アジア・パシフィックオピオイドシンポジウム

A-1

Role of κ-opioid receptor in cardioprotection of ischaemic preconditioning

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We have determined the role of κ-opioid receptor (κ-OR), the predominant OR in the heart, in immediate and delayed cardioprotection of preconditioning with ischaemic and metabolic inhibition, using isolated perfused rat heart and cultured single ventricular myocyte, respectively.

In the isolated perfused rat heart, ischaemia/reperfusion caused infarct. The effect was significantly attenuated by preconditioning of the heart with two cycles of 5 min ischaemia (IP) or perfusion with U50,488H, a selective κ-OR agonist. Nor-binaltorphimime (nor-BNI), a selective κ-OR antagonist, significantly attenuated and abolished the effects of IP and pretreatment with U50,488H, respectively. Blockade of protein kinase C (PKC) or the ATP sensitive potassium channel (K_{ATP}) with specific inhibitors significantly attenuated the effect of U50,488H pretreatment.

Exposure of isolated myocytes to a solution, that produced similated ischaemia, increased production of lactate dehydrogenase (LDH) measured 16 hr later. Both preconditioning with metabolic inhibition (MIP) and pretreatment with U50,488H for 30 min reduced the elevated production of LDH, an effect antagonized by nor-BNI. The LDH production following various treatments was directly correlated with the

production of heat shock protein (HSP) 70.

The results indicate that κ-OR mediates immediate and delayed cardioprotection of IP and MIP, respectively. The immediate protection involves both PKC and K_{ATP}, while delayed protection HSP 70. (Supported by Research Grant Council, HK)

A-2

Opioid sensitivity of mouse trigeminal ganglion neurons is correlated with calcium channel type, presence of VR1 and responses to prostaglandins and nociceptin.

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Modulation of voltage-gated calcium channel currents was examined in acutely isolated mouse trigeminal ganglion neurons. Cells were subdivided into 2 populations based on the presence or absence of low voltage activated 'T' current. The presence of T current was highly correlated with responses to μ-opioid and VR1 receptor agonists. Although PGE2 inhibited ICa in both cell types with an EC50 of 35 nM and kinetics indicative of inhibition by G-protein βγ subunits, pertussis toxin treatment abolished the effects of PGE_2 only in cells with T current, but not in those without. In contrast, cholera toxin did not affect PGE_2 mediated inhibition of ICa in cells with T current, but prevented the effects of PGE_2 in those without. Responses to prostanoids in the two cell types also displayed pharmacological differences. These data indicate that two populations of neurons in trigeminal ganglia differing in there sensitivity to μ-opioids and vanilloid receptor agonists have separate mechanisms of PGE_2 mediated inhibition of calcium channels. Nociceptin also inhibited ICa in a further subclass of these two types of sensory neuron.

A-3

Differential interaction of β-arrestins with distinct functional domains of δ opioid receptor and the molecular determinants of receptor for the direct contact

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β-arrestins have been shown to play a critical role in regulation of analgesic effectiveness and tolerance/dependence of opiate drugs. This study was designed to measure the potential direct interaction between β-arrestins and δ opioid receptor (DOR). The results from co-immunoprecipitation in transfected human embryonic kidney (HEK) 293 cells demonstrated that β-arrestin 1 effectively interacted with DOR in an agonist-enhanced manner and their interaction was unimpaired by truncation of the carboxyl terminal 15 amino acids on DOR. *In vitro* data from Glutathione S-transferase (GST) pull-down assay confirmed that β -arrestins directly bound to either the carboxyl terminal tail (CT) or the third intracellular loop (i3L) of DOR. Surface plasmon resonance (SPR) detection further revealed that distinct sites on β-arrestin 1 appeared to be involved in binding to CT (2.9 mM with one site) or to i3L (7.6 mM with two sites). Moreover, deletion of the last 15 residues on CT and site-directed mutation of several serine/threonine residues on CT or i3L resulted in the complete abrogation

of their binding to β-arrestins. Taken together, this study suggests that as small as 6-7 amino acid domains with two separate serine/threonine residues (S/TX4-5S/T), independent of their phosphorylation, are responsible for interaction of G protein coupled receptors to β-arrestins.

