day 22 (Group 3) and day 3 (Group 4). The brains were rapidly removed, placed on a glass plate on dry ice and stored at −80°C until use. Trunk blood was collected and the plasma concentration of iCa was measured on the same as above and plasma concentrations of leptin were measured using an ELISA kit (YK051 Rat Leptin-HS, Yanaihara Institute, Shizuoka, Japan). The animals used in the experiment were treated in accordance with the ethical guidelines for animal care, handling and termination promulgated by the Chugai Pharmaceutical Co. (Tokyo, Japan).

In situ hybridization histochemistry

Frozen 12-μm-thick coronal brain sections were prepared in a cryostat at −20°C, thawed, and mounted onto gelatin/chrome alum-coated slides. The PVN, Arc and LHA were determined according to coordinates given by the atlas of Paxinos and Watson.[31] The localization of sections from each rat was checked by microscopic observation. Two sections containing the PVN (plate 24; Ref. 29) and four sections containing the Arc (plate 27 and 28; Ref. 29) and LHA (plate 28; Ref. 29) were used from each rat to measure the density of the autoradiography. *In situ* hybridization was performed as previously described.[32] Hybridization was carried out under a Nescofilm coverslip (Bando Chemical IMD, Osaka, Japan). [^{35}S]3′-end-labeled deoxyoligonucleotides complementary to transcripts coding for NPY (5′-GGA GTA GTA TCT GGC CAT GTC CTC TGC TGG CGC GTC-3′), AgRP (5′-CGA CGC GGA GAA CGA GAC TCG CGG TTC TGT GGA TCT AGC ACC TCT GCC-3′), POMC (5′-CTT CTT GCC CAG CGG CTT GCC CCA GCA GAA GTG CTC CAT GGA CTA GGA-3′), CART (5′-TGG GGA CTT GGC CGT ACT TCT TCT CAT AGA TCG GAA TGC-3′), orexin (5′-TTC GTA GAG ACG GCA GGA ACA CGT CTT CTG GCG ACA-3′) and CRH (5′-CAG TTT CCT GTT GCT GTG AGC TTG CTG AGC TAA CTG CTC TGC CCT GGC-3′) were used as the specific probes. The specificity of the probes was described previously.[19,33–36] Total counts of 6×10^5 cpm/slide for NPY, AgPR, POMC, CART and CRH and 4×10^5 cpm/slide for orexin were used. Hybridized sections containing the Arc, the LHA and the PVN were exposed to autoradiography film (Hyperfilm; Amersham, Buckinghamshire, UK) for 4 days for orexin and 7 days for NYP, AgRP, POMC, CART and CRH. The autoradiographic images were quantified using an MCID imaging analyzer (Imaging Research, St. Catherines, ON, Canada). The images were captured by a charge-coupled device camera (Dage-MTI, Michigan City, IN) at 40× magnification. The mean absorbance of the autoradiographs was measured and compared with simultaneously exposed ^{14}C microscale samples (Amersham). The standard curve was fitted by the absorbance of the ^{14}C microscale on the same film.

Statistical analysis

All data are given as mean ± SE calculated from the results of the *in situ* hybridization histochemistry. The results of each experimental animal group were compared with those of the control group. The data were analyzed using a one-way factorial ANOVA followed by a Bonferroni correction for multiple comparisons. The changes in body weight, iCa and arthritis index were also statistically analyzed using one-way ANOVA followed by a Bonferroni correction for multiple comparisons. Statistical significance was defined as $p < 0.05$.

Results

Body weight, arthritis index, iCa and plasma leptin in Normal rats, HHM rats, SEKI rats, AA rats and $1\alpha,25(OH)_2D_3$ rats

Consistent with previous results,[19,20,22,23] the body weight of the HHM rats bearing PTHrP secreting LC-6 significantly decreased after day 30, but significantly increased after the HHM rats were given the anti-PTHrP antibody (Fig. 1*a*). The body weights of the SEKI rats and the AA rats also decreased after day 30 and after day 13, respectively, but significantly increased in the AA rats after they were given indomethacin (Figs. 1*b* and 1*c*). The arthritis index in the AA rats increased sharply from day 10 to 19 and remained the same until day 22, but significantly decreased after they were given indomethacin (Fig. 1*d*). The rats treated with $1\alpha,25(OH)_2D_3$ had a significant decrease in body weight accompanied by an elevation of iCa (Figs. 1*e* and 1*f*). The levels of iCa in the HHM rats were significantly higher than in the normal rats, but it decreased when the HHM rats received the anti-PTHrP antibody (Fig. 1*f*). On the other hand, the SEKI rats had no change in the levels of iCa, though they experienced body weight loss (Figs. 1*b* and 1*f*), and neither did the AA rats (Figs. 1*c* and 1*f*).

Concentration in plasma leptin in HHM rats were significantly lower than that in nontumor-bearing rats and also lower in SEKI and AA rats. Administration of anti-PTHrP antibody to HHM rats increased the plasma leptin level, but it was still lower than that of the nontumor-bearing rats. On the other hand, vitamin D treated rats did not change the plasma leptin concentration (Fig. 2).

Expression of hypothalamic peptides: orexigenic peptides genes in HHM, SEKI, AA and $1\alpha,25(OH)_2D_3$ rats

After the HHM rats developed cachexia, the levels of orexigenic peptide mRNAs, NPY and AgRP in the Arc were significantly higher than in normal rats. Administration of the anti-PTHrP antibody to the HHM rats showed reduced levels of NPY and AgRP mRNAs compared with levels in the untreated HHM rats (Figs. 3*a*–3*d*). Although neither the SEKI rats nor the AA rats showed elevated levels of blood iCa (Fig. 1*f*), similar changes in the mRNA expression of orexigenic peptides were observed in both rat models; the orexigenic peptide mRNAs, such as NPY and AgRP in the Arc, became higher (Figs. 3*a*–3*d*). Administration of indomethacin to the AA rats not only improved their body weight and arthritis index but also restored the mRNA levels

