Melanin-concentrating hormone reduces somatolactin release from cultured goldfish pituitary cells

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Abstract

Melanin-concentrating hormone (MCH)-containing neurons directly innervate the adenohypophysis in the teleost pituitary. We examined immunohistochemically the relationship between MCH-containing nerve fibres or endings and somatolactin (SL)-producing cells in the goldfish pituitary. Nerve fibres or endings with MCH-like immunoreactivity were identified in the neurohypophysis in close proximity to the adenohypophysial cells showing SL-like immunoreactivity. We also examined the effect of MCH on SL release from cultured goldfish pituitary cells and SL synthesis using a cell immunoblot and a real-time PCR method. Treatment of individually dispersed pituitary cells with MCH 10^{-7} M for 3 h decreased the area of SL-like immunoreactivity on immunoblots, and MCH-induced reductions in SL release were blocked by treatment with the mammalian MCH receptor (MCHR) antagonist, compound-30, at a concentration of 10^{-5} M. Treatment with 10^{-7} M MCH for 3 h did not affect sl-α and -β (smtla and -b as given in the Zfin Database) mRNA expression levels. These led us to explore the signal transduction mechanism leading to the inhibition of SL release, for which we examined whether MCH-induced reductions in SL release are mediated by the G_{i/o} or G_{q} protein-coupled signalling pathway. The MCH-induced reductions in SL release were abolished by treatment with the G_{i/o} protein inhibitors, NF023 (10^{-5} M) or pertussis toxin (260 ng/ml), but not by the phospholipase C inhibitor, U-73122 (3×10^{-6} M). These results indicate that MCH can potentially function as a hypothalamic factor suppressing SL release via the MCHR, and subsequently through the G_{q} protein to inhibit the adenylyl cyclase/cAMP/protein kinase A-signalling pathway in goldfish pituitary cells.

Introduction

Melanin-concentrating hormone (MCH) was first isolated from the chum salmon pituitary during an attempt to identify a novel pituitary hormone that could modulate body colour in fish (Kawauchi et al. 1983, Kawazoe et al. 1987). MCH from fish species such as the barfin flounder, bonito, chinook and coho salmon, rainbow trout, Japanese eel, tilapia and goldfish have been shown to be 17-residue peptides that have a cyclic structure due to a disulphide bond linkage, and feature highly conserved amino acid sequences (Kawauchi & Baker 2004, Takahashi et al. 2004, Cerdá-Reverter et al. 2006). Subsequently, MCH was also found in the brain of tetrapod animals (Mouri et al. 1993, Nahon 1994). Phylogenetically, MCH is widely present throughout the chordate phylum, and its primary structure is highly conserved among vertebrates (Kawauchi & Baker 2004, Kawauchi 2006). MCH receptors (MCHR), including MCH receptor-1 (MCHR1) and -2 (MCHR2) (rodents lack MCHR2), were first identified from an orphan G-protein-coupled receptor (called SLC1) and a human bacterial artificial chromosome library whose natural ligand was MCH. These receptors have been shown to be widely expressed in various tissues, especially in the brain (Saito et al. 1999, Wang et al. 2001). While MCH has no evident function in providing pigmentation, it is now recognised to be involved in the regulation of food intake and energy homeostasis in mammals (Qu et al. 1996, Rossi et al. 1997, Shimada et al. 1998, Ludwig et al. 2001, Gomori et al. 2003, Shi 2004).

Several reports have described the distribution and physiological functions of MCH in fish. Neuronal cell bodies with MCH-like immunoreactivity are distributed mainly in the thalamus and hypothalamus including the paraventricular organ (PVO) and the nucleus lateralis tuberis (NLT), and their fibres project to the neurohypophysis of teleosts (Vallarino...
et al. 1989, 1998, Baker et al. 1995, Amano et al. 2003, Pandolfi et al. 2003, Amiya et al. 2007, Matsuda et al. 2009a,b). These reports suggest that nerve fibres containing MCH-like immunoreactivity arise from the thalamic and hypothalamic areas, and terminate mainly in the neurohypophysis, and that MCH acts as a peripheral hormone affecting body colour in fish. MCH also affects α-melanocyte-stimulating hormone (α-MSH) release from cultured tilapia pituitary cells (Gröneveld et al. 1995) and gonadotropin secretion from dispersed goldfish pituitary cells (Cerdá-Reverter et al. 2006). In cichlid fish, MCH-containing nerve fibres or endings seem to innervate the adenohypophysial cells including somatolactin (SL)-producing cells, and MCH affects SL release from cultured pituitaries (Cánepe et al. 2008).

