Published in final edited form as:
Mol Cell Biochem. 2009 July ; 327(1-2): 15–19. doi:10.1007/s11010-009-0036-2.

The identification of a caffeine-induced Ca^{2+} influx pathway in rat primary sensory neurons

João Paulo L. Daher,
Department of Pathology, School of Medicine, Fluminense Federal University, 303 Marquês do Paraná Street, Room 4, Niterói, RJ 24033-900, Brazil

Tony D. Gover,
Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine, Baltimore, MD, USA

Thais H. V. Moreira,
Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine, Baltimore, MD, USA

Vânia G. S. Lopes, and
Department of Pathology, School of Medicine, Fluminense Federal University, 303 Marquês do Paraná Street, Room 4, Niterói, RJ 24033-900, Brazil

Daniel Weinreich
Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine, Baltimore, MD, USA

João Paulo L. Daher: jpldaher@hotmail.com

Abstract

Caffeine-induced Ca^{2+} transients (CICTs) in rabbit nodose ganglion neurons (NGNs) are produced by two distinct mechanisms: release from intracellular stores via ryanodine receptors and Ca^{2+} influx across the plasma membrane, due to activation of an unknown receptor. In isolated rat NGNs, we used single-cell microfluorimetry to measure changes in intracellular Ca^{2+} and to test whether TRPV1 receptors underlie the Ca^{2+} influx pathway. Caffeine (10 mM) evoked CICTs in all NGNs tested ($n = 47$) averaging 365 ± 32 nM. CICTs were partially dependent upon a Ca^{2+} influx pathway that ranged between 33% and 98% of the total Ca^{2+} transient. Application of two selective TRPV1 antagonists significantly attenuated CICTs. The peak average amplitudes of CICTs in Ca^{2+}-free Locke solution and Ca^{2+}-free Locke solution with IRTX or with BCTC were not significantly different from one another ($n = 5$ and 7, respectively). These observations suggest that caffeine can induce Ca^{2+} influx by activating TRPV1 channels.

Keywords

Caffeine; Ca^{2+} Influx; TRPV1; CICR; Nodose ganglia neurons; Vagus nerve

Introduction

Caffeine can produce an elevation in intracellular Ca^{2+} (Ca^{2+} transients), in a variety of peripheral and central neurons [1]. In rabbit primary vagal sensory neurons (nodose ganglion

Correspondence to: João Paulo L. Daher, jpldaher@hotmail.com.

neurons, NGNs), caffeine evokes Ca^{2+} transients by activating two distinct pathways [2]. In all NGNs, caffeine induces Ca^{2+} transients by activating ryanodine receptors (RyR) resulting in Ca^{2+} efflux from the endoplasmic reticulum [2, 3]. In about 50% of the rabbit NGNs, caffeine-induced Ca^{2+} transients (CICTs) are also produced by an additional pathway: a Ca^{2+} influx pathway through the plasma membrane. This pathway remains functional when intracellular Ca^{2+} stores is depleted, or when Ca^{2+}-induced Ca^{2+} release (calcium induced calcium release, CICR) is blocked by ryanodine; it is absent when extracellular Ca^{2+} is nominally zero and it is independent of store-operated Ca^{2+} channels [2]. To date, the nature of this influx pathway has not yet been resolved.

The vanilloid family of transient receptor potential channels (TRPVs) are a good candidate for the Ca^{2+} influx pathway mediated by caffeine. The TRPV1 subfamily is highly permeable to Ca^{2+} and is modulated by a wide range of disparate molecules [4–7]. Moreover, TRPV1 channels are present and functional in 70–85% of rat NGNs [8, 9]. In the present work, we used specific antagonists of TRPV1 channels to test whether TRPV1 might function as an influx pathway activated by caffeine in rat NGNs.