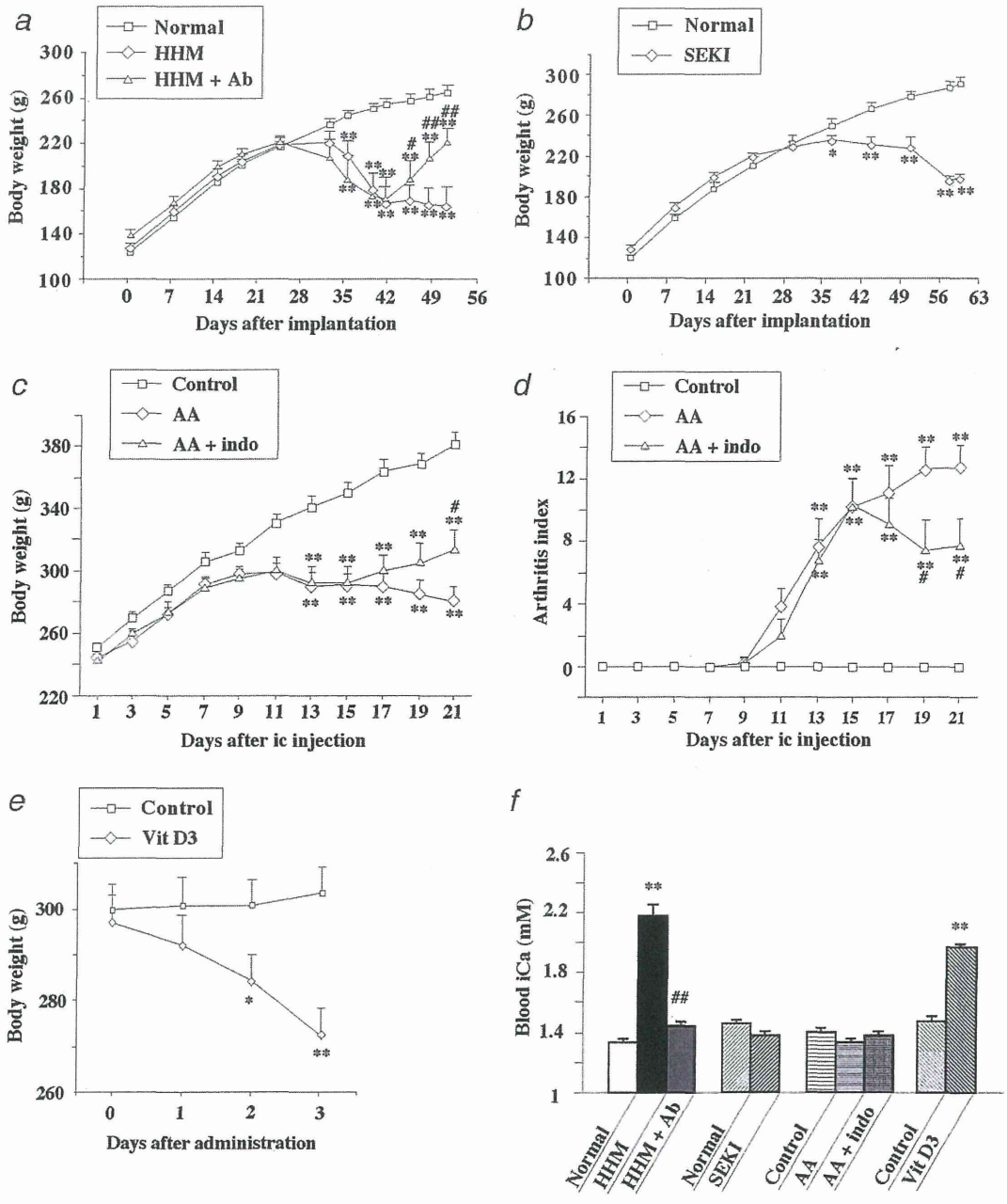

Figure 1. Time course of changes in body weight in Group 1 containing nontumor-bearing rats (Normal), humoral hypercalcemia of malignancy rats (HHM) and HHM rats intravenously (i.v.) injected with anti-PTHrP antibody (HHM + Ab) (*a*), Group 2 containing nontumor-bearing rats (Normal) and SEKI rats (SEKI) (*b*) and Group 3 containing rats injected with vehicle (Control), adjuvant-induced arthritis rats treated with vehicle (AA), and AA rats perorally (po) treated with indomethacin (AA + indo) (*c*). Changes in the arthritis index in Group 3 (*d*). Time course of changes in body weight in Group 4 containing rats treated with vehicle (Control) and rats orally treated with 10 μg/kg of $1\alpha,25(OH)_2D_3$ (Vit. D3) daily from day 0 to 3 (*e*), and changes in iCa of the rats in Group 1 to Group 4 (*f*). For treatment with or without an anti-PTHrP antibody, the rats were given 3 mg/kg of anti-PTHrP antibody or saline i.v. on days 42 and 49 in Group 1. AA rats in Group 3 were not treated or orally administered 1 mg/kg of indomethacin daily from days 15–21. Data points, mean ($n = 6$); bars, SE. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with each control. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with HHM rats or AA rats.

of the hypothalamic feeding peptides. The level of orexin mRNA in the LHA did not change even under cachectic conditions in the HHM rats, but decreased in the SEKI rats and AA rats when they developed cachexia and, in the AA rats, was not increased by administration of indomethacin (Figs. 3*e* and 3*f*).

Because HHM rats bearing LC-6 concurrently develop cachexia and hypercalcemia, there is a possibility that serum

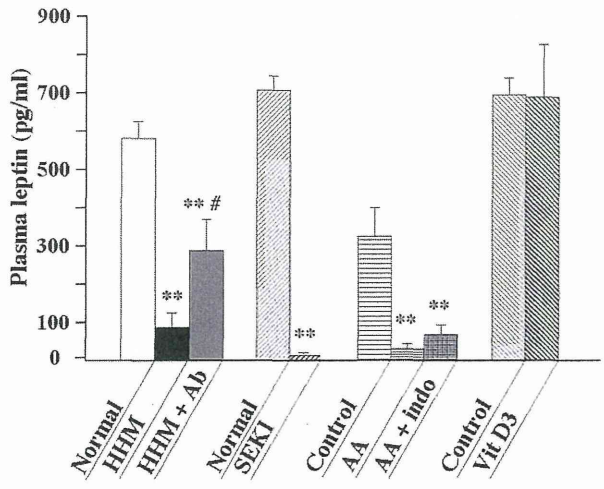

Figure 2. The changes of plasma leptin concentration in Group 1 containing nontumor-bearing rats (Normal), humoral hypercalcemia of malignancy rats (HHM) and HHM rats intravenously (i.v.) injected with anti-PTHrP antibody (HHM + Ab), Group 2 containing nontumor-bearing rats (Normal) and SEKI rats (SEKI), Group 3 containing rats injected with vehicle (Control), adjuvant-induced arthritis rats treated with vehicle (AA) and AA rats perorally (po) treated with indomethacin (AA + indo) and Group 4 containing rats treated with vehicle (Control) and rats orally treated with $1\alpha,25(OH)_2D_3$ (Vit. D3). Data points, mean ($n = 6$); bars, SE. $^{**}p < 0.01$ compared with each control. $^{\#}p < 0.05$ compared with HHM rats.

calcium is also involved in the changes in the expression of hypothalamic feeding-regulating peptides. The SEKI and AA rats showed an increased expression of the orexygenic peptide without an increase in serum iCa. This indicates that changes in the expression of hypothalamic feeding-regulating peptides are not related to an increase in serum calcium. To further confirm the relationship between hypercalcemia and the expression of hypothalamic feeding-regulating peptides, rats were administered a high dose of $1\alpha,25(OH)_2D_3$ to induce hypercalcemia. In the $1\alpha,25(OH)_2D_3$ rats, the serum levels of iCa increased as body weight decreased, but the levels of NPY, AgRP and orexin mRNA did not change significantly.

Expression of hypothalamic peptides: anorexigenic peptides genes in HHM, SEKI, AA and $1\alpha,25(OH)_2D_3$ rats

POMC and CART in the Arc and CRH in the PVN became lower after the HHM rats developed cachexia (Fig. 4). The administration of the anti-PTHrP antibody restored not only their body weight but also restored the mRNA expression of the POMC, CART and CRH.

The SEKI rats and the AA rats also showed similar changes in the mRNA expression of anorexigenic peptides; the mRNA levels of POMC and CART in the Arc and CRH in the PVN were lower after they developed cachexia. On the other hand, $1\alpha,25(OH)_2D_3$ rats did not experience a change in the levels of POMC, CART and CRH mRNAs similar to orexigenic peptides such as NPY, AgRP and orexin mRNAs. Taken together, the results demonstrate that the upregulation of orexigenic peptides and downregulation of anorexigenic peptides are not specific to PTHrP-induced cachexia but rather to other physiological responses under cachectic conditions. Furthermore, such changes in the expression of hypothalamic feeding-regulating peptides occur independently of hypercalcemia.

Discussion

Tumor and host tissues containing macrophages often secrete proinflammatory cytokines and elevated levels of cytokines have been thought to directly or indirectly transmit signals to the hypothalumus, repressing the feeding center and activating the satiety center. In fact, it was reported that macrophage inhibitory cytokine-1 (MIC-1), which causes cachexia in cancer and renal disorders, binds to TGFβ Type II receptors and downregulates NPY and upregulates POMC.[37] In addition, MIC-1 and leptin have similar effects on the expression of hypothalamic feeding peptides that act at different sites of the hypothalamus.

