

Melanocortin receptor-mediated mobilization of intracellular free calcium in HEK293 cells

KATHLEEN G. MOUNTJOY, PHILIP L. KONG, JOHN A. TAYLOR, DERRIL H. WILLARD and WILLIAM O. WILKISON

Physiol. Genomics 5:11-19, 2001.

You might find this additional info useful...

This article cites 35 articles, 17 of which can be accessed free at:

<http://physiolgenomics.physiology.org/content/5/1/11.full.html#ref-list-1>

This article has been cited by 10 other HighWire hosted articles, the first 5 are:

The Melanocortin-4 Receptor: Physiology, Pharmacology, and Pathophysiology

Endocrine Reviews, August, 2010; 31 (4): 506-543.

[Abstract] [Full Text] [PDF]

Binding of Guanylyl Cyclase Activating Protein 1 (GCAP1) to Retinal Guanylyl Cyclase (RetGC1) :

Igor V. Peshenko, Elena V. Olshevskaya and Alexander M. Dizhoo

J. Biol. Chem., August 1, 2008; 283 (31): 21747-21757.

[Abstract] [Full Text] [PDF]

The melanocortin system in leukocyte biology

J Leukoc Biol, February 1, 2007; 81 (2): 383-392.

[Abstract] [Full Text] [PDF]

Peripherally administered desacetyl α -MSH and α -MSH both influence postnatal rat growth and associated rat hypothalamic protein expression

Chia-Shan Jenny Wu, David R. Greenwood, Janine M. Cooney, Dwayne J. Jensen, Michele A.

Tatnell, Garth J. S. Cooper and Kathleen G. Mountjoy

Am J Physiol Endocrinol Metab, December 1, 2006; 291 (6): E1372-E1380.

[Abstract] [Full Text] [PDF]

Adrenocorticotropin Evokes Transient Elevations in Intracellular Free Calcium ($[Ca^{2+}]_i$) and Increases Basal $[Ca^{2+}]_i$ in Resting Chondrocytes through a Phospholipase C-Dependent Mechanism

Jodi F. Evans, Chwan-L. Shen, Simcha Pollack, John F. Aloia and James K. Yeh

Endocrinology, July 1, 2005; 146 (7): 3123-3132.

[Abstract] [Full Text] [PDF]

Updated information and services including high resolution figures, can be found at:

<http://physiolgenomics.physiology.org/content/5/1/11.full.html>

Additional material and information about *Physiological Genomics* can be found at:

<http://www.the-aps.org/publications/pg>

This information is current as of May 19, 2011.

Melanocortin receptor-mediated mobilization of intracellular free calcium in HEK293 cells

KATHLEEN G. MOUNTJOY,¹ PHILIP L. KONG,¹ JOHN A. TAYLOR,²
DERRIL H. WILLARD,³ AND WILLIAM O. WILKISON³

¹Research Centre for Developmental Medicine and Biology, Department of Paediatrics,
and ²School of Biological Sciences, University of Auckland, Auckland 1, New Zealand;
and ³Glaxo Wellcome Research Institute, Research Triangle Park, North Carolina 27709

Received 7 October 1999; accepted in final form 21 November 2000

Mountjoy, Kathleen G., Philip L. Kong, John A. Taylor, Derril H. Willard, and William O. Wilkison. Melanocortin receptor-mediated mobilization of intracellular free calcium in HEK293 cells. *Physiol Genomics* 5: 11–19, 2001.—Mouse melanocortin receptors, MC1-R, MC3-R, MC4-R, and MC5-R, when expressed in HEK293 cells and stimulated with either α -melanocyte-stimulating hormone (α -MSH) or desacetyl- α -MSH, mediate increases in intracellular free calcium concentration ($[Ca^{2+}]_i$) with EC_{50} values between 0.3 and 4.3 nM. The increase in $[Ca^{2+}]_i$ is cholera toxin sensitive and pertussis toxin insensitive. The mechanism involves calcium mobilization from intracellular stores without a transient rise in inositol trisphosphate. Mouse agouti protein (55 nM) is a competitive antagonist of α -MSH (6-fold) and desacetyl- α -MSH (8-fold), coupling the mMC1-R to increased $[Ca^{2+}]_i$. Agouti protein (55 nM) significantly increased the EC_{50} for α -MSH (3-fold), and 550 nM agouti protein significantly increased the EC_{50} for desacetyl- α -MSH (4-fold), coupling the mMC4-R to a rise in $[Ca^{2+}]_i$. However, agouti protein antagonism of the MC4-R may not be competitive since there was a trend for the maximum response to also increase. There was no significant antagonism of the MC3-R and MC5-R by agouti protein (55 nM). Understanding the physiological relevance of the transduction of a calcium signal by melanocortin peptides may be important for future development of therapeutic targeting of the melanocortin receptors.

agouti; desacetyl- α -melanocyte-stimulating hormone; α -melanocyte-stimulating hormone

A FAMILY OF FIVE MELANOCORTIN receptors (MC1-R to MC5-R), through which peptides derived from pro-opiomelanocortin function, have been identified. The MC1-R is expressed in melanocytes and plays a role in the pigmentation response (26). The MC2-R is expressed in adrenal cortex and is involved in the stress response (26). Localization of MC3-R (33) and MC4-R (25) mRNA to regions in adult rat brain indicated both receptors may function in neuroendocrine, cardiovascular, and food and water intake regulation. Targeted

disruption of the gene encoding the MC4-R identified a role for this receptor in appetite control, weight regulation, and regulation of linear growth (15). The MC4-R is probably also involved in autonomic control and higher learning processes. The MC5-R regulates exocrine gland function (4).

Overexpression of G protein-coupled receptors in heterologous cell lines is widely used to begin to understand ligand-induced intracellular signaling pathways. Numerous studies have confirmed that all members of the melanocortin receptor family couple to the adenylyl cyclase/protein kinase A (PKA) intracellular signaling pathway when expressed in heterologous cells (10–12, 19, 25, 26, 33). Mouse agouti protein is a competitive antagonist of the mouse MC1-R (mMC1-R) coupling to the PKA signaling pathway and thereby regulates pigmentation (21). Agouti protein also antagonizes the MC4-R coupling to the PKA signaling pathway (21), but in this case agouti protein decreases both the sensitivity and maximum responsiveness of the mouse MC4-R (mMC4-R) to melanocortin peptides (27). Agouti protein antagonism of MC4-R underlies the obesity and non-insulin-dependent diabetes mellitus (NIDDM) phenotype in mice ectopically expressing agouti protein.

A second melanocortin receptor-mediated intracellular signaling pathway, at least for the MC3-R, has also been described (18). Konda et al. (18) showed that low concentrations of melanocortin peptides stimulate a transient rise in inositol trisphosphate (IP_3), and when the PKA pathway is inhibited, high concentrations of melanocortin peptides increase intracellular calcium concentration ($[Ca^{2+}]_i$) in heterologous cells. Very little else is known about melanocortin peptides coupling their receptors to signaling pathways resulting in changes in $[Ca^{2+}]_i$. Agouti protein, however, is associated with changes in intracellular calcium. Agouti protein induces a slow rise in $[Ca^{2+}]_i$ in heterologous cells overexpressing melanocortin receptors (17) and also in isolated skeletal muscle myocytes from wild-type mice (38), differentiated 3T3-L1 adipocytes (16), and the pancreatic β -cell (37). A link between agouti protein, melanocortin receptors, and calcium signaling was strengthened by the finding that $[Ca^{2+}]_i$ is elevated in

Article published online before print. See web site for date of publication (<http://physiolgenomics.physiology.org>).