Recently, respective orthologues of mammalian MCH-R1 and -R2 were also characterised from zebrafish and pufferfish in silico (Logan et al. 2003). Our recent studies have actually demonstrated that goldfish and barfin flounders possess two orthologues of Mhr1 and -r2 (Takahashi et al. 2007, Mizusawa et al. 2009), and have indicated that, in goldfish Mchr-transfected mammalian cells, the Mchr2 signalling pathway is partially sensitive to pertussis toxin (PTX; Mizusawa et al. 2009). The goldfish has been widely used as an animal model for studies of the effect of hypophysal factors on the release and synthesis of adenohypophysial hormones. Our previous studies have indicated that pituitary adenylate cyclase-activating polypeptide (PACAP), which is a hypothalamic peptide that is present in nerves that innervate the adenohypophysis, induces SL release from cultured goldfish pituitary cells (Matsuda et al. 2008, Azuma et al. 2009). However, there is little information about the effect of MCH on SL release from the pituitary in goldfish. It is well known that SL, recently identified in the pars intermedia (pi) of fish and comprised of two subtypes, SL-α and -β (smtla and smtli as listed in the Zfin Database) (Ono et al. 1990, Rand-Weaver et al. 1992, Kaneko 1996, Zhu et al. 2004), is involved in the regulation of steroidogenesis, water–mineral balance and body pigmentation, although a functional analysis of SL in fish is still under way (Planas et al. 1992, Lu et al. 1995, Kakizawa et al. 1997).

The aim of the present study was to elucidate the functional relationship between MCH and SL in the teleost pituitary using the goldfish. We investigated the morphological relationship between MCH-containing nerve fibres and SL-producing cells in the pituitary, and examined the effect of MCH on SL release from dispersed and cultured pituitary cells using a cell immunoblot method.

Materials and Methods

Animals

Goldfish (Carassius auratus, body weight 10–25 g) of both sexes were purchased from a commercial supplier, and kept for 2 weeks under controlled 12 h light:12 h darkness conditions with the water temperature maintained at 20–24 °C. The fish were fed once per day at noon with a uniform granular diet (containing 32% protein, 5% dietary fat, 2% dietary fibre, 6% minerals and 8% water, Tetragold, Tetra GmbH, Melle, Germany) until used in experiments. All animal experiments were conducted in accordance with the University of Toyama guidelines for the care and use of animals.