A-4

Recent Studies on Proenkephalin Gene Expression Regulation in Glial Cells

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In this study, the regulation of proenkephalin gene expression in glial cells will be discussed. First, the extracellular factors, primarily arachidonic acid and PGE2, affecting proenkephalin gene expression will be dicussed. Next, the roles of second messengers such as PKC and PKA involved in the regulation of proenkephalin gene expression in both C6 rat glioma cells and primary cultured glial cells will be discussed. We found that PMA (a PKC activator) increased proenkephalin mRNA in only primary cultured glial cells, but not C6 rat glioma cells, although PMA was able to induce c-fos and c-jun mRNA and their products, which are known as transcriptional factors involved in proenkephalin gene. PMA also elevated AP-1 DNA binding activity in both primary cultured glial cells and C6 rat glioma cells. Additionally, cycloheximide attenuated PMA-induced increase of proenkephalin mRNA in primary cultured glial cells. cycloheximide also attenuated c-Fos, c-Jun, and Fra proteins induced by PMA, suggesting that c-Fos, c-Jun and Fra proteins may play major roles in the regulation of proenkephalin gene expression when PKC system is stimulated in primary cultured glial cells. On the other hand, the activation of PKA system increased proenkephalin mRNA levels in both primary cultured glial cells and C6 rat glioma cells. In primary cultured glial cells, cycloheximide potentiated

proenkephalin mRNA elevation induced by forskolin, whereas cycloheximide did not affect proenkephalin mRNA level induced by the activation of PKA system in C6 rat glioma cells. The potentiating effect of cycloheximide appears to be mediated by both phosphorylated c-Fos protein and phosphorylated cAMP responsive element binding protein (pCREBP). Dexamethasone, a synthetic glucocorticoid hormone, showed a synergism for the regulation of proenkephalin mRNA expression, when combined with forskolin or norepinephrine in both primary cultured glial cells and C6 rat glioma cells. We investigated the possible roles of immediate early genes and their products, proto-oncoproteins, and pCREBP in this synergistic regulation of proenkephalin gene expression in primary cultured glial cells, and found neither immediate early gene products nor pCREBP is participating in the synergistic interaction between glucocorticoid and PKA system for the regulation of proenkephalin gene expression in primary cultured glial cells. Currently, we are trying to find the molecular mechanisms involved in this synergistic interaction in C6 rat glioma cells.

A-5

Does Nocistatin play physiological roles in pain perception ?

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In 1998 we proposed nocistatin as a new neuropeptide which could be involved in pain transmission. Although nocistatin was processed from prepronociceptin, it blocked allodynia and hyperalgesia evoked by nociceptin and prostagrandin E2. At first, nocistatin was identified in bovine, followed by human (h), rat (r) and mouse (m) brains. Combined HPLC with specific RIAs for nocistatin and nociceptin, the structures of h-, r- and m-nocistatin were confirmed by comparison with the synthetic peptides based on their mRNAs.

The extracts from h-brain showed three nocistatin-like immunoreactivity peaks [NST-IR(1, 2 and 3)] on their HPLC chromatograms, one of which, NST-IR (3), coincided with the putative 30 amino acid h-nocistatin. NST-IR (2) was estimated to be precursor proteins common to both nociceptin and nocistatin since nociceptin-like immunoreactivity was also detected in this peak. The retention times of NST-IR (1) in two HPLC conditions coincided with those of the synthetic C-terminal heptadecapeptide of h-nocistatin, whereas r- and m-brains did not contain this C-terminal fragment.

The extracts of cerebrospinal fluid from various pain state patients also showed the same three NST-IR peaks as those from brain tissues, which might reflect possible physiological and/or pathological roles of nocistatin.

A-6

Changes of Nociceptin level and receptors in fetal hypoxia-treated rats

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The effects of prenatal hypoxia exposure was investigated on brain nociceptin levels in rats. Subjects were offspring of rats that received hypoxic prenatal treatments. Levels of nociceptin were measured in seven brain regions of control and of rats treated with hypoxia at 2, 7, 14, 28 and 56 days after birth. In sham-treated rats, the highest levels of nociceptin in cerebral cortex, olfactory bulb, striatum, medulla and midbrain were observed at p14. In cerebral cortex and hippocampus, the highest levels of nociceptin were reached at p7 and p56, respectively. Prenatal hipoxia treament altered the amount and pattern of developmental changes of nociceptin in various brain regions. Abrupt increase of the levels of nociceptin in medulla and midbrain at p2 were not seen in hypoxia-treated animals in which the rapid changes was observed at p7. The highest levels of nociceptin in cerebral cortex and striatum were seen at p28 instead of p14 as in control rats. The level of nociceptin in striatum of hypoxia-treated rat was more than two fold than that of control rats. Therefore, these data support the regulatory role of nociceptin in development of central nervous system which can be influenced by maternal hypoxia.

