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SCIENCE DIRECT
General and Comparative Endocrinology 145 (2006) 140–147
GENERAL AND COMPARATIVE ENDOCRINOLOGY
www.elsevier.com/locate/ygcen

Receptors for neuropeptide Y, γ-aminobutyric acid and dopamine differentially regulate Ca^{2+} currents in *Xenopus* melanotrope cells via the G_i protein β/γ-subunit

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Received 6 May 2005; revised 25 July 2005; accepted 19 August 2005
Available online 7 October 2005

Abstract

Secretion of α-melanophore-stimulating hormone (αMSH) from pituitary melanotrope cells of the amphibian *Xenopus laevis* is under inhibitory synaptic control by three neurotransmitters produced by the suprachiasmatic nucleus: γ-aminobutyric acid (GABA), neuropeptide Y (NPY) and dopamine (DA). These inhibitory effects occur through G_i-protein-coupled receptors (G_iPCR), and differ in strength: $GABA_B$-receptor-induced inhibition is the weakest, whereas DA (via a D_2-receptor) and NPY (via a Y_1-receptor) strongly inhibit, with NPY having a long-lasting effect. Previously it was shown that DA inhibits two (R- and N-type channel) of the four voltage-operated Ca^{2+} channels in the melanotrope, and that only part of this inhibition is mediated by β/γ-subunits of the G_i protein. We here demonstrate that also the Y_1- and $GABA_B$-receptor inhibit only part of the total Ca^{2+} current (I_{Ca}), with fast activation and inactivation kinetics. However, $GABA_B$-mediated inhibition is weaker than the inhibitions induced via Y_1- and D_2-receptors (−21 versus −27% and −30%, respectively). Using a depolarizing pre-pulse protocol it was demonstrated that $GABA_B$-induced inhibition of I_{Ca} most likely depends on Gβ/γ-subunit activation whereas Y_1- and D_2- induced inhibitions are only partially mediated by Gβ/γ-subunits. No differences were found between the Y_1- and D_2-induced inhibitions. These results imply that activation of different G_iPCR inhibits the I_{Ca} through different mechanisms, a phenomenon that may underlie the different potencies of the suprachiasmatic neurotransmitters to inhibit αMSH secretion.

Keywords: NPY Y_1-receptor; $GABA_B$-receptor; DA D_2-receptor; G_i-protein-coupled receptor; Gβ/γ-modulation; Voltage-operated calcium current; Melanotrope cell; *Xenopus laevis*

The South African toad, *Xenopus laevis*, adapts its skin color to changed environmental light intensities. In skin melanophores, melanin dispersion is stimulated by α-melanophore-stimulating hormone (αMSH) released from the neuroendocrine melanotrope cells in the pars intermedia of the pituitary gland (Jenks et al., 2003; Roubos et al., 2005). Multiple neural messengers converge on the melanotrope cell to regulate αMSH release and biosynthesis of its precursor pro-opiomelanocortin (POMC). Evidence indicates that when *Xenopus laevis* is moved from a black to a white background, the suprachiasmatic nucleus releases three inhibitory factors, dopamine (DA), neuropeptide Y (NPY) and γ-aminobutyric acid (GABA), from axon terminals contacting the melanotropes, to reduce αMSH secretion (e.g., Berghs and Roubos, 1996; De Rijk et al., 1992; Jenks et al., 1998, 2003; Tuinhof et al., 1994; Ubink et al., 1998). DA acts on *Xenopus* melanotrope cells via a D_2-receptor (Verburg-Van Kemenade et al., 1986a,b) while NPY binds an Y_1-receptor and GABA activates both $GABA_A$- and $GABA_B$-receptors (Verburg-Van Kemenade et al., 1986a; Scheenen et al., 1994, 1995; Jenks et al., 1998). The G_i-protein-coupled receptors (G_iPCR), Y_1, $GABA_B$ and D_2 (Jenks et al., 1991; Kongsamut et al., 1991; Shibuya and Douglas, 1993), inhibit the melanotrope cell to different degrees:

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doi:10.1016/j.ygcen.2005.08.006

NPY and DA inhibit αMSH release stronger than GABA, NPY-inhibition lasts much longer than the DA- and GABA-inhibitions (Leenders et al., 1993), and Y_1 and D_2 both potently inhibit POMC biosynthesis whereas $GABA_B$ does not affect POMC biosynthesis at all (Dotman et al., 1996).

αMSH secretion is driven by transient increases in the cytoplasmic Ca^{2+} concentration, the so-called Ca^{2+} oscillations (Scheenen et al., 1994, 2003). All three G_iPCR inhibit these Ca^{2+} oscillations but, similarly to the regulation of POMC-biosynthesis and αMSH secretion, differences in inhibitory strength occur as the $GABA_B$-induced inhibition is completely reversed by elevating the intracellular cAMP concentration whereas the inhibitions by NPY and DA are not (Lieste et al., 1996).