Double immunohistochemistry for MCH and SL

For immunostaining of MCH and SL, we used rabbit primary antisera raised against salmon MCH (anti-MCH serum, diluted 1:8000; Naito et al. 1985) and salmon SL (anti-SL serum, diluted 1:12 000; Rand-Weaver et al. 1992). Amino acid sequence of salmon Mch is identical to that of goldfish Mch (Cerdá-Reverter et al. 2006). Primary structure of salmon SL has 44–72% homology to those of goldfish Sl-α and -β (GenBank accession no. EU580712 and U72940). Previous pre-absorption tests of these antisera resulted in no specific staining; the specificity of each antiserum has been well demonstrated in the teleost pituitary, and checked by immunoblotting analysis, enzyme immunoassay and RIA (Naito et al. 1985, Rand-Weaver et al. 1992). Double-immunostaining for MCH and SL was used for the simultaneous localisation of multiple tissue antigens, and involved repeating the elution and indirect immunohistochemical procedures for each substrate (Nakane 1968). Six goldfish of both sexes were used. In brief, fish were anesthetised with MS-222 (3-aminobenzoic acid ethyl ester, Sigma–Aldrich Co.), and each brain with the pituitary was removed immediately, immersed in Bouin’s fixative at 4 °C for 48 h, trimmed, dehydrated and embedded in paraffin. Sagittal sections (thickness 6-μm) were then cut. Several sections containing the hypothalamo-pituitary region were treated with normal swine serum (diluted 1:50, Dako A/S, Glostrup, Denmark) and then stained with anti-MCH serum at 4 °C overnight. Following a 1-h incubation with biotinylated swine anti-rabbit immunoglobulin (diluted 1:200, Dako), the sections were treated with avidin–biotinylated swine anti-rabbit immunoglobulin (diluted 1:8000; Naito et al. 1985) and salmon SL (anti-SL serum, diluted 1:12 000; Rand-Weaver et al. 1992). Amino acid sequence of salmon Mch is identical to that of goldfish Mch (Cerdá-Reverter et al. 2006). Primary structure of salmon SL has 44–72% homology to those of goldfish Sl-α and -β (GenBank accession no. EU580712 and U72940). Previous pre-absorption tests of these antisera resulted in no specific staining; the specificity of each antiserum has been well demonstrated in the teleost pituitary, and checked by immunoblotting analysis, enzyme immunoassay and RIA (Naito et al. 1985, Rand-Weaver et al. 1992). Double-immunostaining for MCH and SL was used for the simultaneous localisation of multiple tissue antigens, and involved repeating the elution and indirect immunohistochemical procedures for each substrate (Nakane 1968). Six goldfish of both sexes were used. In brief, fish were anesthetised with MS-222 (3-aminobenzoic acid ethyl ester, Sigma–Aldrich Co.), and each brain with the pituitary was removed immediately, immersed in Bouin’s fixative at 4 °C for 48 h, trimmed, dehydrated and embedded in paraffin. Sagittal sections (thickness 6-μm) were then cut. Several sections containing the hypothalamo-pituitary region were treated with normal swine serum (diluted 1:50, Dako A/S, Glostrup, Denmark) and then stained with anti-MCH serum at 4 °C overnight. Following a 1-h incubation with biotinylated swine anti-rabbit immunoglobulin (diluted 1:200, Dako), the sections were treated with avidin–biotinylated peroxidase complex (ABC, Vector Laboratories Inc., Burlingame, CA, USA) for 1.5 h and then incubated with 10 mg 3,3′-diaminobenzidine–4 HCl (DAB, Sigma–Aldrich) and 0.03% H2O2 in 15 ml 0.05 M Tris–HCl buffer (pH 7.6) containing 150 mM NaCl. After immunostaining for MCH, immunocomplex was removed from its antigen in the following manner. In order to remove anti-MCH serum and anti-rabbit immunoglobulin, sections were reacted with 0.1 M glycine HCl buffer (pH 2.2) for 2 h. After washing with 0.01 M PBS (pH 7.4), sections were immunostained with anti-SL serum at 4 °C overnight, treated with alkaline phosphatase-labelled swine anti-rabbit immunoglobulin (diluted 1:50, Dako) for 1 h, and finally treated with 450 μg nitro blue tetrazolium (NBT, Sigma–Aldrich) and 175 μg 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma–Aldrich) in 1 ml 0.1 M Tris–HCl buffer (pH 9.5) containing


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100 mM NaCl and 5 mM MgCl₂. After all immunostaining steps had been completed, the sections were dehydrated, removed the paraffin using xylene, and mounted. The specificity of each immunoreaction in the method was checked by substitution of the respective antiserum with PBS or replacement of each antiserum with non-immune rabbit serum (diluted 1:500, Dako). In all cases, objective immunoreactions were negative, and there was no overlap of colour development by DAB and NBT/BCIP. Immunostained sections were observed with the aid of a light microscope (BH40, Olympus, Tokyo, Japan) and digital images were recorded using a digital camera (CoolPix 995, Nikon, Tokyo, Japan).