Experimental procedure

Dissociation and culture of NGNs

Male Sprague-Dawley rats (120–200 g) were killed by CO_2 inhalation as approved by the Institutional Animal Care and Use Committee of the University of Maryland, Baltimore. The NGNs were dissociated enzymatically and mechanically as described previously [10]. Briefly, ganglia were rapidly dissected from animals and the connective tissue surrounding the ganglia was removed. Whole ganglia were then incubated in an enzyme solution containing 10 mg collagenase type 1A (Sigma Chemical Co., St Louis, MO) and 10 mg dispase II (Sigma Chemical Co.) in 10 ml of Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS). After a 75-min incubation (37°C), NGN were dissociated by trituration with fire-polished Pasteur pipettes of decreasing tip diameters. Cells were collected by centrifugation (3 times 700*g* for 45 s). Enzyme solutions were replaced by L15 culture medium (Gibco, BRL, Rockville, MD) and 10% foetal bovine serum (JRH Bioscience, Lenexa, KN).

The dissociated NGN were re-suspended in culture medium and plated on poly-D-lysine (0.1 mg ml^{-1}, Sigma Chemical Co.) coated circular 25-mm glass coverslips (Fisher, Newark, DE). After 2 h incubation at 37°C, the coverslips were placed in a room temperature incubator to prevent neurite growth. NGNs were used for experiments up to 48 h in culture.

Calcium recordings

Neurons were superfused with a Locke solution (21–24°C) with the following composition (mM): 136 NaCl, 5.6 KCl, 1.2 NaH_2PO_4, 14.3 $NaHCO_3$, 1.2 $MgCl_2$, 2.2 $CaCl_2$, and 10.0 dextrose, equilibrated with 95% O_2–5% CO_2 and adjusted to pH 7.2–7.4 with NaOH. For experiments where nominally Ca^{2+}-free Locke solution was required, $CaCl_2$ was substituted with $MgCl_2$.

Coverslips were placed in custom fabricated recording chamber with a narrow rectangular flow path (200 μl) and superfused via a gravity-flow system (4 ml/min). Solution changes were complete within 14 s, as determined with fluorescent tracers. Prior to recording, coverslips containing NGNs were incubated with 1 μM fura-2 AM for 60 min. The recording chamber was mounted on an inverted microscope (TE200; Nikon, Tokyo, Japan) equipped with a UV-transmitting objective (SuperFluor, 40×, N.A. 1.4, Nikon). Fura-2 was alternately excited by 340 and 380 nm light from monochrometers (Deltascan Illumination System, Photonic Technology International (PTI), South Brunswick, NJ) and fura-2

emission was detected by a photomultiplier tube (PMT, D-104 microscope photomultiplier, PTI). Felix 1.1 software (PTI) was used for control and synchronization of the monochrometers and PMT.

Data analysis

$[Ca^{2+}]_i$ was derived using the ratio method described previously [11]. Data were analysed and plotted using SigmaPlot 2000 (SPSS, Chicago, IL). Statistics were performed with SigmaStat 2.0 (SPSS) and values are presented as mean ± SEM. To determine statistical significance, one-way ANOVAs were performed with Student–Newman–Keuls to determine significance for pair-wise comparisons. $P < 0.05$ indicated statistical significance.

Reagents

Most drugs were dissolved in vehicle at 1000 times the final concentration and kept frozen in aliquots. *N*-(4-t-Butylphenyl)-4-(3-chloropyridin-2-y1) tetrahydropyrazine-1(2H)-carboxamide (BCTC), purchased from Biomol (Plymoouth Meeting, PA), and iodoresiniferatoxin (IRTX), purchased from Tocris (Ellsville, MO), were dissolved in ethanol. All other reagents were purchased from Sigma Chemical Co. (Sigma Chemical Co., St Louis, MO).

