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General Summary

Our main interests are the physiological roles of nucleotide receptors and the mechanisms of regulation of intracellular Ca^{2+} concentration as an intracellular messenger in several kinds of cells, including bovine adrenocortical fasciculate cells (BAFCs), 3T3-L1 preadipocytes, Madin-Darby canine kidney (MDCK) cells, and brain astrocytes. We are also interested in the convulsant activity of quinolones, the anti-inflammatory activity of methylxanthines including theophylline, the neural mechanisms of vagal inspiration-promoting reflex, the visualization of respiratory neural activities, the effect of the urocortin family on the cardiovascular system, and clinical pharmacology.

Research Activities

Cross-talk between ACTH receptors and purinergic receptors in BAFCs

Extracellular adenosine triphosphate (ATP) and uridine triphosphate (UTP) bind to plasma membrane P2 receptors to regulate several cell functions in many kinds of cells. P2 receptors are divided into 2 families: the ligand-gated P2X and the G protein-coupled P2Y. The P2Y family has at least 8 subfamilies. We have previously reported that BAFCs contain Gq-protein-coupled $P2Y_2$. Both extracellular ATP and UTP bind to $P2Y_2$ to stimulate Ca^{2+} influx via IP_3 production from the extracellular space. ACTH is a physiological stimulator of adrenocortrical steroidogenesis via production of cyclic adenosine monophosphate (cAMP). We found that BAFCs express at least 3 types of adenylyl cyclase, the Ca^{2+}-calmodulin-potentiated types I and III, and the β-subunit-potentiated type II. We have reported that ATP and UTP potentiate both ACTH-induced steroidogenesis and cAMP production. Our findings suggest that the Ca^{2+}-influx pathway or the β subunit of Gq protein (which links to $P2Y_2$) or both are involved in the cross-talk between the ACTH receptor and $P2Y_2$. Results under our experimental conditions suggest that the β subunit of Gq protein, but not the Ca^{2+} pathway, participates in the event.

Study of purinergic receptors and steroidogenesis in a human adrenocortical tumor cell line (H295R cells)

The interaction between purinergic receptors, especially the $P2Y_2$, and ACTH receptors, was described above. However, because of the Japanese Public Health ordinance for the prevention of bovine spongiform encephalopathy, obtaining fresh bovine adrenal glands is now impossible. Therefore, preparing large quantities of fresh BAFCs has become difficult. Cells of the H295R line respond to ACTH and produce cortisol (a

physiological glucocorticoid) via a cAMP-dependent system. Thus, this cell line could be an excellent model for adrenocortical cells. We obtained this cell line and established cell culture conditions. We found that H295R cells express functional $P2Y_2$ and that extracellular ATP stimulates cortisol production.

Study of the intracellular Ca^{2+} dynamics

Ca^{2+} is an important regulator in many cellular functions. Therefore, we studied intracellular Ca^{2+} dynamics and its physiological functions in BAFCs, 3T3-L1 preadipocytes, MDCK cells, and brain astrocytes through the use of samples loaded with fluorescent calcium indicators (fura-2 and fluo-4).

1. Store-operated Ca^{2+} entry in BAFCs

Store-operated Ca^{2+} entry (SOCE), *i.e.,* Ca^{2+} entry triggered by the depletion of Ca^{2+} in the endoplasmic reticulum (ER), plays an important physiological role in nonexcitable cells. Both Gq-protein-coupled receptor agonists stimulate IP_3 production followed by Ca^{2+} release from the ER, and cyclopiazonic acid, a sarcoplasmic/endoplasmic reticulum Ca^{2+} (SERCA) pump inhibitor, inhibits Ca^{2+} reuptake and depresses the luminal Ca^{2+} concentration. Then, Ca^{2+} enters from the extracellular space through the activation of SOCE. Three hypotheses for the mechanism of SOCE have been proposed: 1) the conformational coupling model, 2) the exocytotic model, and 3) the diffusible messenger model. We have previously reported that BAFCs have a steroidogenesis-coupled SOCE system that is closely related to the actin network and that the conformational coupling model is the most prominent model in BAFCs. However, the identity of the plasma membrane SOCE channel in BAFCs is still unclear. One candidate for the SOCE channel is the transient receptor potential protein (TRP), especially the TRPC subtype. Our results, however, show that TRPC is not the SOCE channel in BAFCs.