Address for reprint requests and other correspondence: K. G. Mountjoy, Research Centre for Developmental Medicine and Biology, Dept. of Paediatrics, Univ. of Auckland, Auckland 1, New Zealand (E-mail: kmountjoy@auckland.ac.nz).

skeletal muscle in A^{vy} yellow mice compared with non-agouti (*a/a*) mice (38).

To further understand the functions of melanocortin peptides and their receptors, in particular the mechanism by which the MC4-R influences weight regulation, as well as the mechanism by which ectopic agouti protein drives obesity, it is necessary to know whether the melanocortin receptors couple to a signaling pathway that results in alterations of $[Ca^{2+}]_i$ and whether agouti protein can influence this pathway. We have therefore investigated two naturally occurring melanocortin peptides coupling four different melanocortin receptors to a signaling pathway in HEK293 cells that results in elevation of $[Ca^{2+}]_i$, and we tested for antagonism of this pathway by agouti protein.

METHODS

The melanocortin peptides, desacetyl- α -melanocyte-stimulating hormone (desacetyl- α -MSH) and α -MSH, were purchased from Bachem California. The acetoxyethyl ester of fura-2 (fura-2/AM) and the ionophore, A23187, were purchased from Molecular Probes (Eugene, OR). The production of recombinant mouse agouti protein has previously been described (36). Nifedipine and diltiazem were Sigma products and a gift from Dr. D. R. Mason. [³H]inositol and [5,6,8,9,11,12,14,15-³H(N)]arachidonic acid were purchased from Amersham Life Science (Buckinghamshire, UK). Pertussis toxin and forskolin were purchased from Sigma (St. Louis, MO), and cholera toxin and the Rp isomer of adenosine 3',5'-cyclic monophosphorothioate (Rp-cAMPS) were purchased from Calbiochem.

Cell lines. Stably transfected human embryonic kidney HEK293 cells expressing the mMC1-R have previously been described (26). The coding sequences of mMC3-R and mMC4-R were obtained by probing a mouse genomic library, 129 strain (Stratagene), with the corresponding rat receptor DNA sequences. The mMC5-R cDNA was obtained from brown fat cDNA using RT-PCR with primers specific for this gene (19). DNA coding sequences of the mouse melanocortin receptors (mMC3, mMC4, and mMC5) were subcloned into pcDNAIneo vector (Invitrogen), and these were stably transfected into HEK293 cells using a modified calcium phosphate procedure (3). Stably transfected cells were selected in DMEM containing 10% newborn calf serum (NCS) and 1 mg/ml G418, then grown with 500 μ g/ml G418.

Measurement of inositol phosphates. Inositol phosphates were measured according to a previously published method (29). Cells were plated into 24-well plates at a density of 10^5 cells per well, and on *day 3* the media was aspirated and the cells were prelabeled with 0.7 μ Ci *myo*-[2-³H]inositol per well in 250 μ l DMEM, 10% NCS for 48 h at 37°C. Cells were then washed with 1 ml PBS, warmed to 37°C, and incubated in 250 μ l of 0.1% BSA-DMEM containing 10 mM LiCl for 30 min at 37°C. This media was then aspirated, and the cells were incubated with or without agonists in the same medium for 20 min at 37°C. The reaction was stopped by the addition of 250 μ l ice-cold 10% trichloroacetic acid per well, and the cells were scraped and transferred to Eppendorf tubes on ice. Following vortexing and centrifugation, the cell extracts were transferred to clean Eppendorf tubes and desalted by the addition of 600 μ l of a 1:1 mix of 1,1,2-trichlorotrifluoroethane/tri-*n*-octylamine mixture. The upper phase was removed, and inositol phosphates were separated by ion exchange using 0.5-ml columns of Dowex AG 1-X8 formate form (Bio-Rad) ion-exchange resin. Prior to adding the samples,

the columns were washed with 5 ml water. Following the addition of the samples, the columns were washed with 7.5 ml of 5 mM *myo*-inositol in water. Total inositol phosphates were eluted by adding 1.5 ml of 1 M ammonium formate/0.1 M formic acid and then counted using liquid scintillation spectrometry in a beta scintillation counter (LKB 1219 Rack-Beta).

Measurement of $[Ca^{2+}]_i$. Confluent cells from a T75 culture flask were washed briefly with versene and then detached from the plates by incubation with 2 ml versene at 37°C for 3 min. The cells were diluted in 5 ml DMEM containing 10% NCS and pelleted by centrifugation. The cells were resuspended in DMEM containing 0.1% BSA and 25 mM HEPES, then loaded with fura-2/AM (2 μ M) using gentle rocking for 20 min at 37°C. After loading, the cells were pelleted by centrifugation and washed two times with calcium loading buffer (CLB) (composition, in mM: 132 NaCl, 5 KCl, 5 Na₂HPO₄, 1.2 NaH₂PO₄·2H₂O, 1 CaCl₂, 0.8 MgCl₂, and 1 glucose). Cells from each T75 flask were finally resuspended in 12 ml CLB and maintained at room temperature while experiments were carried out on 1.8-ml aliquots at 37°C in a Hitachi model F-4500 dual-wavelength spectrofluorometer equipped with a thermostated cuvette holder, using previously described methods (32). The excitation wavelength was changed once per second between 340 and 380 nm, and emission was recorded at 510 nm. Fluorescence ratios $R = F_{340}/F_{380}$ were recorded, and $[Ca^{2+}]_i$ was calculated using the equation of Grynkiewicz et al. (14), with $K_d = 224$. Values for R_{min} and R_{max} were determined for each experiment by successive addition of ionomycin (20 μ l of 1 mM stock in methanol) and EGTA (20 μ l of 0.5 M stock in water).

Quenching of fura-2 fluorescence by Mn²⁺ was measured by exciting the dye at 360 nm, where emission measured at 510 nm is independent of ambient calcium concentration. This technique detects agonist-stimulated opening of cation channels in different cell types (9). Thapsigargin was used to test for the release of intracellular calcium. A stock solution of thapsigargin (1 μ M) dissolved in dimethyl sulfoxide (DMSO) was prepared. The rapidly exchangeable calcium pool of the endoplasmic reticulum was empty ~150 s following the addition of thapsigargin to the cuvette (final concentration of 10^{-8} M). The cells were then tested for responses to 10^{-6} M α -MSH or 10^{-3} M carbachol.

Representatives of two chemically distinct classes of organic antagonists of L-type voltage-sensitive Ca²⁺ channels (nifedipine and diltiazem) and two inorganic blocking ions (Cd²⁺ and La³⁺) were used to test whether melanocortin peptides stimulate the entry into cells of extracellular calcium through calcium channels. Stock solutions containing the channel blockers were prepared on the day of each experiment. Nifedipine (2 μ M) was dissolved in DMSO, whereas diltiazem (5×10^{-2} M), CdCl₂ (0.5 M), and La(NO₃)₃ (0.5 M) were dissolved in water. Calcium channel blockers were added to the cuvette ~50 s before the addition of 10^{-6} M α -MSH.