Ca^{2+} oscillations depend on voltage-operated Ca^{2+} currents (I_{Ca}) during action potentials (Lieste et al., 1998; Jenks et al., 2003) that are initiated by various types of calcium channel. D_2-receptor activation partially inhibits the I_{Ca}, involving the G_i-protein β/γ-subunit (Zhang et al., 2004, 2005). Since Y_1-, $GABA_B$- and D_2-receptors are all G_i-protein-coupled receptors, we hypothesize that the different strengths with which these neurotransmitters inhibit *Xenopus* melanotrope cells depend, at least in part, on differences in the way the Gβ/γ-subunit of their respective receptors control the I_{Ca}. This hypothesis has been tested by using the whole-cell voltage-clamp patch-clamp technique and a pre-pulse facilitation protocol to assess Gβ/γ-subunit activity (see Zhang et al., 2004).

1. Materials and methods

1.1. Animals

Juvenile *Xenopus laevis* (age: 6 months) were reared in our laboratory under standard conditions and kept in filtered 22 °C tap water under constant illumination. They were fed weekly with beef heart, and adapted to a black background for three weeks before performing experiments. All experiments were carried out under the guidelines of the Dutch law concerning animal welfare.

1.2. Melanotrope cell culture

Cells were prepared as described previously (Scheenen et al., 2003). In short, after anaesthetization, animals were perfused with *Xenopus* Ringer's solution (112 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 15 mM Hepes, 10 mM glucose; pH 7.4) containing 0.025% (w/v) MS222 (Sigma Chemical, St. Louis, MO, USA), to remove blood cells. The neurointermediate lobe of the pituitary gland was rapidly dissected and rinsed four times in *Xenopus laevis* (XL)-L15 culture medium consisting of 67% L15 medium (Invitrogen, Paisley, UK), 1% kanamycin, 1% antibiotic/antimycotic solution (Life Technologies, Rockville, MD, USA), 2 mM $CaCl_2$, 10 mM glucose (pH 7.4) and 31% MilliQ. After incubating for 45 min in *Xenopus* Ringer's solution without $CaCl_2$ but with 0.25% trypsin (Life Technologies), the enzymatic reaction was stopped by adding XL-L15 containing 10% fetal bovine serum (Invitrogen) and lobes were dissociated by gentle trituration. Per cell suspension, four to six lobes were pooled. A suspension was then filtered and centrifuged at 50 g for 10 min. The cell pellet was resuspended in XL-L15 (100 μl/lobe equivalent) and the cells were plated on a round, glass coverslip coated with poly-L-lysine (Mw > 300 kDa; Sigma Chemical). After allowing the cells to attach to the glass surface for 1 h at 22 °C, 2 ml XL-L15 containing 10% fetal bovine serum was added, and cells were kept in a humidified atmosphere, for 3 days at 22 °C, before use.

1.3. Electrophysiological experiments

Electrophysiological recordings were performed using an EPC-9 patch clamp amplifier and Pulse-pulsefit software (v.8.63, HEKA, Lambrecht/Pfaltz, Germany). Data were filtered by a Bessel filter set at 12.9 kHz. Patch pipettes were pulled from Wiretrol II glass capillaries (Drummond Scientific, Broomall, PA, USA) using a PP-83 pipette puller (Narishige Scientific Instrument Laboratories, Tokyo, Japan), and had a resistance between 3 and 5 MΩ after polishing.

To record Ca^{2+} current in the whole-cell voltage-clamp patch-clamp configuration, the intracellular solution contained 100 mM CsCl, 2 mM $CaCl_2$, 10 mM EGTA, 2 mM MgATP, 0.1 mM cAMP and 10 mM Hepes (pH set to 7.2 with CsOH) and the extracellular solution contained 10 mM $CaCl_2$, 15 mM Hepes, 90 mM TEACl and 2 mM $MgCl_2$ (pH set to 7.4 with TEAOH). To investigate the effects of Y_1-, $GABA_B$- and D_2-receptor activations, the total I_{Ca} was elicited by 250 ms test pulses to 10 mV from an holding potential (HP) of −80 mV, applied at 15 s intervals. For checking Gβ/γ-subunit involvement, a pre-pulse protocol including two test pulses as mentioned above and a short, strong depolarization from an HP of −80 to 100 mV, were used at 15 s intervals (Zhang et al., 2004). The strong depolarization lasted 75 ms and was applied 500 ms after the first test pulse and 15 ms before the second test pulse. Currents were recorded and corrected for leak using the internal algorithm of the EPC-9 amplifier ($P/n = 4$). All experiments were carried out at 20 °C.

1.4. Drugs and application

Drug-containing solutions were applied by a 12-channel valve pressure system (ALA DAD-12, Scientific Instruments, New York, NY, USA) through a small tube placed at a distance of 100 μm from the cells. The level of the bath solution was kept constant by means of a suction device. Y_1-, $GABA_B$- and D_2-receptors were activated with *Xenopus* NPY, the $GABA_B$-receptor agonist baclofen and the D_2-receptor agonist apomorphine, respectively (Dotman et al., 1996). Apomorphine was preferred above DA, as it is not rapidly broken down in in vitro conditions. *Xenopus*