2. SOCE in 3T3-L1 preadipocytes

3T3-L1 preadipocytes differentiate to adipose cells under the controlled culture conditions. Intracellular Ca^{2+} mobilization is an important factor in adipodifferentiation. The early stage of adipodifferentiation in 3T3-L1 preadipocytes is reportedly inhibited by Ca^{2+}. Therefore, we studied the mechanism of Ca^{2+} influx in 3T3-L1 preadipocytes. We found that the cells have an SOCE system that is activated by prostaglandin (PG) $F2\alpha$ via Gq-protein-coupled PG receptors and thapsigargin, a SERCA pump inhibitor. 3T3-L1 preadipocytes reportedly have PG receptors and secrete $PGF2\alpha$. These characteristics suggest the possible involvement, in an autocrine/paracrine fashion, of $PGF2\alpha$ in the adipodifferentiation of 3T3-L1 preadipocytes. The SOCE induced by $PGF2\alpha$ and thapsigargin was not abolished by treatment with the actin cytoskeleton modifying agents cytochalasin D and calyculin A. The results suggest that the SOCE model in 3T3-L1 preadipocytes is the diffusible-messenger model. The cells expressed 4 TRPC subtypes. However, these TRPCs did not participate in SOCE in 3T3-L1 preadipocytes under our experimental conditions. Recently, STIM1, an ER membrane protein, and Orai1, a plasma membrane protein, have been suggested to be involved in SOCE. We found STIM1 in 3T3-L1 preadipocytes.

3. Intracellular Ca^{2+} dynamics in MDCK cells

Autosomal dominant polycystic kidney disease is a common genetic disorder. Although the pathogenesis of this disease remains unclear, recent observations suggest that abnormalities of primary cilia play an important role in renal cyst formation. Primary cilia respond to mechanical bending by flow, and increase in the intracellular Ca^{2+}. MDCK cells are often used to study the mechanism of polycystic kidney formation. Therefore, we studied intracellular Ca^{2+} dynamics in MDCK cells. We found that spontaneous calcium oscillations occurred in the cells, even in the absence of primary cilia and extracellular Ca^{2+}. Spontaneous calcium oscillations in MDCK cells are suggested to be initiated by Ca^{2+} release from ryanodine/IP3-sensitive intracellular Ca^{2+} store, an ER. The frequency of calcium oscillations was enhanced by extracellular ATP. However, the oscillations did not disappear in the absence of extracellular ATP. Our results suggest that Ca^{2+} release from the ER is an important process and that ATP is a physiological factor modifying spontaneous calcium oscillations in MDCK cells.

4. Astrocytic calcium oscillations in rat hippocampal slice cultures

An increasing body of evidence indicates that ATP mediates glia-to/from-neuron signaling in the central nervous system (CNS) network. Extracellular ATP has various target proteins in both glia and neurons in the CNS, including ATP receptors and adenosine receptors, which are activated after converted extracellular ATP is rapidly converted to adenosine by ectoenzymes. Activation of purinergic receptors regulates astrocytic calcium oscillations, which are spontaneous events in astrocytes. However, the mechanism is not fully understood. We studied the role of extracellular ATP in the regulation of spontaneous astrocytic calcium oscillation in fluo-4-loaded rat hippocampal slice cultures using calcium imaging. We have reported that the activation of adenosine A_{2B} receptors modulates astrocytic calcium oscillations through acceleration of extracellular ATP breakdown into adenosine in the hippocampus of the rat.