Sensitivity to cholera and pertussis toxin were used to test for G protein-mediated mobilization of $[Ca^{2+}]_i$ by α -MSH, whereas forskolin and Rp-cAMPS, a competitive antagonist of PKA, were used to test for PKA-mediated mobilization of $[Ca^{2+}]_i$. Cholera and pertussis toxin were dissolved in DMEM and added to cells for 16 h at final concentrations of 2 μ g/ml and 100 ng/ml, respectively. Forskolin (10 mM) was dissolved in DMSO. Rp-cAMPS (5 mM) was dissolved in DMEM containing 0.1% BSA and added to the fura-2-preloaded cells 15–45 min prior to testing the cells response for mobilization of $[Ca^{2+}]_i$.

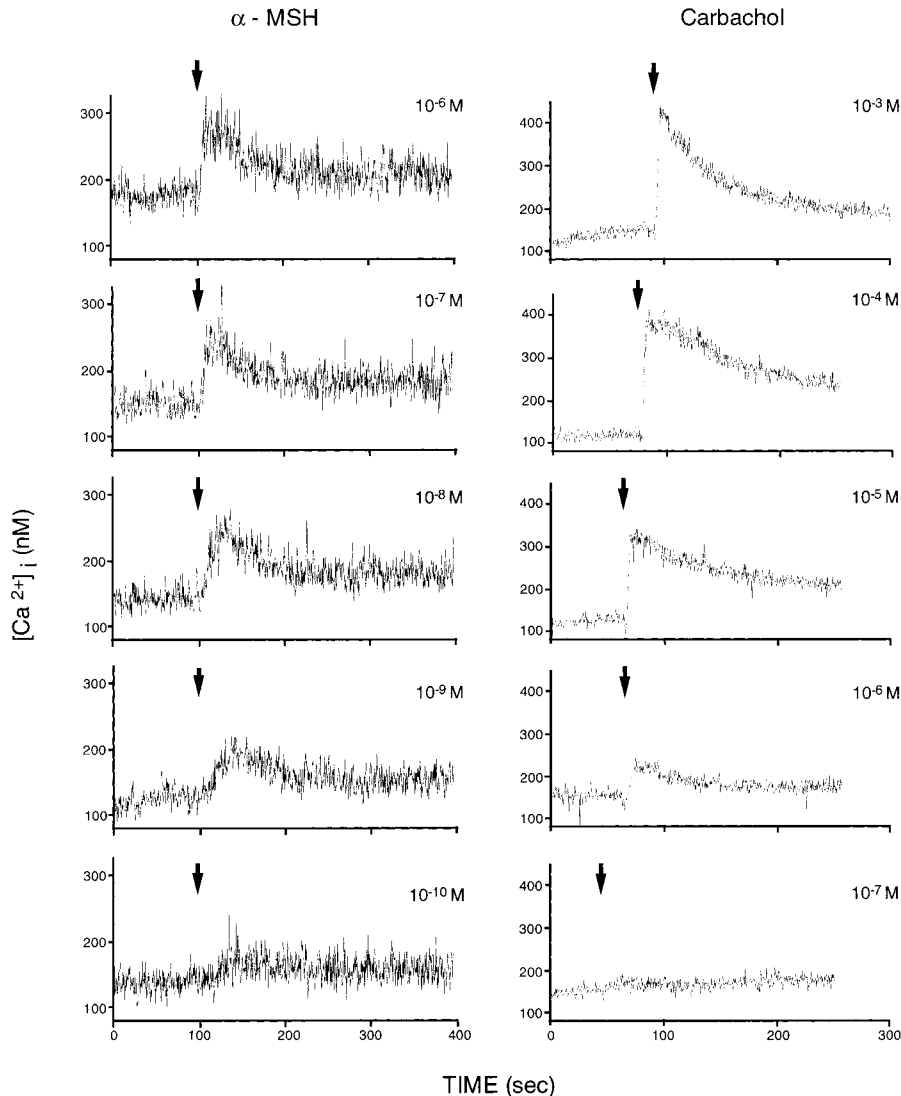


Fig. 1. α -Melanocyte-stimulating hormone (α -MSH, *left*) and carbachol (*right*) stimulate a rise in intracellular free calcium concentration ($[Ca^{2+}]_i$) in HEK293 cells expressing the mouse melanocortin-4 receptor (mMC4-R). The y-axis indicates $[Ca^{2+}]_i$, and the x-axis indicates time. Arrows indicate the time that the agonist was added to the cuvette. Traces shown are representative of >10 separate experiments. Similar data was obtained when HEK293 cells expressing either mMC1, mMC3, or mMC5 receptors were used.

Measurement of agouti antagonism of melanocortin peptide-stimulated increases in $[Ca^{2+}]_i$. Dose response curves were performed pairwise: one without agouti protein and one in the presence of agouti protein. Melanocortin peptides (10^{-10} , 10^{-9} , 10^{-8} , 10^{-7} , and 10^{-6} M) and agouti protein (10, 55, and 550 nM) were diluted DMEM containing 0.1% BSA and 25 mM HEPES. Agouti protein was added 150 s prior to the addition of either α -MSH or desacetyl- α -MSH. The change in $[Ca^{2+}]_i$ for each dose was determined after taking the means of four to seven points at both the baseline and peak responses. The KALEIDAGRAPH software package (Synergy Software, Reading, PA) was used for fitting curves to the data and calculating EC_{50} and maximum response values. The R values for all curves were >0.8.

Statistics. Comparisons between groups were made using Sigmatat (Jandel Scientific) and either one-way ANOVA or the Student's *t*-test.

RESULTS

α -MSH and desacetyl- α -MSH stimulate increases in $[Ca^{2+}]_i$. α -MSH induced a transient rise in $[Ca^{2+}]_i$ in HEK293 cells expressing mMC1, mMC3, mMC4, and mMC5 receptors but not in untransfected HEK293.

This increase was dose dependent, with 10^{-10} M α -MSH having a small effect and a maximum response observed with by 10^{-7} – 10^{-6} M α -MSH (Fig. 1). Desacetyl- α -MSH also coupled each of these receptors to rises in $[Ca^{2+}]_i$. EC_{50} values for the increase in $[Ca^{2+}]_i$ induced by both α -MSH and desacetyl- α -MSH are given in Table 1. Carbachol, through stimulation of endogenous muscarinic receptors in HEK293 cells, also induced a dose-dependent increase in $[Ca^{2+}]_i$ at con-

Table 1. EC_{50} values for melanocortin peptides inducing an increase in mobilization of intracellular calcium in HEK293 cells stably expressing the mouse melanocortin receptors

	EC_{50} , nM			
	MC1-R	MC3-R	MC4-R	MC5-R
α -MSH	$4.3 \pm 1.8(5)$	$1.3 \pm 0.6(4)$	$0.5 \pm 0.3(15)$	$1.3 \pm 0.7(4)$
desacetyl- α -MSH	$2.7 \pm 1.9(6)$	$1.3 \pm 0.3(4)$	$0.3 \pm 0.1(9)$	$1.6 \pm 0.8(4)$

Values are means \pm SD; numbers in parentheses are numbers of dose-response curves. α -MSH, α -melanocyte stimulating hormone.