Results

Nodose ganglion neurons from adult rats revealed a robust Ca^{2+} transient upon brief application of caffeine (10 mM, 15 s) in all cells tested, with a peak average amplitude of 365 ± 32 nM ($n = 47$). The amplitudes of the CICTs were consistent over time. In four neurons, application of caffeine (10 mM) every 250 s produced Ca^{2+} transients that were not significantly different ($P < 0.05$) from one another with average amplitudes of 218 ± 20, 221 ± 24 and 213 ± 35 nM for the first, second and third application, respectively (Fig. 1a). To determine whether extracellular Ca^{2+} contributed to the CICTs in rat NGNs, we stimulated NGNs with caffeine (10 mM) in normal and in Ca^{2+}-free Locke solution. The magnitude of the CICTs was significantly attenuated in nominally Ca^{2+}-free Locke solution: 304 ± 32 vs. 122 ± 29 nM; 54 ± 9% reduction ($n = 6$; Fig. 1b). After washing the NGNs with normal Locke solution, the CICT amplitudes returned to control values, 286.4 ± 28 nM. These results indicate that, like rabbit NGNs, rat NGNs also possess a caffeine-induced Ca^{2+} influx pathway.

To determine if TRPV1 underlies the caffeine-induced Ca^{2+} influx pathway, we used specific antagonists of the TRPV1 channel, iodoresiniferatoxin (IRTX, 100 nM; EC50 ~4 nM) and *N*-(4-t-butylphenyl)-4-(3-chloropyridin-2-yl) tetrahydropyrazine-1(2H)-carboxamide (BCTC, 175 nM; EC50 ~35 nM) [12–14]. Both antagonists inhibited capsaicin-evoked Ca^{2+} transients 100% ($n = 4$ for each antagonist, data not shown). IRTX and BCTC both significantly attenuated the amplitude of the CICTs, 45 ± 8% ($n = 9$) and 33 ± 4% ($n = 8$), respectively (Fig. 2, Table 1). The inhibition of the CICT produced by these TRPV1 antagonists was not significantly different from the inhibition observed by switching to nominally zero Ca^{2+} Locke solution, recorded in the same cell (Fig. 2a, Table 1).

Next, we examined whether TRPV1 antagonists were capable of blocking caffeine-induced Ca^{2+} influx. We compared the amplitudes of CICTs recorded in Ca^{2+}-free Locke solution and in Ca^{2+}-free Locke solution containing either IRTX or BCTC (Fig. 2b). The data shown in Table 1 indicate that the peak average amplitudes of CICTs in Ca^{2+}-free Locke solution and Ca^{2+}-free Locke solution with IRTX or with BCTC were not significantly different from one another. Finally, the percentage inhibition produced by IRTX, BCTC or zero Ca^{2+} (45 ± 8%, 33 ± 4% and 54 ± 9%, respectively) was not significantly different from one

another. These pharmacological data suggest that caffeine-induced Ca^{2+} influx is due to the activation of TRPV1.

Discussion

Calcium induced calcium release is mediated by the activation of RyR and caffeine is a classic pharmacological agonist of the ryanodine receptor [15–17]. In rabbit NGNs, caffeine elicits a Ca^{2+} transient by causing Ca^{2+} release from the endoplasmic reticulum and by triggering Ca^{2+} influx across the plasma membrane [2]. Our results using rat NGNs indicate that caffeine can evoke an influx pathway in this species as well (Fig. 1 and "Results" section). Electrophysiological studies in rabbit NGNs demonstrated that the Ca^{2+} influx pathway was through a non-selective cationic channel [2]. This non-selective cationic channel has not been identified. In the present study, we provide pharmacological evidence suggesting that the non-selective cation channel may be TRPV1.

Using two chemically distinct TRPV1 antagonists, IRTX and BCTC, we demonstrated that the CICTs were significantly attenuated by 45 ± 8% and 33 ± 4%, respectively. The amplitudes of the CICTs recorded in the presence of the TRPV1 antagonists were statistically similar to those elicited in nominally Ca^{2+}-free Locke solution. Moreover, addition of a TRPV1 antagonist to a nominally Ca^{2+}-free Locke solution did not produce a diminution in the CICT amplitude. Thus, we surmise that TRPV1 channels are likely mediators of the caffeine-induced Ca^{2+} influx pathway. To our knowledge, this is the first report of a TRP channel being activated by caffeine. Whether caffeine acts directly or indirectly to gate the TRPV1 channel is not yet known.