**Pharmaceutical reagents**

In order to examine the effect of MCH on SL release from cultured pituitary cells, goldfish/salmon MCH (Asp-Thr-Met-Arg-[Cys-Met-Val-Gly-Arg-Val-Tyr-Arg-Pro-Cys]-Trp-Glu-Val-OH; Peptide Institute Inc., Osaka, Japan) and mammalian MCHR antagonist compound-30 (Ac-Arg-[Cys-Met-Ava-Arg-Val-Tyr-Arg-Cys]-NH₂; Peptide International Inc., Louisville, KY, USA; Shearman et al. 2003, Morens et al. 2005) were purchased commercially, dissolved in ultrapure water at 1 mM for storage, and PTX was dissolved in ultrapure water to 100 mM for storage, and PTX was dissolved in ultrapure water to 100 μg/ml and diluted with ultrapure water before use. In order to examine the signalling pathway for MCH, the G_{i/o} protein inhibitors NFO23 (αs-Ν-(2-phenylcyclopentyl) azacyclotri-dec-1-en-2-amino hydrochloride; Calbiochem, La Jolla, CA, USA) and PTX (Sigma–Aldrich), and the phospholipase C inhibitor U-73122 were dissolved in dimethyl sulphoxide at 0.1% for storage, and PTX was dissolved in ultrapure water at 100 μg/ml and diluted with ultrapure water before use.

**Primary cell culture and cell immunoblot method**

In order to examine the effect of MCH on SL release from goldfish pituitary cells, the cell immunoblot method was carried out according to previous reports (Arita et al. 1993, Cimini et al. 1994, Szabó et al. 2002, 2004) but was modified for use on isolated goldfish pituitary cells (Matsuda et al. 2008, Azuma et al. 2009). This method allowed for the semi-quantitative measurement of SL release from individual cells (Matsuda et al. 2008, Azuma et al. 2009). In brief, goldfish were anesthetised with MS-222, decapitated and the whole pituitaries were cut into small pieces and transferred into Leibovitz’s L-15 medium containing 25 mM HEPES pH 7.4, 10% foetal bovine serum (Sigma–Aldrich), 100 μg/ml streptomycin (MBC, Tokyo, Japan) and 100 U/ml penicillin (MBC), 3 mM EDTA and 2500 U/ml collagenase type I (Worthington Biochemical Co., Lakewood, NJ, USA). After mechanical and enzymatic dispersion, the suspension was centrifuged at 200 g for 10 min and the supernatant removed. Ten pituitaries were used in each experiment. Dispersed cells were then re-suspended in Leibovitz’s L-15 medium containing 25 mM HEPES pH 7.4, 10% foetal bovine serum (Sigma–Aldrich), 100 μg/ml streptomycin (MBC) and 100 U/ml penicillin (MBC) at 27 °C for 24 h to avoid contamination and degradation of the pituitary cells. The number of cells in the suspension was then calculated and the density of cells was adjusted to 5 × 10⁵ cells/500 μl. A total of 500 μl of the suspension were plated in the cassette chamber beneath the PVDF membrane (Hybond-P, polyvinyliden difluoride transfer membrane, GE Healthcare Bio–Sciences Corp., Piscataway, NJ, USA), and MCH, or an equivalent volume of ultrapure water (control), was added to the culture medium at concentrations of 10⁻¹¹, 10⁻⁹ and 10⁻⁷ M. The incubation was continued for 3 h at room temperature in a humidified atmosphere with filtered air. After incubation, the PVDF membrane was collected from each cassette chamber. For immunostaining of the SL in the PVDF membrane, we used anti-SL serum (diluted 1:8000). Previous pre-absorption tests of anti-SL serum resulted in no specific staining; the specificity of this antiserum has been well demonstrated in teleost pituitaries and checked by immunoblotting analysis and RIA (Rand–Weaver et al. 1992, Matsuda et al. 2005a, b, 2008). In brief, each membrane was treated with 10% BSA (Sigma–Aldrich) in 0·1 M Tris–HCl, pH 7·4, 0·1 M NaCl and 0·1% Tween–20 (TBS–T) for 1·5 h, washed with TBS–T, and then stained with anti-SL serum (diluted 1:8000) at 4 °C overnight. Following a 1-h incubation with biotinylated swine anti-rabbit IgG (diluted 1:200), each membrane was treated with ABC for 1·5 h and then incubated with DAB and 0·03% H₂O₂ in 15 ml 0·05 M Tris–HCl buffer (pH 7·6) containing 150 mM NaCl. Immunostained membranes were observed under a light microscope (BH40), and digital images were recorded with a digital camera (CoolPix 995). After converting to grey-coloured images, the density, total area and number of the immunoblots were measured using the Scion Image freeware program (Scion Corporation, Frederick, MD, USA).