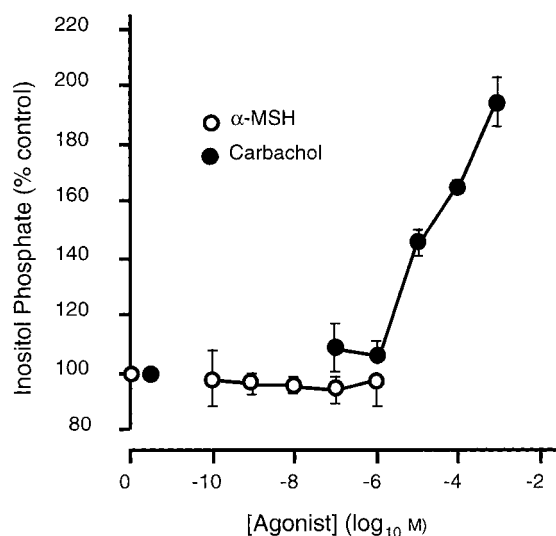


Fig. 2. Carbachol, but not α -MSH, induces inositol phosphates in HEK293 cells expressing the mMC4-R. The y-axis indicates total [3 H]inositol phosphates released after 20 min at 37°C and is expressed as a percentage of control. The x-axis represents the concentration of agonist. The points on the curves are the means \pm SD of 3 separate experiments. For each experiment the points were performed in duplicate. Similar data were obtained when HEK293 cells expressing either mMC1, mMC3, or mMC5 receptors were used.

centrations as low as 10^{-6} M with a peak response observed with 10^{-3} M (Fig. 1). Carbachol induced a classic IP_3 -mediated rise in $[Ca^{2+}]_i$ characterized by a rapid increase, reflecting release from intracellular stores, followed by a subsequent decline. In contrast, the melanocortin peptide stimulation of $[Ca^{2+}]_i$ exhibited a slower increase that was sustained following the maximum response. Examination of inositol phosphate levels in cells following exposure to melanocortin peptides revealed the absence of a detectable increase (Fig. 2).

α -MSH stimulates release of calcium from intracellular stores. The observed rise in $[Ca^{2+}]_i$ (Fig. 1) could be due to release of Ca^{2+} from intracellular stores or reflect an increase in Ca^{2+} influx via plasma membrane channels. Three approaches were taken to distinguish the source of increased cytoplasmic Ca^{2+} in response to melanocortin peptides. First, the Ca^{2+} -ATPase inhibitor, thapsigargin, was used to empty the rapidly exchangeable calcium pool of the endoplasmic reticulum (22). Pretreatment of HEK293 cells with 1 μ M thapsigargin abolished a rise in $[Ca^{2+}]_i$ in response to either α -MSH (10^{-6} M) or carbachol (10^{-3} M) (Fig. 3). Second, we tested the possibility that α -MSH could open Ca^{2+} permeable cation channels on the plasma membrane through which Mn^{2+} (a calcium surrogate) can also enter (22). In the presence of external Mn^{2+} , neither α -MSH nor carbachol induced a decrease in fura-2 fluorescence at its isobestic wavelength (360 nm), whereas each one induced a transient increase in fura-2 fluorescence at 340 nm (Fig. 4). In contrast, ionomycin caused a decrease in fura-2 fluorescence at 360 nm, consistent with quenching of the dye due to Mn^{2+} influx. Third, we tested whether α -MSH caused Ca^{2+} influx through L-type voltage-gated calcium channels in the plasma membrane using antagonist blocking ions, Cd^{2+} and La^{3+} (1 mM), as well as the organic antagonists, nifedipine (10 μ M) and diltiazem (100 μ M). It is unclear why the basal calcium fluctuated for two of these blockers. Importantly, however, all four calcium channel blockers failed to reduce the α -MSH-induced rise of $[Ca^{2+}]_i$, nor did they have any effect on the sustained elevation of calcium levels (Fig. 5).

α -MSH-induced mobilization of $[Ca^{2+}]_i$ is cholera toxin sensitive. Cholera toxin (2 μ g/ml) significantly attenuated 10^{-6} M α -MSH-induced mobilization of $[Ca^{2+}]_i$ in mMC4-R-expressing cells from 105 ± 10 nM (mean \pm SD, $n = 3$) to 20 ± 7 nM ($n = 3$, $P < 0.0003$).

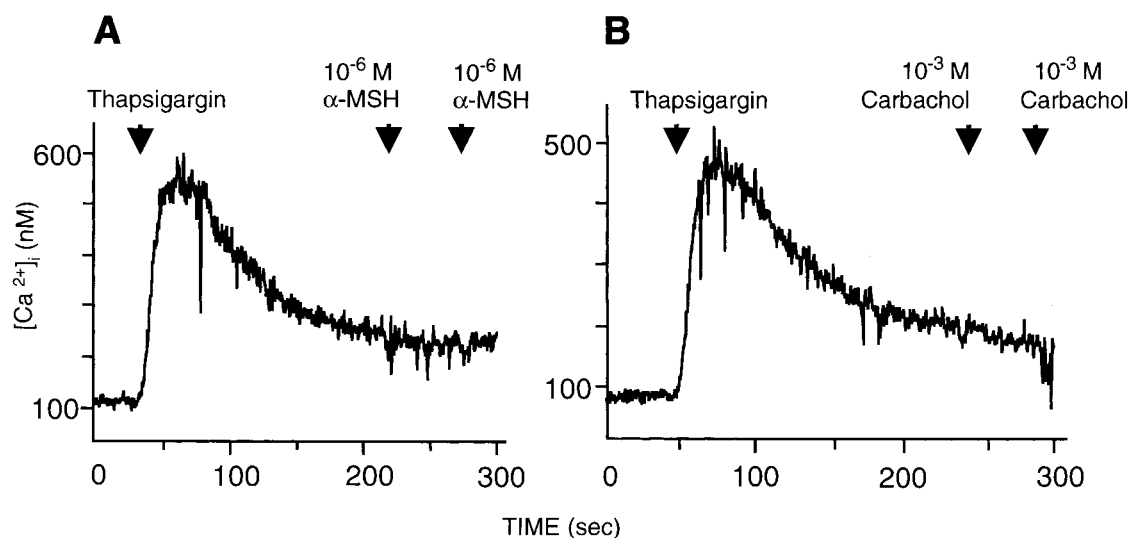


Fig. 3. Thapsigargin pretreatment prevents α -MSH-induced (A) and carbachol-induced (B) changes in $[Ca^{2+}]_i$ in HEK293 cells expressing the mMC4-R. The y-axis represents $[Ca^{2+}]_i$, and the x-axis indicates time. Arrows indicate the time that either thapsigargin (10^{-8} M final concentration) or agonists were added to the cuvette. Curves are representative of 2 separate experiments.