A-7

Leukemia inhibitory factor induced nociceptin/orphanin FQ mRNA in the rat cortical neurons

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Leukemia inhibitory factor is a cytokine that was identified as a glycoprotein that suppressed proliferation of M1 myeloid leukemic cells and induced their differentiation. We showed that mRNA expression of this cytokine was induced mainly in astrocytes of the rat brain after the kainic acid-induced convulsion and that LIF receptor mRNA was expressed in neuronal cells of the normal rat brain. Furthermore, some neurotransmitters, especially, ATP induced significant expression of LIF mRNA in the cultured astrocytes. These results suggest that LIF plays an important role in the neuro-glia interaction in the brain. Next, we examined the effects of LIF on the expression of mRNAs for neuropeptides and synthetases of neurotransmitters in the cultured cortical neurons. Interestingly, LIF specifically increased nociceptin/orphanin FQ mRNA. The expression of mRNAs for enkephalin, somatostatin, galanin, choline acetyltransferase and glutamic acid decarboxylase was not altered. Because the receptor for nociceptin/orphanin FQ is reportedly expressed in the neuronal cells of the various brain regions, nociceptin/orphanin FQ induced by LIF possibly acts on the neuronal cells. These findings suggest that nociceptin/orphanin FQ, together with LIF and ATP, functions as a mediator in the neuro-glia interaction.

A-8

PKC-mediated inhibition of μ-opioid receptor internalization and morphine acute tolerance

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As reported elsewhere, the incubation of μ-opioid receptor (MOR)-expressing CHO cells with 1 μM DAMGO, a peptide MOR agonist, caused a marked internalization of the receptor 30 min after the agonist challenge, detected by immunocytochemistry. However, the incubation with 10 μM morphine did not affect the receptor dynamics. When calphostin C, a protein kinase C inhibitor was treated with morphine, MOR was internalized at as early as 10 min after the agonist challenge. Both internalization by DAMGO alone and morphine plus calphostin C were abolished by the pretreatment with adenovirus expressing K44A dynamin negative mutant. On the other hand, in the peripheral nociception test in mice, nociceptive flexor responses by intraplantar injection of bradykinin (BK) were blocked by similar application with morphine or DAMGO. When morphine was given again at 4-hr later after the initial challenge with morphine, morphine-induced analgesia was markedly reduced (acute tolerance). The acute tolerance was inhibited by calphostin C-pretreatment. Acute tolerance was not observed with the similar treatment with DAMGO, however, it was elicited by the K44A adenovirus pretreatment and then rescued by calphostin C. These results suggest that the acute tolerance is mediated by PKC-dependent inhibition of receptor internalization.

binding by μ-agonists. Concomitant administration of PKC inhibitors with DAMGO restored the desensitization of μ-agonist-activated G-proteins induced by chronic DAMGO treatment. Using Western blotting, the up-regulation of PKCγ, but not α, βI and βII, was observed in spinal cord membranes from DAMGO-tolerant mice. On the contrary, chronic naloxone treatment resulted in the decrease in PKCγ. These findings indicate that translocation of PKCγ isoform to membranes may be the key factor to induce the desensitization of μ-agonist-activated G-proteins following chronic μ-agonist treatment.

A-10

Study the action of U-50,488 to prevent the development of morphine tolerance

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U-50,488, a selective κ-opioid receptor agonist, has been reported to inhibit the development of tolerance to morphine. However the mechanisms involved are not clear. In rat hippocampal slice model, we used Schaffer-Collateral stimulation induced population spikes in CA1 area to study the effects of morphine. We found morphine (10 μM) increased the amplitude of population spikes (200-300%) and tolerance developed after continuous superfusion of morphine for 2-6 h. Meanwhile the NO level was increased about 250%. When U-50,488 (200 nM) was co-superfused with morphine, the development of morphine tolerance was blocked and the NO level decreased to 100-150% of the control level. L-arginine (500 nM) significantly reversed the effect of U-50,488 to block the development of morphine tolerance and also the effect on the NO level.

In guinea pig model, we chronically treated animals with morphine or morphine + U-50,488 *in vivo* and removed spinal cords to do *in vitro* slices experiments including glutamate release, NMDA receptor binding and NO level determination. Our results indicated the increase of glutamate release, down-regulation of NMDA receptors and increase of NO after chronic morphine treatment. The action of U-50,488 to prevent morphine tolerance may be due to its effect on inhibiting glutamate release which stimulated by chronic morphine. Furthermore, U-50,488 also inhibits the overproduction of NO by chronic morphine and this may also contribute to its effect on preventing morphine tolerance.