**Effect of mammalian MCHR antagonist on MCH-reduced SL release**

In order to examine the effect of mammalian MCHR antagonist on MCH-reduced SL release from goldfish pituitary cells, a cell immunoblot method was carried out as described above. MCH (10⁻⁸ M) and/or compound-30 (10⁻⁵ M) or an equivalent volume of ultrapure water (control), were simultaneously added to the culture medium and the cells incubated for 3 h. The incubation concentration of compound-30 had been determined in one of our previous experiments using melanin granules in the melano-phores of goldfish scales (Matsuda et al. 2006), and we examined whether compound-30 at graded concentrations of 10⁻⁷, 10⁻⁶ and 10⁻⁵ M alone affects spontaneous
SL release from cultured pituitary cells. After incubation, each PVDF membrane was collected from each cassette chamber and immunostained with the anti-SL serum as described above.

Effect of the G_{i/o} protein inhibitors or the PLC inhibitor on MCH-reduced SL release

In order to examine the effects of the G_{i/o} protein inhibitors and the PLC inhibitor on MCH-reduced SL release from goldfish pituitary cells, the cell immunoblot method was carried out as described above. MCH (10^{-7} M) and/or NF023 (10^{-5} M), PTX (260 ng/ml) or U-73122 (3×10^{-6} M) or an equivalent volume of ultrapure water or vehicle (same concentration of dimethyl sulphoxide diluted with ultrapure water) (control), were added to the culture medium and the incubation continued for 3 h. The incubation concentrations of NF023, PTX and U-73122 have been determined in previous studies (Beindl et al. 1996, Tipsmark et al. 2005, Mizusawa et al. 2009). After incubation, each PVDF membrane was collected from each cassette chamber and immunostained with the anti-SL serum as described above.

Effect of MCH on Sl-α and -β mRNA expression in cultured pituitary cells

Goldfish were anesthetised and their pituitaries were removed. Thirteen pituitaries were dissected out and the pituitary cells were dispersed in Leibovitz’s L-15 medium. In order to examine the effect of MCH on the expression of sl-α and -β mRNAs, cultured cells (6×10^5 cells) were treated with 10^{-7} M MCH or saline for 3 h. After the treatment, the total RNA was extracted from 6×10^5 cells with Isogen (a solution containing phenol and guanidinium thiocyanate; Nippon Gene, Tokyo, Japan). For amplification and quantitation of the cDNA fragments encoding sl-α, -β and β-actin, an one-step reverse transcription PCR (RT-PCR) method (SYBR Green RT-PCR Reagents Kit, Applied Biosystems, Foster City, CA, USA) was used. Reactions (including 5 μM primers, 2×SYBR Green PCR master mix, 6.25 U MultiScribe RT, 2.5 U RNase inhibitor, RNA template and water) were set-up in a 96-well reaction plate and placed in a sequence detection system for cycling (ABI Prism 7000, Applied Biosystems). RT was carried out at 48°C for 30 min and the resulting cDNA subsequently amplified using 40 cycles of 95°C for 15 s followed by 60°C for 60 s. The PCR products in each cycle were monitored using SYBR Green I fluorescence dye (Applied Biosystems). The gene-specific primers for amplification of the sl cDNA fragment were based on the cDNA sequence of goldfish sl-α (GenBank accession no. EU580712). PCR, with the sense primer 5’-CGT CAT CCA ACA CGC AGA GC-3’ and the anti-sense primer 5’-GTT GTG ACA CAT GGT TCC TCC C-3’ yielded a 124-bp product encoding a part of the goldfish sl-α cDNA. The gene-specific primers for amplification of the sl-β cDNA fragment were based on the cDNA sequence of goldfish sl-β (GenBank accession no. U72940). PCR with the sense primer 5’-ACG AGA TGC TCA TTT CAT TCG G-3’ and the anti-sense primer 5’-TGA AGG AGC CAT TTG TCG GA-3’ yielded a 130-bp product encoding part of the goldfish sl-β cDNA. Goldfish β-actin gene-specific primers were used as an internal control for PCR amplification (GenBank accession no. AB039726). Using these primers (sense primer, 5’-CTA CTG ACC GTA TGC AGA-3’; anti-sense primer, 5’-ATG GAG CCA CCA ATC CAG ACA -3’), a 112-bp product encoding part of goldfish β-actin cDNA was amplified. The expression levels of sl-α and -β mRNAs were calculated quantitatively as a ratio to β-actin mRNA expression.