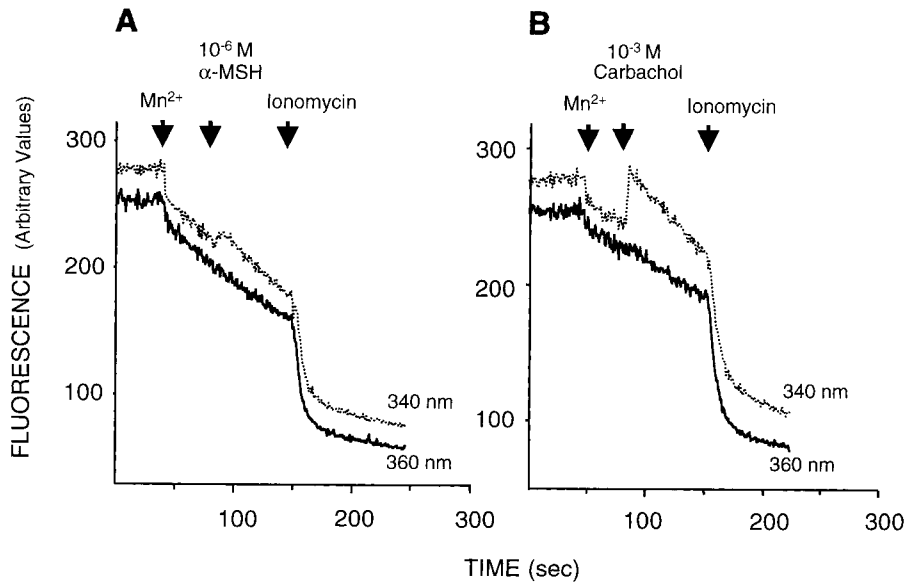


Fig. 4. Manganese does not quench α -MSH- (A) or carbachol-induced (B) changes in $[Ca^{2+}]_i$ in HEK293 cells expressing the mMC4-R. The y-axis indicates the fluorescence at 360 nm (solid line) or 340 nm (dotted line), and the x-axis indicates the time. Arrows indicate the times of the addition of Mn^{2+} (1 mM), α -MSH, carbachol, or ionomycin (11 μ M). Traces shown are representative of 2 separate experiments.

Pertussis toxin (100 ng/ml) had no effect on α -MSH-induced mobilization of $[Ca^{2+}]_i$ in these cells (no pretreatment, 77 ± 32 nM; and 109 ± 25 nM for pertussis toxin treatment; means \pm SD, $n = 3$).

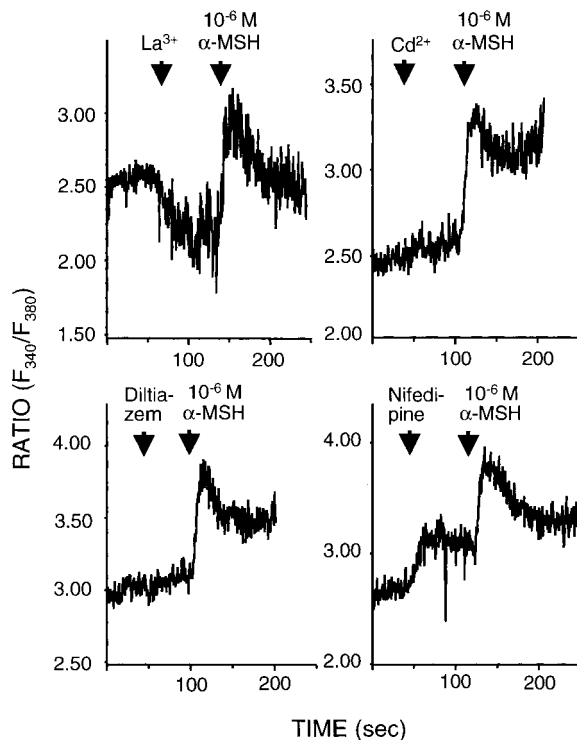


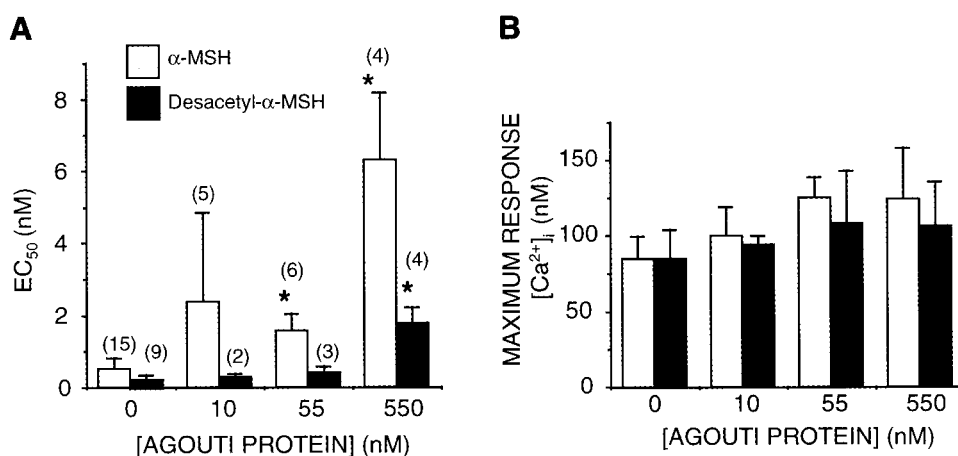
Fig. 5. Blockers of voltage-gated plasma membrane calcium channels did not prevent α -MSH-stimulated increases in $[Ca^{2+}]_i$ in HEK293 cells expressing the mMC4-R. The y-axis indicates the ratio of the fluorescence at 340 nm to the fluorescence at 380 nm. This ratio is directly proportional to the $[Ca^{2+}]_i$. The x-axis indicates the time, and the arrows indicate when the calcium channel blockers and α -MSH were added. The curves are representative of 2 separate experiments (1 mM La^{3+} , 1 mM Cd^{2+} , 10^{-4} M diltiazem, and 10^{-5} M nifedipine).

Rp-cAMPS (5×10^{-5} M) appeared to slightly attenuate 10^{-8} M α -MSH-induced mobilization of $[Ca^{2+}]_i$ in mMC4-R-expressing cells (no treatment, 75 ± 5 nM; and Rp-cAMPS treatment, 66 ± 26 nM; means \pm SD, $n = 3$), but this was not significant. This dose of Rp-cAMPS, however, significantly attenuated 10^{-8} M forskolin-induced mobilization of $[Ca^{2+}]_i$ (no treatment, 36 ± 9 nM; and Rp-cAMPS treatment, 18 ± 2 nM; means \pm SD, $n = 3$, $P < 0.02$).

Mouse agouti protein influences the α -MSH and desacetyl- α -MSH-induced increases in $[Ca^{2+}]_i$ through the mMC1 and mMC4 receptors but not through the mMC3 or mMC5 receptors. Mouse agouti protein (55–550 nM) significantly decreased the sensitivity of α -MSH-mediated increased $[Ca^{2+}]_i$ via mMC4-R (Fig. 6). A relatively high concentration of agouti protein (550 nM) also significantly increased the EC_{50} for desacetyl- α -MSH coupling the mMC4-R to mobilization of $[Ca^{2+}]_i$. The R_{max} values achieved for both α -MSH and desacetyl- α -MSH in the presence of agouti protein, however, appeared to increase but were not significantly different from that achieved in the absence of agouti protein (Fig. 6). The sensitivity of α -MSH and desacetyl- α -MSH coupling of the mMC4-R to the $[Ca^{2+}]_i$ pathway was decreased up to 12- and 4-fold, respectively, by 550 nM mouse agouti protein (Fig. 6). The trend for agouti protein to increase the R_{max} would contribute to the agouti-induced decrease in sensitivity and suggests that agouti protein may not be a competitive antagonist of the mMC4-R, but it influences, and possibly enhances, the ability of melanocortin peptides to couple the MC4-R to the calcium signaling pathway.