In a separate series of experiments, we studied the effects of caffeine in HEK293 cells stably transfected with TRPV1. These cells did not show CICTs, although they responded to the TRPV1 agonist capsaicin (data not shown). These results suggest that accessory proteins or lipids resident to NGNs, but not to HEK293 cells, might make TRPV1 channels responsive to caffeine (for a recent example of accessory proteins and lipids controlling TRP channels function, see references) [7, 18]. Alternatively, another TRP channel sensitive to IRTX and BCTC could underlie caffeine-induced Ca^{2+} influx in NGNs. In this regard, it is noteworthy that in the CNS of TRPV1 −/− knockout mice ^{3}H-RTX binding sites are greatly reduced but not eliminated, suggesting the existence of other vanilloid receptors [19].

We observed that 100% of the NGNs tested had CICT indicating that 100% of the cells should contain TRPV1 receptors and respond to capsaicin. Indeed, in our sample of six NGNs all responded to capsaicin. The literature reveals a lower percentage of rat NGNs responding to capsaicin. In isolated NGNs, ~60% of the cells showed capsaicin-evoked Ca^{2+} transients [20]. Using caged capsaicin analogs, we observed that ~85% of isolated adult NGNs produced Ca^{2+} transients [9]. Finally, in intact nodose ganglia 60–70% of the cells were depolarized by capsaicin [8]. The larger percentage of NGNs responding to capsaicin in our study may be due to choosing NGNs with small diameters which have been reported to show a greater response to capsaicin than larger cells [20]. Additionally, our dissociation techniques could have favoured the survival of small diameter neurons.

Acknowledgments

We would like to thank Drs. Carlos Magno Daher, Salim Kanaan and K.·S. Jagannatha Rao for general support for this project. This work was supported by NIH grant NS-22069 (D.W.).

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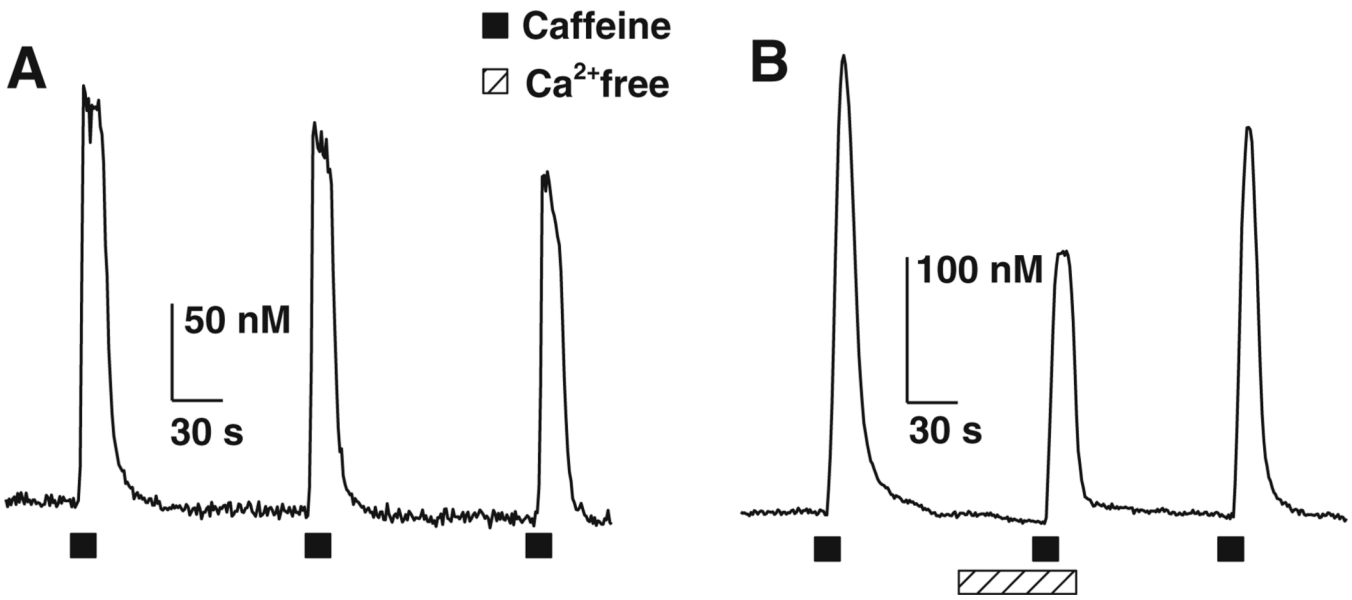

