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Neuroscience Letters 377 (2005) 125–129
Neuroscience Letters
www.elsevier.com/locate/neulet

The extracellular calcium-sensing receptor increases the number of calcium steps and action currents in pituitary melanotrope cells

Maarten J.J. van den Hurk*, Bruce G. Jenks, Eric W. Roubos, Wim J.J.M. Scheenen

Department of Cellular Animal Physiology, Institute for Neuroscience, Radboud University Nijmegen, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands

Received 4 October 2004; received in revised form 19 November 2004; accepted 26 November 2004

Abstract

Secretion of α-melanophore-stimulating hormone (α-MSH) from the neuroendocrine melanotrope cells in the intermediate lobe of the pituitary gland of the clawed frog *Xenopus laevis* is regulated by various inhibitory, stimulatory and autocrine factors. The neuropeptide sauvagine stimulates α-MSH secretion by changing the pattern of intracellular Ca^{2+} oscillations and the electrical properties of the cell membrane. In the present study we investigated whether another secreto-stimulator, the extracellular Ca^{2+}-sensing receptor (CaR), also affects the Ca^{2+} oscillatory pattern and electrical membrane properties. Using high-speed dynamic video-imaging we show that activation of the CaR with the specific agonist L-phenylalanine (L-Phe) changes the Ca^{2+} oscillatory pattern by increasing the number of Ca^{2+} steps, which are the "building blocks" of the oscillations. Moreover, using patch-clamp electrophysiology it is demonstrated that L-Phe affects membrane properties by increasing frequency and duration of action currents. Compared to sauvagine, the CaR has different effects on the action current parameters, suggesting that multiple mechanisms regulate the electrical properties of the melanotrope cell membrane and, thereby, the Ca^{2+} oscillation-dependent level of α-MSH secretion.

Keywords: Calcium-sensing receptor; Calcium oscillations; Action current firing; Excitability; Melanotropes; Pituitary

The South African clawed toad *Xenopus laevis* adapts its skin color to the light intensity of the environment. This background adaptation process is regulated by α-melanophore-stimulating hormone (α-MSH), secreted by the neuroendocrine melanotrope cells in the pars intermedia of the pituitary gland. Melanotrope cells are under extensive regulation by a large number of stimulatory, inhibitory and autocrine factors [11,16], and their secretory activity can be stimulated by various neurotransmitters, such as thyrotropin-releasing hormone, noradrenalin, acetylcholine, brain-derived neurotrophic factor, corticotropin-releasing factor (CRF), urocortin 1 and sauvagine, an amphibian homologue of mammalian CRF [11,16]. α-MSH secretion by *Xenopus* melanotrope cells is driven by intracellular Ca^{2+} oscillations that depend on Ca^{2+} influx through voltage-operated Ca^{2+} channels [9,18]. The Ca^{2+} oscillations are built up by Ca^{2+} steps that are generated by burst-like firing of action currents [12,13]. We have previously shown that sauvagine increases the number of Ca^{2+} steps in an oscillation but does not affect other step parameters, like step amplitude and the time interval between steps [5]. Sauvagine also increases frequency and duration of the action currents [5].

Recently, we demonstrated that the *Xenopus* melanotrope cell expresses the extracellular Ca^{2+}-sensing receptor (CaR) and that activation of this receptor stimulates secretion of α-MSH [21]. The CaR is a G protein-coupled receptor that is expressed in a wide variety of organs, including the parathyroids, thyroid, kidney, intestine, bone, brain and pituitary gland [2]. The CaR regulates numerous processes, such as cell proliferation, secretion and excitability [2]. In neurons, the CaR modulates excitability by changing the activities of different types of ion channels, such as Ca^{2+}-activated K^+ channels and non-selective cation channels (NCCs) in rat hippocampal pyramidal neurons [22,24,25]. In several types of endocrine cell the CaR modulates hormone secretion by

* Corresponding author. Tel.: +31 24 3652705; fax: +31 24 3652714.
E-mail address: M.vandenHurk@science.ru.nl (M.J.J. van den Hurk).

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doi:10.1016/j.neulet.2004.11.084

changing intracellular Ca^{2+} levels and excitability. In G-cells of the stomach, beta-cells of the pancreas and parafollicular cells of the thyroid, the CaR increases secretion of gastrin, insulin and serotonin, respectively, by stimulating NCCs [3,14,20]. In addition, in parathyroid cells the CaR inhibits parathyroid hormone secretion by inhibiting K^+ channels [10,23]. In somatotropes, melanotropes and tumor cells of the pituitary gland the CaR is thought to stimulate hormone secretion by increasing intracellular Ca^{2+} levels, but if this receptor affects excitability is unclear [6,7,15,19,21,27].

The aim of the present study was to determine if the CaR affects the Ca^{2+} step pattern and action current firing of the *Xenopus* melanotrope cell similarly or differently to sauvagine. For this purpose, studies on single melanotrope cells were carried out, using high-speed dynamic video-imaging and patch clamp electrophysiology.

Young adult (6 months) specimens of *X. laevis* were bred and reared under standard conditions in the aquatic facility of the Department of Animal Physiology, Radboud University Nijmegen. Prior to the experiments, animals were adapted to a black background for three weeks, under constant illumination, at 22 °C. All experiments were carried out under the guidelines of the Dutch law concerning animal welfare.