Mouse agouti protein (55 nM) antagonized α -MSH-mediated and desacetyl- α -MSH-mediated increases in $[Ca^{2+}]_i$ via mMC1-R (Fig. 7). This antagonism, however, exhibited competitive kinetics, since the R_{max} achieved in the presence of agouti protein was similar

Fig. 6. Agouti protein influences α -MSH and desacetyl- α -MSH coupling the mMC4-R to mobilization of $[Ca^{2+}]_i$. Dose response curves for measuring $[Ca^{2+}]_i$ using α -MSH or desacetyl- α -MSH were performed in the absence or presence of 10, 55, and 550 nM mouse agouti protein, and the resulting curves were analyzed using KALEIDAGRAPH software. EC_{50} (A) and maximum response (B) data points are means \pm SD of multiple separate experiments. The number of experiments is shown in parentheses for each point. Significant differences between the EC_{50} values generated in the absence or presence of agouti protein were determined using one-way ANOVA. * $P < 0.05$.



to that achieved in the absence of agouti. The sensitivity of α -MSH and desacetyl- α -MSH coupling the mMC1-R to the $[Ca^{2+}]_i$ pathway was significantly decreased by six- and eightfold, respectively, by 55 nM mouse agouti protein (Fig. 7). Mouse agouti protein did not significantly antagonize α -MSH or desacetyl- α -MSH coupling the mMC3 and mMC5 receptors to the $[Ca^{2+}]_i$ pathway (Fig. 7).

Mouse agouti protein alone, or DMEM containing 0.1% BSA and 25 mM HEPES alone, induced a small increase in $[Ca^{2+}]_i$ (<30 nM) that was independent of the agouti dose.

DISCUSSION

We show that mouse MC1, MC3, MC4, and MC5 receptors when stably expressed in HEK293 cells and stimulated with either α -MSH or desacetyl- α -MSH, mediate increases in $[Ca^{2+}]_i$ in addition to activating adenylyl cyclase. Desacetyl- α -MSH is more abundant than α -MSH in the brain (5, 28), the fetus (8, 30), human blood (7), and amniotic fluid (23), and the functional responses to desacetyl- α -MSH and α -MSH differ in vivo. For example, subcutaneous injections of desacetyl- α -MSH do not affect pigmentation, and desacetyl- α -MSH is more potent at stimulating growth in vivo than is α -MSH (24, 35). There is, however, little information on desacetyl- α -MSH interactions with melanocortin receptors in vitro. In this study, we have compared and contrasted the ability of desacetyl- α -MSH and α -MSH to couple melanocortin receptors to a calcium mobilization signaling pathway. In contrast to the different in vivo effects of these peptides, desacetyl- α -MSH and α -MSH similarly activate MC1, MC3, MC4, and MC5 receptors coupling to the calcium signaling pathway with EC_{50} values between 0.3 and 4.3 nM.

The increase in $[Ca^{2+}]_i$ is cholera toxin sensitive and pertussis toxin insensitive and is due to mobilization from intracellular stores by a mechanism that does not involve a transient increase in IP_3 . The absence of increased $[IP_3]$, despite a clear increase in $[Ca^{2+}]_i$ in response to α -MSH, suggests the involvement of an alternative second messenger. The parathyroid hor-

mone (34) and dopamine D₁ (20) receptors expressed in HEK293 cells also couple to an intracellular signaling pathway that does not involve IP_3 , but results in Ca^{2+} release from intracellular stores (34). Interestingly, forskolin increases $[Ca^{2+}]_i$ in HEK293 cells, implicating cAMP as the second messenger that mobilizes calcium in these cells (21), and since the forskolin response is attenuated by prestimulation with carbachol, the cAMP-dependent store is considered part of the IP_3 -sensitive calcium pool (20). In hepatocytes, cAMP has also been shown to sensitize IP_3 -mediated release of calcium stores (2). Our studies, however, do not provide strong evidence for the involvement of cAMP as a second messenger for α -MSH-induced mobilization of calcium. First, prestimulation with carbachol (10^{-4} M) did not affect the calcium response to α -MSH (10^{-8} M) (data not shown), and second, 5×10^{-5} M Rp-cAMPS did not significantly attenuate α -MSH-mediated (10^{-8} M) calcium mobilization in HEK293 cells.

In addition to the intracellular Ca^{2+} release channels (IP_3 and ryanodine receptors), Ca^{2+} can be mobilized by arachidonic acid, a product of phospholipase A₂ (PLA₂) activity (1), and also by a novel Ca^{2+} release mechanism activated by nicotinic acid adenine dinucleotide phosphate (NAADP) (6, 13). In this study, we were unable to show any melanocortin peptide-stimulated increase of [³H]arachidonic acid (data not shown), which suggests that melanocortin receptors do not couple to PLA₂ in HEK293 cells. Furthermore, it is unlikely that NAADP is the signaling pathway that melanocortin receptors use to mobilize Ca^{2+} , since NAADP mobilizes this ion from a pool distinct from the endoplasmic reticulum.

A similar response to that reported in our study, namely, a rapid transient elevation of $[Ca^{2+}]_i$ followed by a sustained elevation of $[Ca^{2+}]_i$, was also observed when HEK293 cells expressing human MC1 (hMC1), hMC3, or hMC5 receptors were stimulated with the superpotent synthetic analog of α -MSH, NDP- α -MSH (17). In contrast to our data that compare activation of this pathway by two naturally occurring melanocortin peptides, no dose response curves or EC_{50} values were

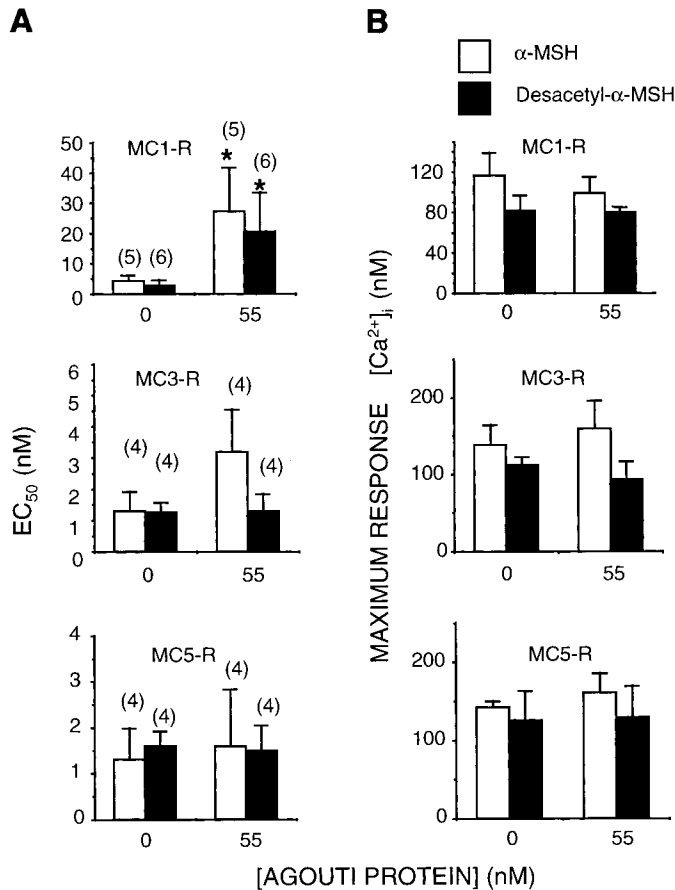


Fig. 7. Agouti protein antagonizes α -MSH and desacetyl- α -MSH coupling the mMC1-R to mobilization of $[Ca^{2+}]_i$ but does not antagonize mMC3-R and mMC5-R. Dose response curves for measuring $[Ca^{2+}]_i$ using α -MSH or desacetyl- α -MSH were performed in the absence or presence of 55 nM mouse agouti protein, and the resulting curves were analyzed using KALEIDAGRAPH software. EC_{50} (A) and maximum response (B) data points are means \pm SD of multiple separate experiments. The number of experiments is shown in parentheses for each point. Significant differences between the EC_{50} generated in the absence or presence of agouti protein were determined using one-way ANOVA. * $P < 0.05$.

reported and nor was agouti antagonism of this pathway studied. This study by Kim et al. (17), however, also characterized a different signaling pathway from that activated by melanocortin peptides: one in which agouti protein induces a slow, gradual rise in $[Ca^{2+}]_i$ that can be blocked with calcium channel blockers (17, 37). In contrast to the transient rise in $[Ca^{2+}]_i$ at ~ 50 s we observed following melanocortin peptide administration, the peak calcium concentration Kim et al. (17) measured was at ~ 400 s after agouti administration. It is unclear whether there is any role for the melanocortin receptors in this agouti-induced elevation of calcium.