However, in this study, the expression of orexigenic peptide mRNAs was upregulated and anorexigenic peptide mRNAs expression was downregulated in several cachectic models. Particularly, nontumor-bearing cachectic AA rats revealed the same mRNA changes in the hypothalamus as did HHM and SEKI rats. In addition to the observation that those mRNA changes were restored in HHM rats after they were treated with an anti-PTHrP antibody, the AA rats treated with indomethacin, which suppressed the synthesis of prostaglandins, had a partial restoration of those mRNA changes accompanied by a decrease of the arthritis index. It is possible that under cachectic conditions, the feeding center is activated and the satiety center is repressed, and yet, inflammatory cytokines, hormones and bioactive substances affect the orexigenic and anorexigenic peptide mRNA expression downstream of the feeding and satiety centers. In addition, we have previously reported that the body weight loss in HHM rats was accompanied by reduced amounts of muscle as well as fat.[23] Indeed, the concentration of plasma leptin in the HHM rats was significantly lower and administration of the anti-PTHrP antibody increased the plasma leptin level. A recessive mutation in the mouse *ob* genes results in obesity, and the *ob* gene encodes a hormone leptin that is expressed in adipose tissue.[38] Leptin regulates energy balance in part by suppressing NPY neurons and activating POMC neurons in the Arc[39] and it would be possible that leptin deficiency partly affects the changes of the gene expression in this study.

Although the mechanisms that explain the differences in the expression of hypothalamic feeding-regulating peptides between MIC-1-induced cachexia and our models remain to be elucidated, one possibility is that downstream processes of

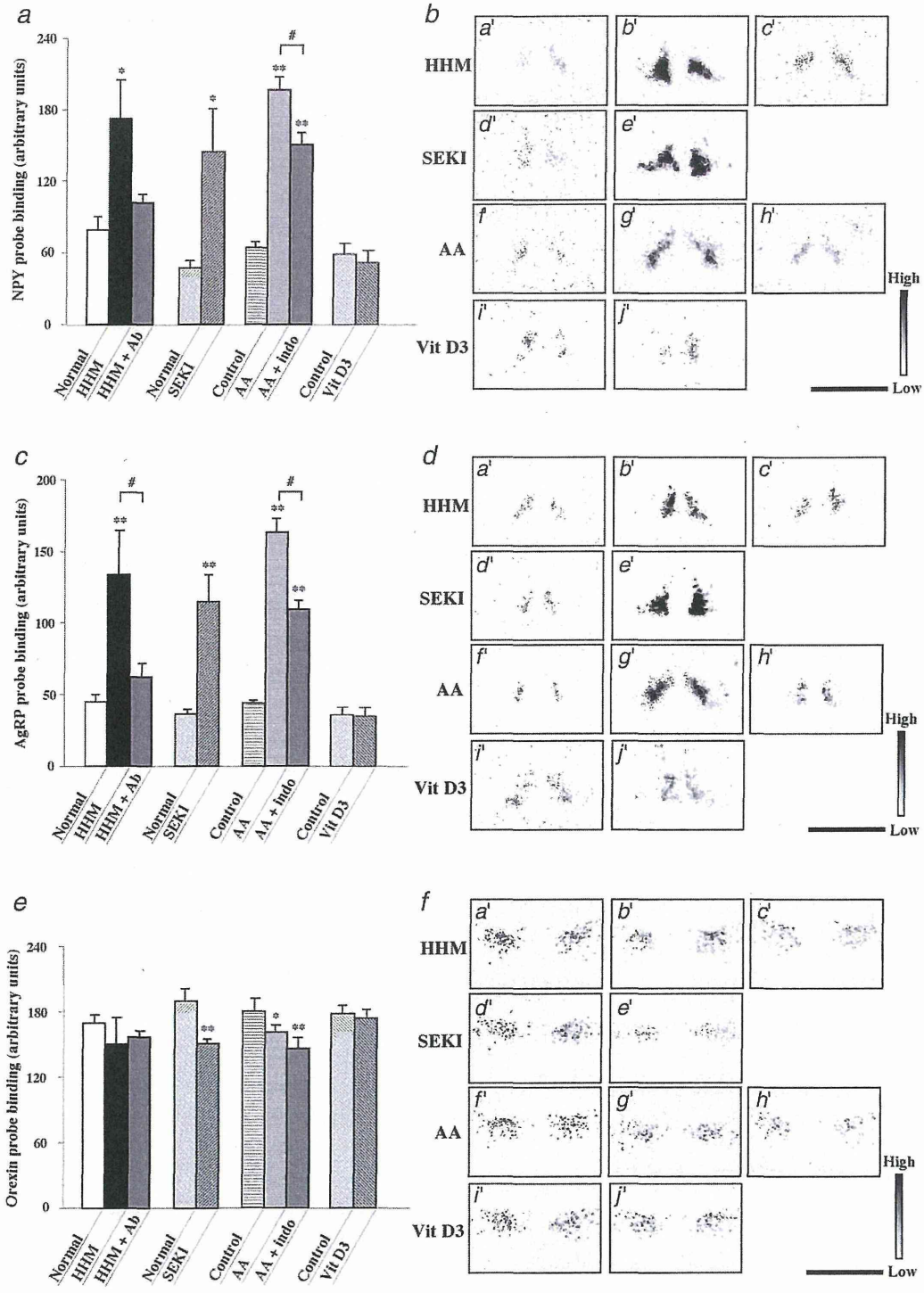

Figure 3. Expression of mRNA for neuropeptide Y (NPY) (*a* and *b*), agouti-related protein (AgRP) (*c* and *d*) in the arcuate nucleus (Arc) and orexin (*e* and *f*) in the lateral hypothalamic area (LHA) of Group 1 containing nontumor-bearing rats (Normal; *b-a′*, *d-a′*, *f-a′*), HHM rats (HHM; *b-b′*, *d-b′*, *f-b′*) and HHM rats injected with anti-PTHrP antibody (HHM + Ab; *b-c′*, *d-c′*, *f-c′*), Group 2 containing nontumor-bearing rats (Normal; *b-d′*, *d-d′*, *f-d′*) and SEKI rats (SEKI; *b-e′*, *d-e′*, *f-e′*), Group 3 containing rats injected with vehicle (Control; *b-f′*, *d-f′*, *f-f′*), adjuvant-induced arthritis rats treated with vehicle (AA; *b-g′*, *d-g′*, *f-g′*), and AA rats treated with indomethacin (AA + indo; *b-h′*, *d-h′*, *f-h′*) and Group 4 containing rats treated with vehicle (Control; *b-i′*, *d-i′*, *f-i′*) and rats treated with $1\alpha,25(OH)_2D_3$ (Vit. D3; *b-j′*, *d-j′*, *f-j′*). Representative autoradiographs of sections hybridized by a ^{35}S-labeled oligodeoxynucleotide probe complementary to mRNA for NPY (*b-a′-j′*), AgRP (*d-a′-j′*) and orexin (*f-a′-j′*). Signal intensity ranges from high (black) to low (white). Bar, 1 mm. Columns, mean ($n = 6$); bars, SE. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with each control. $^{\#}p < 0.05$ compared with HHM rats or AA rats.