Data analysis

All results are expressed as the mean ± S.E.M. Statistical analysis was performed using one-way or two-way ANOVA with Tukey’s method. Statistical significance was determined at the 5% level.

Results

Double-immunostaining for Mc and sl in the pituitary

A sagittal section view of the pituitary in Fig. 1A shows the structure of the goldfish adenohypophysis and neurohypophysis. Figure 1B and C shows details of the double-immunostaining experiments carried out to identify Mch and sl in the goldfish pituitary. Nerve fibres with Mch-like immunoreactivity were identified throughout the neurohypophysis, with nerve endings of these neurons also identified in regions of the adenohypophysis, such as the rostral pars distalis, proximal pars distalis, caudal pars distalis and pi. The cells showing sl-like immunoreactivity were detected in the pi, and nerve fibres and endings with Mc-like immunoreactivity were identified in close proximity to these cells.

Effects of Mc on Sl release from cultured pituitary cells and of mammalian Mchr antagonist on Mc-reduced Sl release

Figure 2 shows the effect of Mc on Sl release from cultured pituitary cells, and effect of mammalian Mchr antagonist on Mc-reduced Sl release. Incubation of the pituitary cells for 3 h with graded concentrations of Mc at concentrations of 10^{-11}, 10^{-9} and 10^{-7} M induced a dose-dependent reduction in the Sl-like immunoreactive area on the immunoblots. Treatment with 10^{-7} M Mc reduced a significant reduction in the Sl-like immunoreactive area on the immunoblots (Fig. 2A). Treatment with Mc (10^{-7} M) alone for 3 h reduced, relative to treatment with ultrapure


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water (control) or compound-30 alone at $10^{-5}$ M, the Sl-like immunoreactive area on the immunoblots. Incubation of the cells with $10^{-7}$ M Mch alone also produced a significant decrease in the immunoblot area for Sl-like immunoreactivity than was observed with $10^{-7}$ M Mch and $10^{-5}$ M compound-30. Incubation of cells with the same concentration of compound-30 alone had no effect compared to the control (Fig. 2B). Interaction between Mch and compound-30 was shown to be significant by two-way ANOVA with Tukey’s method (df, F and P values, 1, 4.32 and 0.038 respectively), and simple effects were also significant (df, F and P values, 1, 10.65 and 0.001 respectively). The incubation concentration of $10^{-5}$ M for compound-30 was used, because treatment with compound-30 at graded concentrations of $10^{-7}$, $10^{-6}$ and $10^{-5}$ M alone did not affect the spontaneous SL release from cultured pituitary cells (Fig. 2C).