Animals were anaesthetized in tap water containing 1 g/l MS222 (Sigma, St. Louis, MO, USA) and 1.5 g/l $NaHCO_3$ (pH 7.4). Blood cells were removed by perfusion with *Xenopus* Ringer's solution (112 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 15 mM Ultral-HEPES, 2 mg/ml glucose, pH 7.4) containing 0.25 mg/ml MS222. Neurointermediate pituitary lobes were dissected and collected in 1 ml *X. laevis* Leibovitz's culture medium (XL15, containing 67% Leibovitz's culture medium (Life Technologies, Paisley, UK), 33% Milli Q water, 10 mg/ml kanamycine, 10 mg/ml antibiotic/antimitotic (Life Technologies), 2 mM $CaCl_2$ and 10 mM glucose; pH 7.4). After washing the lobes 4 times with XL15, they were incubated for 45 min in 1 ml Ringer's solution without $CaCl_2$, containing 0.25% trypsin. Trypsin action was stopped by adding 9 ml XL15 containing 10% fetal calf serum (FCS). Lobes were dispersed by gentle trituration using a siliconized Pasteur's pipette. The suspension was filtered through a nylon gauze (pore size 150 μm) to remove undissociated neural lobe tissue, and isolated cells were collected by centrifugation (50 g, 10 min). For high-speed dynamic video-imaging studies, the pellet was resuspended in 20 μl XL15 per lobe equivalent, and cells were plated in the middle of a LabTek Chambered Coverglass (Nalge Nunc Int., Naperville, IL, USA) coated with poly-L-lysine. For electrophysiology, cells were plated on a Ø15 mm coverslip (Menzel-Gläser, Braunschweig, Germany) coated with poly-L-lysine. After cells had been allowed to attach to the glass for an hour, 2 ml XL15/10% FCS was added to each chamber/coverslip and cells were cultured for 2 days in a humidified atmosphere, at 22 °C.

Cells were washed with Ringer's solution and loaded for 30 min with 6 μM fura-2/AM (Molecular Probes Inc., Eugene, OR, USA) in Ringer's solution containing 0.3 mg/ml BSA and 1 μM Pluronic F127 (Molecular Probes Inc.). After loading, cells were washed with Ringer's solution for at least 20 min to allow complete de-esterification of the dye, and placed on an inverted microscope (Axiovert 135 TV; Zeiss, Göttingen, Germany). Then, the chamber was connected to a superfusion system (0.6 ml/min). Cells were imaged using a 40× oil-immersion objective (Zeiss Fluar, N.A. 1.30). They were alternately excited by 340 and 380 nm light for 50 ms, generated by a 150 W Xenon lamp (Ushio UXL S150, Cypress, CA, USA) connected to a monochromator (Polychrome IV, Till Photonics, Martinsried, Germany). Emission light was directed through an LP440 filter (Till Photonics) and imaged with a monochrome digital camera (Coolsnap fx, Roper Scientific, Tucson, AZ, USA). Data were collected and analyzed with Metafluor imaging software (Universal Imaging Corporation, Downingtown, PA, USA). For this, individual cells were selected using areas of interest and the intensities of the fluorescent emitted light were stored, and off-line processed using Origin 6.0 (Microcal Software Inc., Northampton, MA, USA). Changes in intracellular Ca^{2+}-concentrations were expressed as ratio values of the 340/380 nm fluorescence intensity.

Exogenous Ca^{2+} buffers like fura-2 may alter the amplitude and kinetics of the Ca^{2+} oscillations [8,17]. In a previous study we investigated the possible effect of fura-2 on global Ca^{2+} step amplitude [13] and found only a very small decrease in relative amplitude during loading with fura-2 in concentrations up to 100 μM. Furthermore, a depolarising potassium pulse appears to induce a much higher Ca^{2+} oscillation peak compared to a control oscillation [13]. These findings indicate that changes in $[Ca^{2+}]_i$ in *Xenopus* melanotropes lie within the dynamic range of the fura-2 signaling, meaning that the fura-2 fluorescence signal is not saturated during the global Ca^{2+} oscillations, and can be used to reveal changes in $[Ca^{2+}]_i$.

Cell-attached patch-clamp experiments were recorded on an EPC-9 patch clamp amplifier controlled by pulse-pulsefit software (v. 8.53; HEKA, Lambrecht/Pfaltz, Germany). Data was filtered by a Bessel filter set at 12 kHz. Patch pipettes were pulled from Wiretrol II glass capillaries (Drummond Scientific, Broomall, PA, USA) using a Narishige PP-83 pipette puller (Narishige Scientific Instrument Laboratories, Tokyo, Japan) and had a resistance of 3–6 MΩ after polishing. Action currents were recorded in the cell-attached recording configuration of the patch-clamp technique, as described previously [13]. During recordings the pipette potential was set to 0 mV. Patch pipettes were filled with Ringer's solution.

Analysis of the fluorescence ratio values of the Ca^{2+} imaging experiments was performed with Origin 6.0 (Microcal Software Inc.) and Excel software (Microsoft Corp.). Three parameters of the Ca^{2+} steps were analyzed: (1) Ca^{2+} step frequency (number of Ca^{2+} steps per min), (2) Ca^{2+} step amplitude (highest value minus lowest value of Ca^{2+} step), (3) Ca^{2+} step time interval (period between the starts of two successive steps). For each cell, the mean step amplitude and mean step interval were calculated on the basis of 20 steps