Convulsant activity of new quinolones

New quinolones have been reported to have potent convulsant activity. We compared the convulsant activity of new quinolones in young mice and adult mice. New quinolones induced convulsions in a dose-dependent manner in young mice, and the ED_{50}values in young mice were similar to those in adult mice. Furthermore, co-administration with anti-inflammatory drugs enhanced the convulsant activity of new quinolones in both young mice and adult mice. The interaction of new quinolones and anti-inflammatory drugs did not differ between young mice and adult mice.

Anti-inflammatory activity of theophylline

Because theophylline has been reported to have anti-inflammatory activity, we studied the effects of theophylline and other methylxanthines on carrageenan-induced edema in rats. Theophylline and caffeine inhibited carrageenan-induced edema in rats. The inhibition of carrageenan-induced edema was suppressed by pretreatment with mefepristone or aminogluthetimide. These results suggest that theophylline has anti-inflammatory activity that is involved in the glucocorticoid-glucocorticoid receptor

system.

Visualization of the spatiotemporal pattern of respiratory neural activities in the isolated frog brainstem

We visualized the spatiotemporal activity of respiratory-related neurons in the frog by means of an isolated brainstem spinal cord preparation. We recorded optical signals from the ventral surface of the medulla with a voltage-sensitive dye and calculated cross-correlations with the integrated respiratory activity of the trigeminal nerve. Lung-burst-related depolarizing optical signals were observed bilaterally as longitudinal columns in the ventrolateral medulla between the levels of the trigeminal and hypoglossal rootlets, mostly caudal to the vagal nerve rootlet. However, we could not differentiate between neurons involved in rhythm generation and motoneurons. Extracellular recordings of respiration-related neurons verified the optically identified area. Strychnine disrupted the spatiotemporal organization of optical signals, although trigeminal periodic bursts persisted. We conclude that the pattern generator, but not the rhythm generator, of the lung burst in the frog involves glycinergic mechanisms and lies in longitudinal columns in the reticular formation of the ventrolateral medulla. (Collaboration with the Department of Physiology, Hyogo College of Medicine, Hyogo, and the Department of Medicine, Keio University Tsukigase Rehabilitation Center).

Neurophysiological and neuropharmacological studies on the central respiratory mechanism

1. Neural mechanisms of the vagal inspiration-promoting reflex and involvement of P2X receptors in nucleus of the solitary tract

To understand how respiratory movements are optimized in response to changes in internal and external environments, the research group for mammalian respiratory function has been studying neural mechanisms underlying various types of respiratory reflexes in mammals (in collaboration with the Laboratory of Neurophysiology, Department of Neuroscience). We have demonstrated that a local microinjection of pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), a P2X receptor antagonist, and kynurenic acid, a glutamate receptor antagonist, into the caudal nucleus of the solitary tract (NTS) of the anesthetized rabbit. The inspiration-promotion reflex of Hering-Breuer was attenuated by PPADS injection, and the inspiration-suppression reflex of Hering-Breuer was inhibited by kynurenic acid. Histological analysis of the site of action of PPADS showed that the marked reduction of the vagal inspiratory promotion reflex occurs most often when the site of PPADS injection, as identified with staining, contained the caudal part of the lateral NTS. This result argues clearly for a pivotal role of P2X receptors in the caudal NTS in the inspiration-promoting responses activated by decreased pulmonary stretch-receptor inputs.

Studies of the effects of cardiovascular regulatory substances on rat cardiomyocyte function

We have studied the effects of cardiovascular regulatory factors and agents on primary cultured neonatal rat cardiomyocytes. However, some factors and agents have little

effect on isolated cardiomyocytes but have more prominent effects on cardiomyocytes in the diseased human heart or in animal models. In pathological conditions of the heart, the ratio of noncardiac myocytes to cardiomyocytes increases. Therefore, the cross-talk between cardiomyocytes and noncardiac myocytes should be clarified. We developed a cardiomyocyte/noncardiac myocyte co-culture system using rat neonatal cardiocytes to address the difficulties of evaluating the effects of cardiovascular agents on cardiomyocytes and reported the fundamental data of this co-culture system. In addition, we investigated the intracellular dynamics of urocortin and its related peptides using HL-1 cardiomyocytes and some agents affecting rat cardiomyocytes.

