receptor in dorsal root ganglion cells, it was 1000-fold more potent than CAP in the rat isolated mesenteric arterial bed [37]. Taken altogether, these observations and our results suggest the probable existence of multiple vanilloid receptor subtypes.

Previous studies showed than acid-sensitive ion channels (ASIC) and TRPV1 played a role in signalling acid pain [39]. This observation suggests that the pharmacology of these two acid-sensitive proteins is important to investigate new therapeutic pathways. Effectively, NSAIDs inhibit the activity of ASIC in nociceptors [40] and also significantly attenuated acid-evoked pain suggesting a specific interaction between NSAID and acid sensor [41]. In addition, TRPV1, perhaps the most important signal integrator in sensory nociceptors, is well established as a novel target for the treatment of pain [34,42,43]. Extensive profiling of inhibitors of TRPV1 provides intriguing evidence that TRPV1 blockade can be a useful therapeutic approach for inflammatory, cancer and neuropathic pain [42,43]. Moreover, NSAIDs decrease pain caused by inflammation and a low pH environment around sites of inflammation have been proposed as a major contributing factor of inflammatory pain [39]. The standard hypothesis for the mechanism of action of NSAID is by inhibiting cyclooxygenase (COX) enzymes responsible for the production of prostaglandins. While it is possible that these actions may be responsible for the observed effect of ibuprofen on ASCC, we cannot exclude a direct interaction of ibuprofen on ASCC. Nevertheless, we also need to further explore a potential implication of COX enzymes in ibuprofen-induced ASCC regulation by using COX1 and COX2 inhibitors.

However, other mechanisms have been proposed such as interactions of NSAIDs, particularly ibuprofen, with anandamide, an endogenous cannabinoid neurotransmitter with antinociceptive properties [44] and also another agonist of

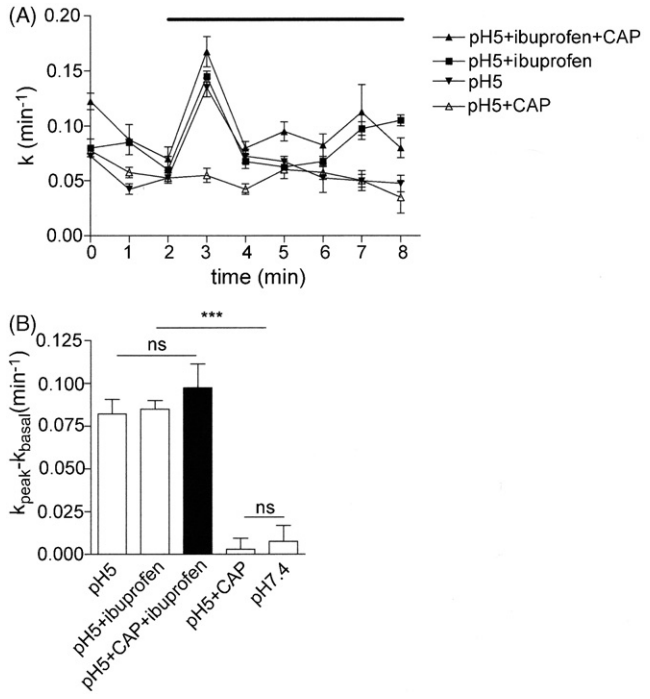

Fig. 5 – Effect of ibuprofen on the activity of ASCC and TRPV1. (A) Time-dependent iodide efflux with cells bathed in a NaCl-rich solution at pH 5 with or without 1 μM CAP or 100 μM ibuprofen as indicated. (B) Mean of rate of efflux for each experimental conditions as in (A). *n* = 4 for each conditions.

TRPV1 [45]. Anandamide has been described to be released by Sertoli cells and could play a role in the hormonal regulation and reproductive functions [11,46]. Moreover, another study has shown that an NSAID like ibuprofen inhibited in a pH-sensitive manner the fatty acid amide hydrolase (FAAH), an enzyme that hydrolyzes anandamide, that could enhance anadamide concentration [47]. Since anandamide has been shown to activate TRPV1 [45], it might be hypothesized that ibuprofen-induced FAAH inhibition would increase the anandamide level and possibly increase TRPV1 activity in Sertoli cells. Likewise, we observed a pH-dependent mechanism in ibuprofen effects; we will continue the study by measuring anandamide in the supernatants derived from Sertoli cells following ibuprofen exposure. Moreover, the fact that ibuprofen abolishes CAP-inhibitory effect suggests an action of ibuprofen on TRPV1 but further experiments will be needed to determine the effects of ibuprofen on intracellular calcium concentration in the presence or absence of CAP and to understand the possible relationship between ibuprofen, TRPV1 and ASCC.

In conclusion, we describe in the present study the first evidence for the regulation of ASCC by TRPV1 in rat Sertoli cells. A relationship was found between the activation of TRPV1 and the inhibition of ASCC by CAP, and conversely, between the inhibition of TRPV1 and the potentiation of ASCC by CPZ. Moreover the NSAID ibuprofen prevents the effect of capsaicin on ASCC. These results suggest that TRPV1 may be considered as a physiological regulator of ASCC in these cells and, in regard of the results obtained with ibuprofen, as a potential target for the pharmacological treatment of reproductive disorders.

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