A melanocortin receptor-mediated intracellular signaling pathway different from that presented in this report has previously been described. Konda et al. (18) demonstrated that MC3-R stably expressed in Hepa, L, or Chinese hamster ovary cells coupled to a signaling pathway in response to α -MSH, which resulted in a

transient increase in inositol phosphates but no change in $[Ca^{2+}]_i$. Furthermore, when the PKA signaling pathway was inhibited in these cell types, activation of MC3-R by melanocortin peptides produced a sustained rise in $[IP_3]$ at $\geq 10^{-11}$ M α -MSH and an increase in $[Ca^{2+}]_i$ at $\geq 10^{-8}$ M α -MSH. This observation clearly differs from the response we have observed in HEK293 cells, where as little as 10^{-10} M α -MSH increases $[Ca^{2+}]_i$ in the absence of an increase in inositol phosphates.

Mouse agouti protein is a competitive antagonist of mMC1-R coupling to a calcium signaling pathway in response to either α -MSH or desacetyl- α -MSH. Furthermore, agouti protein antagonizes both α -MSH and desacetyl- α -MSH coupling mMC1-R to the Ca^{2+} and PKA signaling pathways similarly (27). In contrast to this finding for the MC1-R, we have previously shown agouti antagonism of mMC4-R coupling to the PKA signaling pathway not to be competitive (maximum response is decreased) and also to be more potent in the presence of desacetyl- α -MSH than with α -MSH (27). It is of interest, therefore, that agouti protein also influences the mMC4-R coupling to the calcium pathway differently from its interaction with the MC1-R and also differently from the way it antagonizes MC4-R coupling to the PKA pathway. First, mouse agouti protein alters both α -MSH and desacetyl- α -MSH coupling mMC4-R to increases in $[Ca^{2+}]_i$ but is more effective with α -MSH. Second, although mouse agouti protein decreases the sensitivity of melanocortin peptides coupling the MC4-R to the calcium signaling pathway, there is a dose-dependent trend for it to increase maximum responsiveness. It is unclear from our data, therefore, whether agouti protein is a competitive antagonist, or an enhancer, of the MC4-R coupling to the calcium signaling pathway.

The phenotypes of the *e* recessive (31) and MC4-R knockout (15) mice strongly support agouti protein antagonism of α -MSH coupling to the MC1-R inhibiting pigmentation, as well as agouti protein antagonism of desacetyl- α -MSH or α -MSH coupling to the MC4-R resulting in obesity and type 2 diabetes. An additional role for agouti protein driving the obesity, hyperinsulinemia phenotype in the yellow obese mouse has been proposed: one in which agouti protein stimulates calcium signaling independent of melanocortin receptor antagonism (17, 37, 38). This agouti protein stimulation of calcium signaling is clearly a mechanism different from the melanocortin peptide-stimulated rise in $[Ca^{2+}]_i$ reported in this study.

The mechanism of melanocortin receptor antagonism responsible for the yellow obese mouse phenotype is believed to be agouti antagonism of MSH peptides coupling the MC1-R and MC4-R to increased cAMP with fivefold and three- to fivefold shifts, respectively, in their EC_{50} values (27). We show here that melanocortin peptides also couple the MC1-R and MC4-R to a second intracellular signaling pathway that results in elevations in $[Ca^{2+}]_i$. The physiological consequences of agouti protein-induced six- and threefold shifts in EC_{50} values for α -MSH coupling MC1-R and MC4-R,

respectively, to increased $[Ca^{2+}]_i$ are currently unknown, but the magnitude of the shifts in EC_{50} are similar to those for agouti antagonism of melanocortin peptides increasing cAMP. Therefore, the physiological relevance of the transduction of a calcium signal by melanocortin peptides and melanocortin receptors, as well as their interactions with agouti protein, requires further investigation.

We are grateful to Professor Michael Hanley, UC Davis, for helpful discussions, to Professor Garth Cooper, University of Auckland, for the use of the Hitachi F-4500 dual-wavelength spectrofluorometer, and to B. Stickney for technical support.

K. G. Mountjoy is supported by a Wellcome Trust Senior Research Fellowship of New Zealand, Health Research Council of New Zealand, National Child Health Foundation, and the New Zealand Lottery Board.

Present address of W. O. Wilkison: Zen-Bio, PO Box 12503, Research Triangle Park, NC 27709.