**Effect of Gi/o inhibitors or PLC inhibitor on MCH-reduced SL release in cultured pituitary cells**

Figure 3 shows the effect of the Gi/o inhibitors and the PLC inhibitor on Mch-reduced Sl release from cultured pituitary cells. The Mch-induced decrease in Sl-like immunoreactive area on the immunoblots was blocked by treatment with $10^{-5}$ M NF023 (Fig. 3A). Interaction between Mch and NF023 was shown to be significant by two-way ANOVA with Tukey’s method (df, F and P values, 1, 10.05 and 0.0015 respectively), and simple effects were also significant (df, F and P values, 1, 16.02 and 0.0001 respectively). The Mch-induced decrease in Sl-like immunoreactive area was also abolished by treatment with 260 ng/ml PTX (Fig. 3B). Interaction between Mch and PTX was shown to be significant by two-way ANOVA with Tukey’s method (df, F and P values, 1, 3.85 and 0.0008 respectively). Simple effects were also significant (df, F and P values, 1, 8.87 and 0.003 respectively). In contrast, the Mch-induced decrease in the area of the immunoblots representing Sl-like immunoreactivity was not affected by treatment with U-73122 ($3 \times 10^{-6}$ M; Fig. 3C).

**Effect of Mch on the expression of sl-α and -β mRNAs in cultured pituitary cells**

Figure 4 shows the effect of Mch on the expression of sl-α and -β mRNAs in cultured pituitary cells. The levels of sl-α and -β mRNAs were analyzed by real-time PCR and expressed as ratios relative to the expression of β-actin mRNA. In control and experimental groups, the mean level of sl-β mRNA expression was approximately three times that of sl-α mRNA expression. Treatment with Mch at $10^{-7}$ M, which significantly reduces Sl release, did not affect sl-α and -β mRNA expression levels.
Discussion

This is the first report to describe the physiological and pharmacological details of the inhibitory effect of Mch on Sl release from cultured goldfish pituitary cells. In this species, the distribution of Mch in the brain has been well characterised: Mch-containing neuronal cell bodies are localised in the thalamus and the hypothalamus including the NLT and the PVO, and Mch-containing fibres or endings are distributed in the telencephalon, mesencephalon, diencephalon and neurohypophysis (Huesa et al. 2005, Matsuda et al. 2007, 2009a,b, Shimakura et al. 2008a,b). Mch-containing neurons that originate in the NLT form a magnocellular population and also seem to innervate the pituitary to regulate body colour (Matsuda et al. 2006, 2007). Hypothalamic Mch is also implicated in the regulation of food intake in goldfish (Matsuda et al. 2006, 2007, 2009a,b, Shimakura et al. 2008a,b, Matsuda 2009). The present study indicates that, in this species, Mch-containing nerve fibres or endings which originate from the hypothalamus are found throughout the neurohypophysis and even the adenohypophysis, and, to be more specific, are in close proximity to Sl-producing cells in the pituitary, suggesting that pituitary cells including Sl-producing cells are modulated by Mch. In fact, Mch is able to stimulate gonadotropin release from cultured goldfish pituitary cells (Cerdà-Reverter et al. 2006). In other teleost fish, Mch (10^{-8}–10^{-6} M) inhibits α-Msh release from cultured tilapia pituitary cells while Mch at the relatively high concentrations of 10^{-5}–3.5\times10^{-5} M stimulates α-Msh release, suggesting that under physiological conditions the inhibitory action of Mch on α-Msh release dominates (Gröneveld et al. 1995). A previous study reported a histological relationship between Mch and Sl in the cichlid pituitary and found that Mch at 10^{-7}–10^{-5} M induced Sl release from cultured cichlid pituitaries (Cánepe et al. 2008). There is a discrepancy between results obtained by us and the above report, suggesting that major differences may occur in MCH action between these species, or that it may be due to different culture system.

In the present study, we used a cell immunoblot method to examine the effect of Mch on Sl release from cultured goldfish pituitary cells. This method has been used to examine the regulation of pituitary hormone secretion (Arita et al. 1993, Figure 2).
hormone levels in the goldfish pituitary, we have previously modified this method to examine if PACAP stimulates Sl or Prl release in dispersed and cultured pituitary cells (Matsuda et al. 2008, Azuma et al. 2009). In the present study, we also applied this method to examine whether Mch affects Sl release from cultured goldfish pituitary cells.