Study of blood lactate level to evaluate the athletic performance

Many studies have examined muscle performance by measuring blood lactate concentrations during high-intensity exercise in athletes, and blood lactate is considered the metabolic variable that best reflects the capability of muscles for athletic performance. However, few studies have been performed in nonathletes, and the blood lactate concentration has not been recognized as a standard variable. Therefore, we investigated the effect of a 10-minute light bicycle load on the blood lactate concentration during the athletic performance of nonathletes. The blood lactate concentration during loading in nonathletes increased more quickly and markedly than in athletes but by 30 minutes after loading had returned to the pre-exercise level. In conclusion, even with light exercise, athletic performance can be evaluated by measuring blood lactate concentrations in a time-dependent manner.

Publications

Okada Y[1], Kuwana S[2], Masumiya H[3], Kimura N, Chen Z[4], Oku Y[3] ([1]Keio Univ, [2]Teikyo Univ, [3]Hyogo Col Med, [4]Glaxo Smith Kline). Chemosensitive neuronal network organization in the ventral medulla analyzed by dynamic voltage-imaging. *Adv Expl Med Biol* 2007; **605:** 353–7.

Kanai H, Marushima H, Kimura N, Iwaki T, Saito M, Maehashi H, Shimizu K, Muto M, Masaki T, Ohkawa K, Yokoyama K, Nakayama M, Harada T, Hano H, Hataba Y, Fukuda T, Nakamura M, Totsuka N, Ishikawa S, Unemura Y, Ishii Y, Yanaga K, Matsuura T. Extracorporeal bioartifitial liver using the radial-flow bioreactor in treatment of fatal experimental hepatic encephalopathy. *Artif Organs* 2007; **31:** 148–51.

Kubota Y[1], Tsuchiya M[1], Yamakami J[1], Terajima T[1], Hori S, Kizu J[1] ([1]Kyoritsu Univ Pharm). Pharmaceutical and pharmacological edvaluation of "Indomethacin M Ointment" as a pharmacy preparation. *Jpn J Pharm Health Care Sci* 2008; **34:** 174–80.

Kurihara Y[1], Kizu J[1], Hori S ([1]Kyoritsu Univ Pharm). Simple and rapid determination of serum carbapenem concentrations by high-performance liquid chromatography. *J Infect Chemother* 2008; **14:** 30–4.

Watanabe S[1], Terajima T[1], Kizu J[1], Hori S ([1]Kyoritsu Univ Pharm). Inhibitory effect of theophylline and methylxanthines on carrageenan-induced edema in rats and their Structure-activity Relationship. *Jikeikai Med J* 2008; **55:** 15–8.

Reviews

Hori S. PK/PD analysis and proper dosage of antimicrobial agents for effective and safe treatment of infectious diseases (in Japanese). *Yakugaku Zasshi* 2007; **127:** 931–7.

Hori S. Ketolide and its clinical evaluation based on PK/PD analysis (in Japanese). *J Jpn Hosp Pharmacists* 2007; **43:** 771–5.

Hori S. Macrolides: pharmacokinetics and safety (in Japanese). *Biomed Ther* 2007; **41:** 453–8.

Hori S. Antimicrobial chemotherapy based on PK/PD analysis (in Japanese). *Med Frontline* 2007; **62:** 1683–9.

Kimura N. Comparative respiration physiology (in Japanese). *Respiration* 2007; **26:** 719–25.

Hori S. Anti MRSA drugs and their re-evaluation (in Japanese). *Infection* 2007; **37:** 89–93.