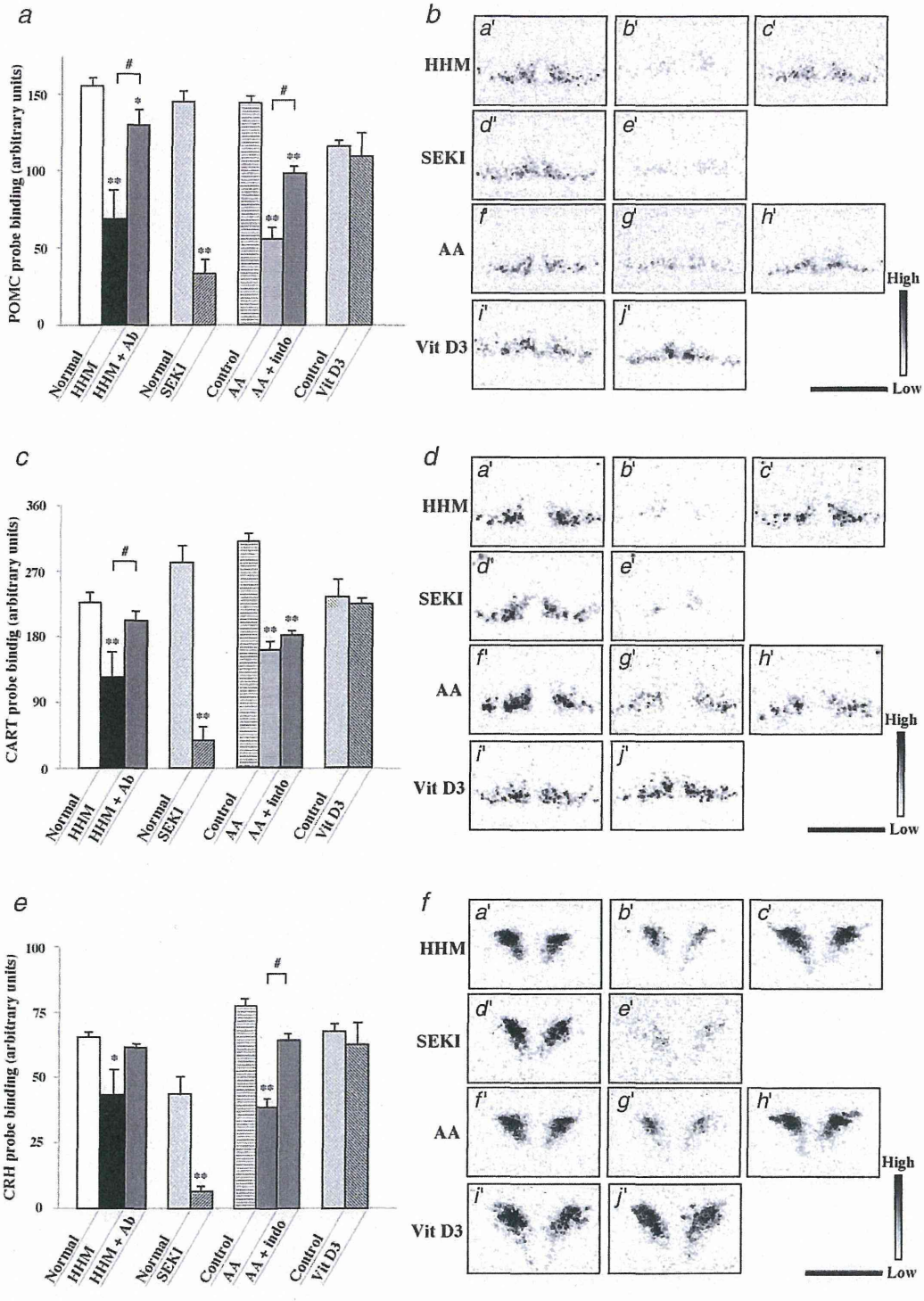

Figure 4. Expression of mRNA for proopiomelanocortin (POMC) (*a* and *b*) and cocaine-and amphetamine-regulated transcript (CART) (*c* and *d*) in the arcuate nucleus (Arc) and corticotropin-releasing hormone (CRH) (*e* and *f*) in the paraventricular nucleus (PVN) of Group 1 containing nontumor-bearing rats (Normal; *b-a′*, *d-a′*, *f-a′*), HHM rats (HHM; *b-b′*, *d-b′*, *f-b′*) and HHM rats injected with anti-PTHrP antibody (HHM + Ab; *b-c′*, *d-c′*, *f-c′*), Group 2 containing nontumor-bearing rats (Normal; *b-d′*, *d-d′*, *f-d′*) and SEKI rats (SEKI; *b-e′*, *d-e′*, *f-e′*), Group 3 containing rats injected with vehicle (Control; *b-f′*, *d-f′*, *f-f′*), adjuvant-induced arthritis rats treated with vehicle (AA; *b-g′*, *d-g′*, *f-g′*) and AA rats treated with indomethacin (AA + indo; *b-h′*, *d-h′*, *f-h′*) and Group 4 containing rats treated with vehicle (Control; *b-i′*, *d-i′*, *f-i′*) and rats treated with $1\alpha,25(OH)_2D_3$ (Vit. D3; *b-j′*, *d-j′*, *f-j′*). Representative autoradiographs of sections hybridized by a ^{35}S-labeled oligodeoxynucleotide probe complementary to mRNA for POMC (*b-a′-j′*), CART (*d-a′-j′*) and CRH (*f-a′-j′*). Bar, 1 mm. Columns, mean ($n = 6$); bars, SE. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with each control. $^{\#}p < 0.05$ compared with HHM rats or AA rats.

NPY action are affected by humoral factors such as cytokines and tumor-derived factors.[40] In fact, intracerebroventricular injection of TNF-α reportedly increased the NPY mRNA level in the hypothalamus but reduced food intake,[41] and proinflammatory signals decreased the secretion of AgRP but increased the transcription of the AgRP gene.[42]

On the other hand, the rats treated with $1\alpha,25(OH)_2D_3$ had reduced body weight without changes in feeding-regulating peptide gene expression. One possible explanation is that changes of metabolic rates and locomotor activity related to $1\alpha,25(OH)_2D_3$-induced hypercalcemia may be involved in the decrease of body weight without affecting feeding. Another possible explanation is that body fluid balance related to drinking and urine volume will change and cause dehydration. The reason why body weight was reduced after $1\alpha,25(OH)_2D_3$ treatment without affecting the feeding-regulating peptide genes should be clarified by further study.

Previously, using HHM rats treated with anti-PTHrP antibody, we have demonstrated: (*i*) body weight gain accompanied by restoration of locomotor activity and food and water intake, (*ii*) restoration of plasma calcium levels and (*iii*) restoration of feeding-regulating peptide genes.[19,23] It could be possible that proinflammatory cytokines such as IL-1, IL-6 and TNFα are responsible for the changes in feeding-regulating peptide gene expression, to be sure, but PTHrP might also be responsible for those changes. In this study, there were no effects of hypercalcemia induced by $1\alpha,25(OH)_2D_3$ treatment on the hypothalamic feeding-regulating peptide gene expression. Consequently, not PTHrP-induced hypercalcemia but hormonal effects of PTHrP might have brought about the changes in feeding-regulating peptide gene expression.

In the HHM rats, the level of orexin mRNA that enhanced feeding was not significantly increased but rather decreased in the LHA. Because orexin is involved not only in feeding behavior but also in sleep regulation and narcolepsy,[43–45] orexin expression may be regulated in a more complex manner. Especially, orexin increases the proportion of time spent awake through projecting fibers for the locus coeruleus that is a key modulator of attentional state.[46] Previously, Onuma *et al.* reported that there was an approximately double increase in the locomotor activity of the HHM rats after they received the anti-PTHrP antibody.[23] Consequently, in tumor-bearing cachextic rats and AA rats it can be presumed that locomotor activity and waking state are reduced and result in reduced orexin gene expression. Further studies are necessary both to clearly understand the mechanisms of orexigenic and anorexigenic peptide regulations in response to cachectic conditions and the mechanisms by which orexigenic and anorexigenic peptide regulations could cause the cachectic conditions.

Acknowledgements

We thank Ms. Yoshimi Asao and Ms. Kumiko Nasu for their technical assistance. We thank Mr. F. Ford for critical reading and language editing of the manuscript. This paper was supported in part by The Naito Foundation to (Y.U.), Yamaguchi Endocrine Research Foundation to (Y.U.), a Grant-in-Aid for Scientific Research (C), No19591771, to (H.O.) and a Grant-in-Aid for Scientific Research on Priority Areas, No18077006, to (Y.U.) from the Ministry Education, Culture, Sports, Science and Technology, Japan.