We have clearly demonstrated here that Mch reduces Sl release from cultured goldfish pituitary cells. We have also indicated that the Mch-induced reduction in Sl release is blocked by treatment with compound-30, a known mammalian Mchr1 antagonist (Shearman et al. 2003, Morens et al. 2005), showing that compound-30 at graded concentrations of 10⁻⁷, 10⁻⁶ and 10⁻⁵ M alone does not affect spontaneous Sl release from cultured pituitary cells. Our previous study analysed the expression levels of Mchr1 and -r2 mRNAs in goldfish and found that the Mchr1 transcript was detected in all tissues tested, i.e. in the pituitary, brain, eyeball, heart, liver, spleen, intestine, kidney, skin, muscle and fat, and that the Mchr2 transcript was detected in all tissues excluding the liver, spleen and intestine (Mizusawa et al. 2009). We have also demonstrated that the Mch-induced increase in the intracellular calcium concentration in goldfish Mchr2, but not Mchr1, expressing human embryonic kidney (HEK) 293T cells (HEK293T-gfMchr1 and -r2 cells) are partially attenuated by treatment with PTX, suggesting that goldfish Mchr2 is possibly coupled both to the G_i/o and G_q proteins (Mizusawa et al. 2009). Furthermore, our preliminary experiments using HEK293T-gfMchr1 and -r2 cells showed that simultaneous treatment with MCH and compound-30 led to a reduction in the calcium mobilisation through gfMchr2 but not gfMchr1.

There is little published information available about the action and signal transduction of Mch on adenohypophysial hormone release from pituitary cells in goldfish. In the HEK293T-gfMchr2 cells, calcium mobilisation is evoked by MCH via both the PTX-sensitive G_i/o protein as described above and insensitive G_q protein (Mizusawa et al. 2009), suggesting that the goldfish MCH-R2 is coupled to the G_i protein inhibiting the adenylate cyclase (AC)/cAMP/protein kinase A (PKA)-signalling pathway or to the G_q protein subsequently acting through the PLC/inositol 1,4,5-trisphosphate (IP3)/protein kinase C (PKC)-signalling pathway. Therefore, we examined whether the G_i/o protein inhibitors, NF023 and PTX, or the PLC inhibitor, U-73122, affects Mch-reduced Sl release from cultured pituitary cells.

**Figure 3** Effect of G_i/o protein inhibitors (A and B) and PLC inhibitor (C) on Mch-reduced Sl release in cultured goldfish pituitary cells. Each column and bar are expressed as the mean±S.E.M. respectively, and the numbers in parentheses in each panel indicate the number of immunoblots in each experimental or control group. Significance of differences between each group was evaluated by two-way ANOVA with the Tukey’s test (*P<0.05, **P<0.01).

A photomicrograph in each column indicates representative cell immunoblots in each experimental or control group. Scale bar in the photomicrographs is 500 μm.
coupled to PTX-sensitive Gi/o protein and subsequently that through the Mchr, perhaps Mchr2 system, which is coupled to Gs and Gq proteins and subsequently through the PTX-sensitive Gi/o protein. We found that treatment with NF023 or PTX, but not U-73122, abolishes Mch-reduced Sl release from cultured goldfish pituitary cells. Further investigations are warranted. The primary structure of Mch is conserved among vertebrates (Kawauchi & Baker 2004), while PACAP is known to be a most ancient peptide in the glucagon-secretin superfamily of peptides (Lee et al. 2007). This raises the question of why Mch and PACAP regulate the release of Sl, which is an adenohypophysial hormone identified only in the pi of the fish pituitary (Ono et al. 1990, Rand-Weaver et al. 1992).

In conclusion, our investigation indicates that Mch can potentially act as a hypothalamic factor for inhibition of Sl release in the goldfish pituitary. The present study clearly supports the theory that Mch-induced reduction in Sl release is mediated by the Mchr, perhaps Mchr2 system and subsequently through the PTX-sensitive Gs/o protein inhibiting the AC/cAMP/PKA-signaling pathway in cultured goldfish pituitary cells. Further investigations to clarify the regulatory mechanism of Sl release by Mch are warranted.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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