Fig. 1.

Caffeine-induced Ca^{2+} transients (CICTs) are partially dependent upon extracellular Ca^{2+}. **a** Reproducibility of CICTs. Three representative CICTs evoked by three 15-s pulses of 10 mM caffeine in normal Locke solution. The CICTs had an average peak amplitude of 218 ± 20, 221 ± 24 and 212 ± 34 nM ($n = 4$) for the first, second and third application of caffeine, respectively. **b** CICTs elicited by caffeine (10 mM) in the presence or in the absence of extracellular Ca^{2+}. The first and third CICTs were evoked by caffeine in normal Locke solution. The middle CICT was evoked by caffeine in a Locke solution containing nominally zero Ca^{2+}. The difference in amplitude between the averaged control CICTs and the second CICTs represents the magnitude of Ca^{2+} influx across the plasma membrane. The amplitude of the CICTs recorded in nominally Ca^{2+}-free Locke solution averaged 45 ± 9% of the amplitude of control CICTs ($n = 6$) recorded in normal Lock solution. *Solid squares* depict time of caffeine application; *hatched bar* depicts time when the neurons were superfused with nominally Ca^{2+}-free Locke solution

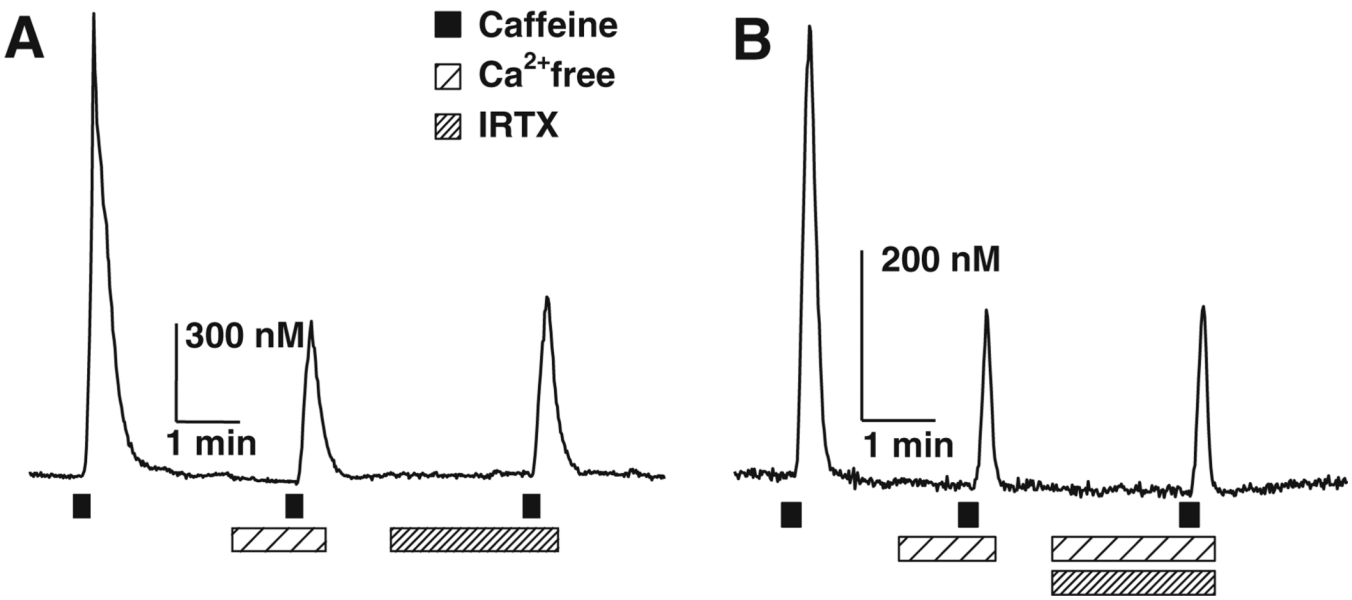

Fig. 2.

Effect of IRTX, a TRPV1 selective antagonist on the Ca^{2+} influx component of caffeine-induced Ca^{2+} transients (CICTs). **a** CICTs elicited by 10 mM caffeine in the presence or absence of IRTX (100 nM). IRTX significantly attenuated CICTs. The average CICT amplitude recorded in IRTX was 45 ± 8% of the control CICTs recorded in normal Ca^{2+} Locke solution ($P = 0.001$, $n = 9$). **b** CICTs elicited by 10 mM caffeine in nominally Ca^{2+}-free Locke containing IRTX. Addition of IRTX did not significantly reduce the amplitude of CICT beyond that produced by nominally Ca^{+}-free Locke solution alone ($n = 5$)

Table 1

Effect of nominally zero Ca^{2+} and TRPV1 antagonists on caffeine-evoked Ca^{2+} transients recorded in isolated nodose ganglion neurons