References

1. Larkin M. Thwarting the dwindling progression of cachexia. *Lancet* 1998;351: 1336.
2. Tisdale MJ. Biology of cachexia. *J Natl Cancer Inst* 1997;89:1763–73.
3. Bruera E. ABC of palliative care. Anorexia, cachexia, and nutrition. *BMJ* 1997;315: 1219–22.
4. Tisdale MJ. Cancer anorexia and cachexia. *Nutrition* 2001;17:438–42.
5. Endo K, Katsumata K, Iguchi H, Kubodera N, Teramoto T, Ikeda K, Fujita T, Ogata E. Effect of combination treatment with a vitamin D analog (OCT) and a bisphosphonate (AHPrBP) in a nude mouse model of cancer-associated hypercalcemia. *J Bone Miner Res* 1998;13: 1378–83.
6. Broadus AE, Mangin M, Ikeda K, Insogna KL, Weir EC, Burtis WJ, Stewart AF. Humoral hypercalcemia of cancer. Identification of a novel parathyroid hormone-like peptide. *N Engl J Med* 1988; 319:556–63.
7. Cone RD. Anatomy and regulation of the central melanocortin system. *Nat Neurosci* 2005;8:571–8.
8. Morton GJ, Cummings DE, Baskin DG, Barsh GS, Schwartz MW. Central nervous system control of food intake and body weight. *Nature* 2006;443:289–95.
9. Oomura Y. Input-output organization in the hypothalamus relating to food intake behavior. In: Marcel D, ed. Handbook of the hypothalamus, vol. 2. New York, 1980. 557–620.
10. Chen HY, Trumbauer ME, Chen AS, Weingarth DT, Adams JR, Frazier EG, Shen Z, Marsh DJ, Feighner SD, Guan XM, Ye Z, Nargund RP, et al. Orexigenic action of peripheral ghrelin is mediated by neuropeptide Y and agouti-related protein. *Endocrinology* 2004;145:2607–12.
11. Ariyasu H, Takaya K, Tagami T, Ogawa Y, Hosoda K, Akamizu T, Suda M, Koh T, Natsui K, Toyooka S, Shirakami G, Usui T, et al. Stomach is a major source of circulating ghrelin, and feeding state determines plasma ghrelin-like immunoreactivity levels in humans. *J Clin Endocrinol Metab* 2001;86:4753–8.
12. Morrison CD, Morton GJ, Niswender KD, Gelling RW, Schwartz MW. Leptin inhibits hypothalamic Npy and Agrp gene expression via a mechanism that requires phosphatidylinositol 3-OH-kinase signaling. *Am J Physiol Endocrinol Metab* 2005;289:E1051–E1057.
13. Ollmann MM, Wilson BD, Yang YK, Kerns JA, Chen Y, Gantz I, Barsh GS. Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. *Science* 1997;278:135–8.
14. Maffei M, Halaas J, Ravussin E, Pratley RE, Lee GH, Zhang Y, Fei H, Kim S, Lallone R, Ranganathan S, Kern PA, Friedman JM. Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. *Nat Med* 1995;1:1155–61.
15. Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, Williams SC, Richardson JA, Kozlowski GP, Wilson S, Arch JR, Buckingham RE, et al. Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell* 1998;92:573–85.
16. da Silva AA, Kuo JJ, Hall JE. Role of hypothalamic melanocortin 3/4-receptors in mediating chronic cardiovascular, renal,

and metabolic actions of leptin. *Hypertension* 2004;43:1312–17.
17. Cummings DE, Schwartz MW. Genetics and pathophysiology of human obesity. *Annu Rev Med* 2003;54:453–71.
18. Kalra SP, Dube MG, Pu S, Xu B, Horvath TL, Kalra PS. Interacting appetite-regulating pathways in the hypothalamic regulation of body weight. *Endocr Rev* 1999;20:68–100.
19. Hashimoto H, Azuma Y, Kawasaki M, Fujihara H, Onuma E, Yamada-Okabe H, Takuwa Y, Ogata E, Ueta Y. Parathyroid hormone-related protein induces cachectic syndromes without directly modulating the expression of hypothalamic feeding-regulating peptides. *Clin Cancer Res* 2007;13:292–8.
20. Iguchi H, Onuma E, Sato K, Ogata E. Involvement of parathyroid hormone-related protein in experimental cachexia induced by a human lung cancer-derived cell line established from a bone metastasis specimen. *Int J Cancer* 2001;94:24–7.
21. Onuma E, Sato K, Saito H, Tsunenari T, Ishii K, Esaki K, Yabuta N, Wakahara Y, Yamada-Okabe H, Ogata E. Generation of a humanized monoclonal antibody against human parathyroid hormone-related protein and its efficacy against humoral hypercalcemia of malignancy. *Anticancer Res* 2004;24:2665–73.
22. Onuma E, Azuma Y, Saito H, Tsunenari T, Watanabe T, Hirabayashi M, Sato K, Yamada-Okabe H, Ogata E. Increased renal calcium reabsorption by parathyroid hormone-related protein is a causative factor in the development of humoral hypercalcemia of malignancy refractory to osteoclastic bone resorption inhibitors. *Clin Cancer Res* 2005;11:4198–203.
23. Onuma E, Tsunenari T, Saito H, Sato K, Yamada-Okabe H, Ogata E. Parathyroid hormone-related protein (PTHrP) as a causative factor of cancer-associated wasting: possible involvement of PTHrP in the repression of locomotor activity in rats bearing human tumor xenografts. *Int J Cancer* 2005;116:471–8.
24. Iseki H, Kajimura N, Ohue C, Tanaka R, Akiyama Y, Yamaguchi K. Cytokine production in five tumor cell lines with activity to induce cancer cachexia syndrome in nude mice. *Jpn J Cancer Res* 1995;86:562–7.
25. Hanada T, Toshinai K, Kajimura N, Nara-Ashizawa N, Tsukada T, Hayashi Y, Osuye K, Kangawa K, Matsukura S, Nakazato M. Anti-cachectic effect of ghrelin in nude mice bearing human melanoma cells. *Biochem Biophys Res Commun* 2003;301:275–9.
26. Harbuz MS, Jessop DS. Dissociation between c-fos mRNA in the paraventricular nucleus and corticosterone secretion in rats with adjuvant-induced arthritis. *J Endocrinol* 1999;163:107–13.
27. Suzuki H, Kawasaki M, Ohnishi H, Nakamura T, Ueta Y. Regulatory mechanism of the arginine vasopressin-enhanced green fluorescent protein fusion gene expression in acute and chronic stress. *Peptides* 2009;30:1763–70.
28. Suzuki H, Onaka T, Kasai M, Kawasaki M, Ohnishi H, Otsubo H, Saito T, Hashimoto H, Yokoyama T, Fujihara H, Dayanithi G, Murphy D, et al. Response of arginine vasopressin-enhanced green fluorescent protein fusion gene in the hypothalamus of adjuvant-induced arthritic rats. *J Neuroendocrinol* 2009;21:183–90.
29. Tanaka H, Ueta Y, Yamashita U, Kannan H, Yamashita H. Biphasic changes in behavioral, endocrine, and sympathetic systems in adjuvant arthritis in Lewis rats. *Brain Res Bull* 1996;39:33–7.
30. Hogervorst EJ, Wagenaar JP, Boog CJ, van der Zee R, van Embden JD, van Eden W. Adjuvant arthritis and immunity to the mycobacterial 65 kDa heat shock protein. *Int Immunol* 1992;4:719–27.
31. Paxinos G, Watson C. The rat brain in stereotaxic coordinatesed. Sydney: Academic Press, 1982.
32. Ueta Y, Levy A, Chowdrey HS, Lightman SL. Hypothalamic nitric oxide synthase gene expression is regulated by thyroid hormones. *Endocrinology* 1995;136:4182–7.
33. Hanada R, Teranishi H, Pearson JT, Kurokawa M, Hosoda H, Fukushima N, Fukue Y, Serino R, Fujihara H, Ueta Y, Ikawa M, Okabe M, et al. Neuromedin U has a novel anorexigenic effect independent of the leptin signaling pathway. *Nat Med* 2004;10:1067–73.
34. Harbuz MS, Chalmers J, De Souza L, Lightman SL. Stress-induced activation of CRF and c-fos mRNAs in the paraventricular nucleus are not affected by serotonin depletion. *Brain Res* 1993;609:167–73.
35. Nomura M, Ueta Y, Serino R, Kabashima N, Shibuya I, Yamashita H. PACAP type I receptor gene expression in the paraventricular and supraoptic nuclei of rats. *Neuroreport* 1996;8:67–70.
36. Yamamoto Y, Ueta Y, Yamashita H, Asayama K, Shirahata A. Expressions of the prepro-orexin and orexin type 2 receptor genes in obese rat. *Peptides* 2002;23:1689–96.
37. Johnen H, Lin S, Kuffner T, Brown DA, Tsai VW, Bauskin AR, Wu L, Pankhurst G, Jiang L, Junankar S, Hunter M, Fairlie WD, et al. Tumor-induced anorexia and weight loss are mediated by the TGF-beta superfamily cytokine MIC-1. *Nat Med* 2007;13:1333–40.
38. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. *Nature* 1994;372:425–32.
39. Pinto S, Roseberry AG, Liu H, Diano S, Shanabrough M, Cai X, Friedman JM, Horvath TL. Rapid rewiring of arcuate nucleus feeding circuits by leptin. *Science* 2004;304:110–15.
40. McCarthy HD, McKibbin PE, Perkins AV, Linton EA, Williams G. Alterations in hypothalamic NPY and CRF in anorexic tumor-bearing rats. *Am J Physiol* 1993;264:E638–E643.
41. Amaral ME, Barbuio R, Milanski M, Romanatto T, Barbosa HC, Nadruz W, Bertolo MB, Boschero AC, Saad MJ, Franchini KG, Velloso LA. Tumor necrosis factor-alpha activates signal transduction in hypothalamus and modulates the expression of pro-inflammatory proteins and orexigenic/anorexigenic neurotransmitters. *J Neurochem* 2006;98:203–12.
42. Scarlett JM, Zhu X, Enriori PJ, Bowe DD, Batra AK, Levasseur PR, Grant WF, Meguid MM, Cowley MA, Marks DL. Regulation of agouti-related protein messenger ribonucleic acid transcription and peptide secretion by acute and chronic inflammation. *Endocrinology* 2008;149:4837–45.
43. Chemelli RM, Willie JT, Sinton CM, Elmquist JK, Scammell T, Lee C, Richardson JA, Williams SC, Xiong Y, Kisanuki Y, Fitch TE, Nakazato M, et al. Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. *Cell* 1999;98:437–51.
44. Hara J, Beuckmann CT, Nambu T, Willie JT, Chemelli RM, Sinton CM, Sugiyama F, Yagami K, Goto K, Yanagisawa M, Sakurai T. Genetic ablation of orexin neurons in mice results in narcolepsy, hypophagia, and obesity. *Neuron* 2001;30:345–54.
45. Peyron C, Faraco J, Rogers W, Ripley B, Overeem S, Charnay Y, Nevsimalova S, Aldrich M, Reynolds D, Albin R, Li R, Hungs M, et al. A mutation in a case of early onset narcolepsy and a generalized absence of hypocretin peptides in human narcoleptic brains. *Nat Med* 2000;6:991–7.
46. Hagan JJ, Leslie RA, Patel S, Evans ML, Wattam TA, Holmes S, Benham CD, Taylor SG, Routledge C, Hemmati P, Munton RP, Ashmeade TE, et al. Orexin A activates locus coeruleus cell firing and increases arousal in the rat. *Proc Natl Acad Sci USA* 1999;96:10911–16.