REFERENCES

1. Axelrod J. Receptor-mediated activation of phospholipase A2 and arachidonic acid release in signal transduction. *Biochem Soc Trans* 18: 503–507, 1990.
2. Bird GS, Burgess GM, and Putney JWJ. Sulfhydryl reagents and cAMP-dependent kinase increase the sensitivity of the inositol 1,4,5-triphosphate receptor in hepatocytes. *J Biol Chem* 268: 17917–17923, 1993.
3. Chen C and Okayama H. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol Cell Biol* 7: 2745–2752, 1987.
4. Chen W, Kelly MA, Opitz-Araya X, Thomas RE, Low MJ, and Cone RD. Exocrine gland dysfunction in MC5-R-deficient mice: Evidence for coordinated regulation of exocrine gland function by melanocortin peptides. *Cell* 91: 789–798, 1997.
5. Chretien M, Seidah NG, and Dennis M. Processing of precursor polyproteins in rat brain: regional differences in acetylation of POMC peptides. In: *Central and Peripheral Endorphins: Basic and Clinical Aspects*, edited by Muller EE and Genazzi AR. New York: Raven, 1984, p. 27–58.
6. Dousa T, Chini EN, and Beers KW. Adenine nucleotide diphosphates: emerging second messengers acting via intracellular Ca^{2+} release. *Am J Physiol Cell Physiol* 271: C1007–C1024, 1996.
7. Facchinetti F, Bernasconi S, Iughetti L, Genazzani AD, Ghizzoni L, and Genazzani AR. Changes in dopaminergic control of circulating melanocyte-stimulating hormone-related peptides at puberty. *Pediatr Res* 38: 91–94, 1995.
8. Facchinetti F, Storchi AR, Furani S, Radi D, and Genazzani AR. Pituitary changes of desacetyl- α -melanocyte stimulating hormone throughout development. *Biol Neonate* 54: 86–92, 1988.
9. Fasolato C, Hoth M, Matthews G, and Penner R. Ca^{2+} and Mn^{2+} influx through receptor-mediated activation of nonspecific cation channels in mast cells. *Proc Natl Acad Sci USA* 90: 3068–3072, 1993.
10. Gantz I, Konda K, Tashiro T, Shimoto Y, Miwa H, Munzert G, Watson SJ, DelValle J, and Yamada T. Molecular cloning of a novel melanocortin receptor. *J Biol Chem* 265: 8246–8250, 1993.
11. Gantz I, Miwa H, Konda Y, Shimoto Y, Tashiro T, Watson SJ, DelValle J, and Yamada T. Molecular cloning, expression, and gene localization of a fourth melanocortin receptor. *J Biol Chem* 268: 15174–15179, 1993.
12. Gantz I, Shimoto Y, Konda Y, Miwa H, Dickson CJ, and Yamada T. Molecular cloning, expression, and characterization of a fifth melanocortin receptor. *Biochem Biophys Res Commun* 200: 1214–1220, 1994.
13. Genazzani AA and Galione A. A Ca^{2+} release mechanism gated by the novel pyridine nucleotide, NAADP. *Trends Pharmacol Sci* 18: 108–110, 1997.
14. Grynkiewicz G, Poenie M, and Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescent properties. *J Biol Chem* 260: 3440–3450, 1985.
15. Huszar D, Lynch CA, Fairchild-Huntress V, Dunmore JH, Fang Q, Berkemeier LR, Gu W, Kesterson RA, Boston BA, Cone RD, Smith FJ, Campfield LA, Burn P, and Lee F. Targeted disruption of the melanocortin-4 receptor results in obesity in mice. *Cell* 88: 131–141, 1997.
16. Jones BH, Kim JH, Zemel MB, Woychik RP, Michaud EJ, Wilkison WO, and Moustaid N. Upregulation of adipocyte metabolism by agouti protein: possible paracrine actions in yellow mouse obesity. *Am J Physiol Endocrinol Metab* 270: E192–E196, 1996.
17. Kim JH, Kiefer LL, Woychik RP, Wilkison WO, Truesdale A, Ittoop O, Willard D, Nichols J, and Zemel MB. Agouti regulation of intracellular calcium: role of melanocortin receptors. *Am J Physiol Endocrinol Metab* 272: E379–E384, 1997.
18. Konda Y, Gantz I, DelValle J, Shimoto Y, Miwa H, and Yamada T. Interaction of dual intracellular signaling pathways activated by the melanocortin-3 receptor. *J Biol Chem* 269: 13162–13166, 1994.
19. Labbe O, Desarnaud F, Eggerickx D, Vassart G, and Parmentier M. Molecular cloning of a mouse melanocortin 5 receptor gene widely expressed in peripheral tissues. *Biochemistry* 33: 4543–4549, 1994.
20. Lin CW, Miller TR, Witte DG, Bianchi BR, Stashko M, Manelli AM, and Frail DE. Characterization of cloned human dopamine D1 receptor-mediated calcium release in 293 cells. *Mol Pharmacol* 47: 131–139, 1995.
21. Lu D, Willard D, Patel IR, Kadwell S, Overton L, Kost T, Luther M, Chen W, Woychik RP, Wilkison WO, and Cone RD. Agouti protein is an antagonist of the melanocyte-stimulating-hormone receptor. *Nature* 371: 799–802, 1994.
22. Lytton J, Westlin M, and Hanley MR. Thapsigargin inhibits the sarcoplasmic or endoplasmic reticulum Ca-ATPase family of calcium pumps. *J Biol Chem* 266: 17067–17071, 1991.
23. Mauri A, Argiolas A, Melis MR, Lai L, Fratta W, Carso A, Lanzona A, Serri F, Caminiti F, Mancuso S, and Gessa GL. Des-acetyl- α -MSH and not α -MSH is the major form of α -MSH in amniotic fluid. *J Endocrinol Invest* 11: 345–349, 1988.
24. Mauri A, Melis MR, Deiana P, Loviselli A, Volpe A, and Argiolas A. Melanocortins and opioids modulate early postnatal growth in rats. *Regul Pept* 59: 59–66, 1995.
25. Mountjoy KG, Mortrud MT, Low MJ, Simerly RB, and Cone RD. Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the brain. *Mol Endocrinol* 8: 1298–1308, 1994.
26. Mountjoy KG, Robbins LS, Mortrud MT, and Cone RD. The cloning of a family of genes that encode the melanocortin receptors. *Science* 257: 543–546, 1992.
27. Mountjoy KG, Willard DH, and Wilkison WO. Agouti antagonism of melanocortin-4 receptor: greater effect with desacetyl- α -MSH than with α -MSH. *Endocrinology* 140: 2167–2172, 1999.
28. O'Donohue TL, Handelsmann GE, Miller RL, and Jacobowitz DM. N-Acetylation regulates the behavioral activity of α -melanotropin in a multineurotransmitter neuron. *Science* 215: 1125–1127, 1982.
29. Patterson SI, Jackson TR, Dreher M, and Hanley MR. Second messenger responses to neuropeptides. In: *Neuropeptides: A Methodology*, edited by Fink G and Harmar AJ. New York: Wiley, 1989, p. 259–265.
30. Rius RA, Chikuma T, and Peng Loh Y. Prenatal processing of pro-opiomelanocortin in the brain and pituitary of mouse embryos. *Dev Brain Res* 60: 179–185, 1991.
31. Robbins LS, Nadeau JH, Johnson KR, Kelly MA, Roselli-Rehffuss L, Backe E, Mountjoy KG, and Cone RD. Pigmentation phenotypes of variant extension locus alleles result from point mutations that alter MSH receptor function. *Cell* 72: 827–834.
32. Roe MW, Lemasters JJ, and Herman B. Assessment of fura-2 for measurements of cytosolic free calcium. *Cell Calcium* 11: 63–73, 1990.

33. **Roselli-Reh fuss L, Mountjoy KG, Robbins LS, Mortrud MT, Low M, Tatro JB, Entwistle ML, Simerly R, and Cone RD.** Identification of a receptor for γ melanotropin and other proopiomelanocortin peptides in the hypothalamus and limbic system. *Proc Natl Acad Sci USA* 90: 8856–8860, 1993.
34. **Seuwen K and Boddeke HGWM.** Heparin-insensitive calcium release from intracellular stores triggered by the recombinant human parathyroid hormone receptor. *Br J Pharmacol* 114: 1613–1620, 1995.
35. **Shimizu H, Shargill NS, Bray GA, Yen TT, and Gesellchen PD.** Effects of MSH on food intake, body weight and coat color of the yellow obese mouse. *Life Sci* 45: 543–552, 1989.
36. **Willard DH, Bodnar W, Harris C, Nichols J, Kiefer LL, Hoffman C, Moyer M, Burkhart W, Weiel J, Wilkison WO, and Rocque W.** Agouti structure and function: characterization of a potent alpha-melanocyte stimulating hormone receptor antagonist. *Biochemistry* 34: 12341–12346, 1995.
37. **Xue BZ, Wilkison WO, Mynatt RL, Moustaid N, Goldman M, and Zemel MB.** The *agouti* gene product stimulates pancreatic β -cell Ca^{2+} signaling and insulin release. *Physiol Genomics* 1: 11–19, 1999.
38. **Zemel MB, Han Kim J, Woychik RP, Michaud EJ, Kadwell SH, Patel IR, and Wilkison WO.** Agouti regulation of intracellular calcium: Role in the insulin resistance of viable yellow mice. *Proc Natl Acad Sci USA* 92: 4733–4737, 1995.