Control	**IRTX**	**Zero Ca^{2+} Locke**	**Zero Ca^{2+} Locke + IRTX**	***n***
304 ± 32	–	122 ± 29		6
405 ± 114	205 ± 53	220 ± 55		9
398 ± 73	–	244 ± 53	231 ± 53	5
Control	BCTC	Zero Ca^{2+} Locke	Zero Ca^{2+} Locke + BCTC	*n*
572 ± 73	395 ± 64	382 ± 59		8
395 ± 33	–	253 ± 25	245 ± 35	7

Each row represents a separate experimental series. TRPV1 antagonists, IRTX and BCTC, were used at 100 and 175 nM, respectively. Caffeine (10 mM) was applied for 15 s to evoke caffeine-induced Ca^{2+} transients (CICTs) measured with fura-2. Caffeine was applied at 1.5–3 min intervals. Drug or nominally zero Ca^{2+} Locke solution preceded the caffeine pulses by 0.5 and 2 min, respectively (see Figs. 1 and 2). The values are in nM. Zero Ca^{2+} significantly reduced the amplitude of CICT, row 1. The amplitude of the CICT in the presence of IRTX was not significantly different from that recorded in zero Ca^{2+} and both treatments were significantly different from control values, row 2. Addition of IRTX to zero Ca^{2+} Locke solution did not significantly change the amplitude of CICT. The amplitude of CICT recorded in zero Ca^{2+} Locke or IRTX plus zero Ca^{2+} Locke solution was significantly different from control values, row 3. BCTC significantly reduced the amplitude of CICT and its effect was not significantly different from that measured in zero Ca^{2+} Locke solution, row 4. The amplitude of the CICT in the presence of BTCT was not significantly different from that recorded in zero Ca^{2+} Locke solution, but both treatments were significantly different from control values, row 4. Addition of BCTC to zero Ca^{2+} Locke solution did not significantly reduce the amplitude of the CICTs, row 5. Statistical significance was assessed with an one-way ANOVA performed with Student–Newman–Keuls post hoc test to determine significance for pair-wise comparisons. $P < 0.05$ indicated statistical significance. Data in row 1 were compared with a paired Student *t* test; *n* represents the number of nodose ganglion cells studied in each row