European Journal of Pharmacology 655 (2011) 31–37

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Neuropharmacology and Analgesia

Allyl isothiocyanates and cinnamaldehyde potentiate miniature excitatory postsynaptic inputs in the supraoptic nucleus in rats

Toru Yokoyama [a,b], Toyoaki Ohbuchi [a], Takeshi Saito [a], Yuka Sudo [b,c], Hiroaki Fujihara [a], Kouichiro Minami [a,b], Toshihisa Nagatomo [a], Yasuhito Uezono [b], Yoichi Ueta [a,*]

[a] Department of Physiology, School of Medicine, University of Occupational and Environmental Health, Kitakyushu 807-8555, Japan
[b] Cancer Pathophysiology Division, National Cancer Center Reseach Institute, Tokyo 104-0045, Japan
[c] Department of Molecular and Cellular Biology, Nagasaki University School of Biomedical Science, Nagasaki 852-8523, Japan

ARTICLE INFO

Article history:
Received 2 July 2010
Received in revised form 11 January 2011
Accepted 12 January 2011
Available online 23 January 2011

Keywords:
TRPA1
EPSC
IPSC
Slice patch clamp
Supraoptic nucleus

ABSTRACT

Allyl isothiocyanates (AITC) and cinnamaldehyde are pungent compounds present in mustard oil and cinnamon oil, respectively. These compounds are well known as transient receptor potential ankyrin 1 (TRPA1) agonists. TRPA1 is activated by low temperature stimuli, mechanosensation and pungent irritants such as AITC and cinnamaldehyde. TRPA1 is often co-expressed in TRPV1. Recent study showed that hypertonic solution activated TRPA1 as well as TRPV1. TRPV1 is involved in excitatory synaptic inputs to the magnocellular neurosecretory cells (MNCs) that produce vasopressin in the supraoptic nucleus (SON). However, it remains unclear whether TRPA1 may be involved in this activation. In the present study, we examined the role of TRPA1 on the synaptic inputs to the MNCs in *in vitro* rat brain slice preparations, using whole-cell patch-clamp recordings. In the presence of tetrodotoxin, AITC (50 μM) and cinnamaldehyde (30 μM) increased the frequency of miniature excitatory postsynaptic currents without affecting the amplitude. This effect was significantly attenuated by previous exposure to ruthenium red (10 μM), non-specific TRP channels blocker, high concentration of menthol (300 μM) and HC-030031 (10 μM), which are known to antagonize the effects of TRPA1 agonists. These results suggest that TRPA1 may exist at presynaptic terminals to the MNCs and enhance glutamate release in the SON.

1. Introduction

Allyl isothiocyanates (AITC) and cinnamaldehyde are pungent compounds present in mustard oil and cinnamon oil, respectively (Jordt et al., 2004; Bandell et al., 2004). Transient receptor potential ankyrin 1 (TRPA1) (formerly ANKTM1) is the only mammalian member of the TRPA subfamily, and belongs to the TRP superfamily (Story et al., 2003). TRPA1 is a non-selective cation channel expressed widely, encompassing 20–35% of sensory neurones (Jordt et al., 2004; Nagata et al., 2005). TRPA1 is activated by a variety of noxious stimuli, including cold temperatures (below 17 °C), alkaline pH (Story et al., 2003; Fujita et al., 2008) and mechanosensation (Kwan et al., 2006, 2009). Surprisingly, a recent study demonstrated that hypertonic solution activates TRPA1 channels in human embryonic kidney 293 cells transiently expressing rat TRPA1 (Zhang et al., 2008).

TRPA1 is found in a subset of primary sensory neurons of dorsal root ganglia (DRG) that coexpressed with noxious heat-sensing TRPV1 (Story et al., 2003; Kobayashi et al., 2005). A recent finding has indicated that an N-terminal variant of the TRPV1 channel is required for osmosensory transduction in mouse supraoptic nucleus (SON) neurones (Sharif Naeini et al., 2006). The release of arginine vasopressin (AVP) from the magnocellular neurosecretory cells (MNCs) in the SON is crucial for body fluid homeostasis. The neuronal activity of the MNCs and AVP release is modulated by excitatory and inhibitory synaptic inputs and humoral factors such as osmotic change in plasma and many different endogenous factors (Mason, 1980; Leng et al., 1982; Bourque, 1989; Nagatomo et al., 1995). Excitatory synaptic inputs to the SON in rats are activated by hyperosmotic stimulation (Inenaga et al., 1997). To our knowledge, it is unknown whether TRPA1 is involved in modulating excitatory and inhibitory synaptic inputs to the SON or whether TPPA1 expresses in the SON. Therefore, we examined the effects of AITC and cinnamaldehyde on excitatory and inhibitory synaptic inputs in the SON in rats.

2. Materials and methods

2.1. Animals

Experiments were performed on male Wistar rats weighting 100–200 g. All procedures described in the present study were carried out

* Corresponding author at: Department of Physiology, School of Medicine, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu, 807-8555, Japan. Tel.: +81 93 691 7420; fax: +81 93 692 1711.
E-mail address: yoichi@med.uoeh-u.ac.jp (Y. Ueta).

0014-2999/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2011.01.011

in accordance with the guidelines on the use and care of laboratory animals as set out by the Physiological Society of Japan and under the control of the Ethics Committee of Animal Care and Experimentation, University of Occupational and Environmental Health, Japan.

2.2. Slice preparations

Rats were sacrificed by decapitation. We ensured the absence of gross contusion and hemorrhage after removal of each brain from the skull. The brains were rapidly removed and cooled in a perfusion medium at 4 °C for 1 min. A block containing the hypothalamus was cut and glued to the stage of a vibratome-type slicer (DSK Linearslicer™ PRO7; DSK, Kyoto, Japan). After careful removal of the meninges, coronal slices (150-μm thick) containing the SON were cut as described previously (Nagatomo et al., 1995). The slices were carefully trimmed with a circular punch (inner diameter 1.8 mm) and preincubated in the perfusion medium at room temperature for at least 1 h, after which they were transferred to the recording chamber.

2.3. Solutions and drugs

The perfusion medium contained (in mM): NaCl 124; KCl 5; KH_2PO_4 1.24; $CaCl_2$ 2; $NaHCO_3$ 25.9; and glucose 10. For Ca^{2+}-free solution, Ca^{2+} was replaced by Mg^{2+} and the osmolality was adjusted by lowering the Na^+ concentration. The pH was adjusted to 7.3, and the osmolality of all the solutions ranged between 298 and 303 mOsmol/kg. AITC was purchased from Wako (Osaka, Japan). Cinnamaldehyde, HC-030031 and ruthenium red were purchased from Sigma (St. Louis, MO, USA). Tetrodotoxin (TTX) was obtained from Sankyo Co. (Tokyo, Japan). Menthol was purchased from Nacalai (Kyoto, Japan). For stock solution, AITC, cinnamaldehyde and HC-030031 were dissolved in dimethyl sulfoxide (DMSO). Menthol was dissolved in ethanol. TTX and ruthenium red were dissolved in distilled water, and then all the drugs were dissolved into a working solution, with the final concentration of the solvents not being more than 0.1%. All the solutions used in this experiment were bubbled with a mixture of 95% O_2–5% CO_2. The pipette solution used in the recording electrodes contained (in mM): K-gluconate 140; $MgCl_2$ 1, $CaCl_2$ 1; EGTA 10; and Mg-ATP 2 (pH 7.3 with Tris base). TTX was present in all experiments except for Ca^{2+}-free solution.

2.4. Whole-cell recordings and data analyses

The slices were fixed in a recording chamber as described previously (Kabashima et al., 1997). Briefly, the slices were placed onto a glass-bottomed chamber and fixed with a grid of parallel nylon threads supported by a U-shaped stainless steel weight. The volume of the recording chamber was 1 ml, and the perfusion rate was 1.4 ml/min. The solution level was kept constant by a low-pressure aspiration system. To identify magnocellular neurones in the SON, we used an upright microscope (BX-50, Olympus, Japan) with Nomarski optics (×400). The drugs were applied to the slice preparation by switching the perfusion solution using a two-way valve (HV 4-4, Hamilton, Reno, NV, USA). The dead space washing time was excluded from the calculations.

The electrodes used in this study were triple-pulled with a puller (P-87, Sutter Instrument Co., Novoto, CA, USA) from a glass capillary, and had a final resistance of 5–9 MΩ when filled with the electrode solution. Electrophysiological recordings were carried out at 32–33 °C. Whole-cell recordings were made from microscopically identified SON neurones in the upper surface layers of the slices. Recordings of postsynaptic currents began 5 min after membrane rupture when the current reached a steady state. Currents and voltages were recorded with an EPC-10 amplifier (HEKA, Lambrecht, Germany). Signals were filtered at 3 kHz, digitised at 1 kHz with an analogue-to-digital converter (Mac lab/v. 3.5, Castle Hill, NSW, Australia), and stored on the hard disk of a personal computer. For quantitative analysis of the synaptic currents, only the AC components (using a 1-Hz high pass filter) were used for analysis with software (AxoGraph V.3.6.1, Axon Instruments, Foster Hill, CA, USA). Spontaneous events were automatically screened using an amplitude threshold of 15 pA and then were visually accepted or rejected based on the rise time and decay time. Recordings included for data analysis were collected during periods of stable series resistance (10–20 MΩ with no compensation).

2.5. Statistical analysis

Data are expressed as mean ± S.E.M. with n representing the number of neurones tested. Differences between two groups were examined for statistical significance using the paired t-test and between multiple groups by one-way ANOVA. A P value less than 0.05 denoted the presence of a statistically significant difference.

3. Results

Spontaneous synaptic currents were recorded from a total of 97 MNCs that were identified microscopically in thin punch-out SON slice preparations from 42 rats. As reported previously (Kabashima et al., 1997), excitatory postsynaptic currents (EPSCs) and inhibitory postsynaptic currents (IPSCs) were observed under basal conditions (without any stimulus). The EPSCs and IPSCs could be recorded selectively by setting the holding potential at −70 mV for the EPSCs and at −20 mV for the IPSCs. The EPSCs were abolished by the application of 6-cyano-7-nitroquin-oxaline-2,3-dione (CNQX; a blocker of non-NMDA receptors), and the IPSCs were abolished by the application of picrotoxin (a blocker of $GABA_A$ receptor-gated Cl^- channels), indicating that EPSCs and IPSCs reflect glutamate and GABA release, respectively. Spontaneous EPSCs and IPSCs (sEPSCs and sIPSCs) were insensitive to the Na^+ channel blocker TTX (1 μM), indicating that the sEPSCs and sIPSCs recorded from the thin punch-out slice preparations were miniature EPSCs and miniature IPSCs (mEPSCs and mIPSCs) that reflected the spontaneous quantal release of glutamate and GABA, respectively.

3.1. The effects of AITC and cinnamaldehyde on the mEPSCs

For this analysis, the mEPSCs recorded during 3-min periods under and after the application of AITC and cinnamaldehyde were compared with the mEPSCs recorded during 3-min periods before AITC and cinnamaldehyde application. AITC (50 μM) potentiated the mEPSCs frequency significantly (149 ± 4.6% of control, $P<0.01$, $n=9$). The effects of AITC were selective on the frequency of the mEPSCs, and the amplitude remained virtually unaffected (99.6 ± 1.4% of control, $P>0.05$, $n=9$) (Fig. 1). AITC significantly potentiated the frequency of the mEPSCs in a dose-dependent manner when tested at three concentrations (10, 30 and 50 μM) (Fig. 3A). AITC increased the mEPSC frequency to 106 ± 4.7%, 133 ± 7.8% and 149 ± 4.6% of the control values (n = 5–9). The increases of mEPSC were significant at 30 μM and 50 μM. The application of cinnamaldehyde (30 μM) significantly increased the frequency of the mEPSCs without affecting the amplitude, like AITC (frequency 143 ± 9.1% of control, $P<0.01$, $n=9$, amplitude 100 ± 1.5% of control, $P>0.05$, $n=9$) (Fig. 2). Like AITC, cinnamaldehyde also significantly potentiated the frequency of the mEPSCs in a dose-dependent manner when tested at three concentrations (10, 20 and 30 μM) (Fig. 2B). Cinnamaldehyde increased the mEPSC frequency to 102 ± 4.1%, 127 ± 8.2% and 143 ± 9.1% of the control values (n = 5–9). The increases of mEPSC were significant at 20 μM and 30 μM.

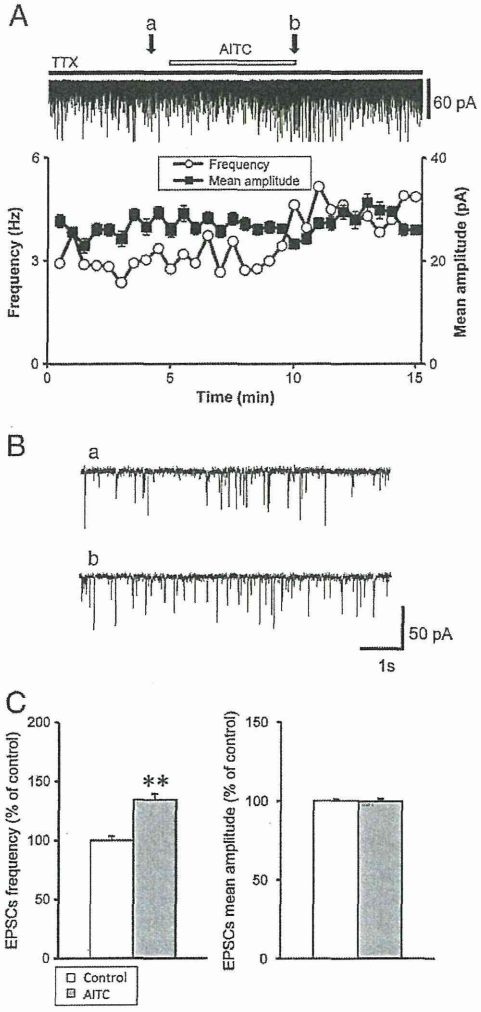

Fig. 1. Effect of AITC and related substances on miniature excitatory postsynaptic currents (mEPSCs) in the SON. (A) Representative example of AITC (50 μM) on mEPSCs. EPSCs were recorded in the presence of TTX (1 μM). The holding potential was −70 mV. Plots of frequency are single measurement, whereas plots of amplitude are mean ± S.E.M. over 30 s. (B) Consecutive trace of mEPSCs is shown in an expanded scale in time, (a) before and (b) during the action of AITC. (C) Summary of the effect of AITC on the frequency and amplitude of mEPSCs ($n=9$). The values are percentage changes (± S.E.M.) from control values obtained during a 3-min period at the beginning of the experiments (before adding AITC). $^{**}P<0.01$ versus control.

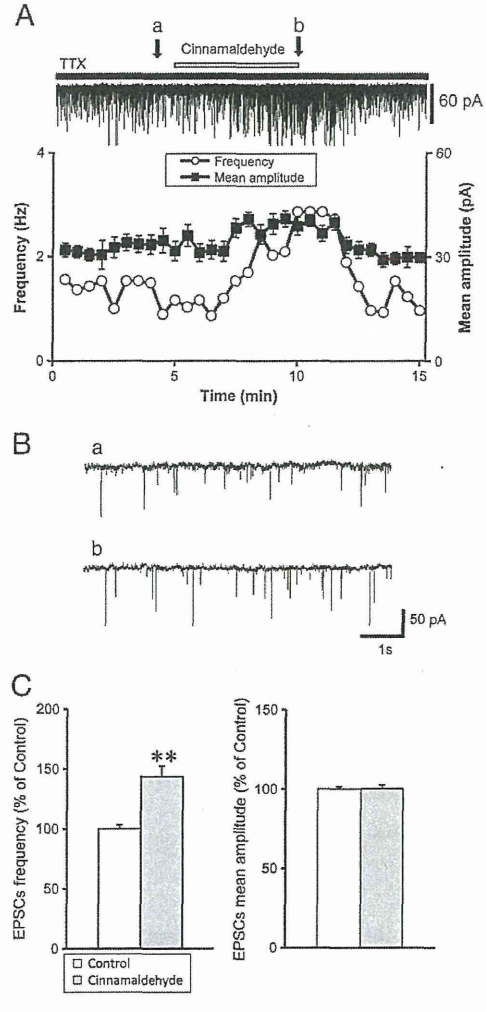

Fig. 2. Effect of cinnamaldehyde and related substances on miniature excitatory postsynaptic currents (mEPSCs) in the SON. (A) Representative example of cinnamaldehyde (30 μM) on mEPSCs. EPSCs were recorded in the presence of TTX (1 μM). The holding potential was −70 mV. Plots of frequency are single measurement, whereas plots of amplitude are mean ± S.E.M. over 30 s. (B) Consecutive trace of mEPSCs is shown in an expanded scale in time, (a) before and (b) during the action of cinnamaldehyde. (C) Summary of the effect of cinnamaldehyde on the frequency and amplitude of mEPSCs ($n=9$). The values are percentage changes (± S.E.M.) from control values obtained during a 3-min period at the beginning of the experiments (before adding cinnamaldehyde). $^{**}P<0.01$ versus control.

3.2. The effects of AITC and cinnamaldehyde on the mIPSCs

In contrast to the effect on the mEPSCs, the application of AITC (50 μM) did not have significant effects (frequency 102 ± 3.6%, amplitude 106 ± 3.6% of control, $P>0.05$, $n=7$) on mIPSCs. In the same way as AITC, cinnamaldehyde (30 μM) did not have significant effects on the mIPSCs (frequency 100 ± 2.3%, amplitude 101 ± 1.3% of control, $P>0.05$, $n=6$).

3.3. Effects of TRP blocker on AITC- and cinnamaldehyde-induced potentiation of mEPSCs

To examine whether the effects of AITC and cinnamaldehyde are mediated by TRP channels, we used 10 μM ruthenium red, a non-specific TRP channel blocker. Figs. 4A and 5A show the representative examples of the effects of ruthenium red. Pre-exposure to ruthenium red attenuated the potentiation of mEPSCs by AITC (50 μM) and cinnamaldehyde (30 μM). Figs. 4B and 5B show the summary data for the effects of ruthenium red on the amplitude and frequency. Ruthenium red almost completely abolished the AITC- and cinnamaldehyde-induced increase in mEPSCs frequency, but had no effect on the amplitude of mEPSCs (AITC: frequency 102 ± 8.1%, amplitude 103 ± 1.8% of control, $n=6$; cinnamaldehyde: frequency 103 ± 6.3%, amplitude 104 ± 1.9% of control, $n=6$). These results suggest the possible involvement of TRP channels in both AITC- and cinnamaldehyde-induced potentiation of the mEPSCs.

3.4. Effects of TRPA1 antagonists on AITC- and cinnamaldehyde-induced potentiation of mEPSCs

To determine whether the effects of AITC and cinnamaldehyde on the mEPSCs are involved in TRPA1, we examined the pre-exposure high concentration of menthol and HC-030031 on AITC- and cinnamaldehyde-induced potentiation of mEPSCs. A previous study demonstrated that a low concentration of menthol activates TRPA1