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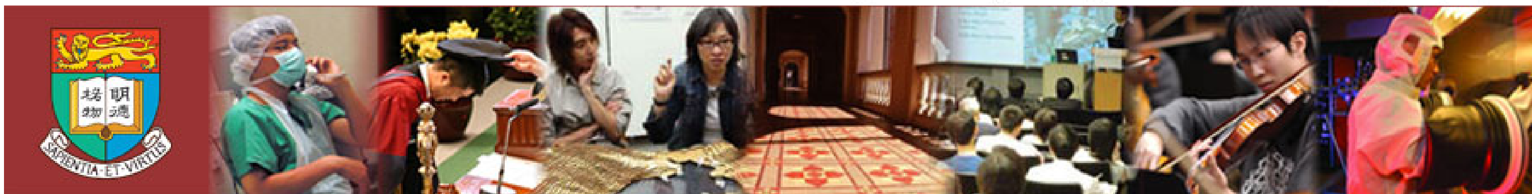

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Lithium ion alter Ins(1,4,5)P_3 production and mobilisation of intracellular free calcium in *k*-agonist stimulated rat ventricular myocytes.

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ABSTRACT

The effect of lithium ion on the generation of inositol phosphates and the mobilisation of intracellular free calcium in isolated adult rat ventricular myocytes stimulated with *k*-agonist was studied. Dynorphin $_{1-13}$ stimulated an increase in the levels of Inositol 1,4,5-trisphosphate (InsP_3) and intracellular free calcium ($[Ca^{2+}]_i$). Preincubation of the ventricular myocytes with 10mM Li^+ for 20 minutes prior to stimulation by dynorphin $_{1-13}$ reduce the increase of both InsP_3 and $[Ca^{2+}]_i$. This effect of Li^+ was not seen if 5mM inositol was also present during preincubation. It is concluded that (1) the inhibition of the *k*-agonist stimulated increase in InsP_3 and $[Ca^{2+}]_i$ in Li^+-pretreated ventricular myocytes was a result of a depletion of intracellular inositol, due probably to the inhibition by Li^+ of inositol monophosphatase which participates in the recycling of intracellular inositol, and (2) the *k*-agonist induced increase in $[Ca^{2+}]_i$ is a consequence of the production of InsP_3 since altering the kinetics of production of this compound also altered $[Ca^{2+}]_i$.

Key words: lithium, dynorphin$_{1-13}$, inositol 1,4,5-trisphosphate, calcium, myocytes

INTRODUCTION

It has been demonstrated that for stimulation with opioid agonists two intracellular events could be detected in cells either of neuronal or cardiac origin: the intracellular mobilisation of calcium ions and the production of inositol 1,4,5-trisphosphate (Jin, *et al.*,1994; Ventura *et.al.*,1992). This observation strongly suggests that inositol 1,4,5-trisphosphate is a second messenger mediating the effect of *k*-opioid on the regulation of intracellular calcium level.

Receptor-stimulated metabolism of inositol lipids is strongly influenced by lithium ion which inhibits inositol monophosphatase in a uncompetitive manner (reviewed by Nahorski *et. al.*, 1992). In the presence of lithium, prolonged stimulation of receptors which were coupled to the production of Ins(1,4,5)P_3 eventually resulted in an impairment of Ins(1,4,5)P_3 production and an accumulation of CMP-phosphatidate (Jenkinson *et al.*, 1994, Kennedy *et al.*,1990, Godfrey, 1989). This is probably due

to an inhibition by lithium of inositol monophatase which was rendered incapable of producing free inositol from inositol monophosphates for the resynthesis of phosphatidylinositol, the precursor for phosphatidylinositol 4,5-bisphosphate. This action of Li has been strongly suggested as the molecular basis for its therapeutic effect in the treatment of manic-depression (Berridge *et al.*, 1982, 1989). The recent findings that *k*-agonist causes an increased production of Ins(1,4,5)P_3 in cells suggested that lithium may also be able to perturb the signalling mechanism of *k*-agonist. Lithium would thus be useful both as a tool for the further investigation of the physiological action of *k*-agonist and as a potential therapeutic agent. In the present study, we had utilised freshly isolated adult rat cardiomyocyte to study the kinetics of Ins(1,4,5)P_3 production and intracellular calcium mobilisation caused by stimulation with the *k*-agonist dynorphin$_{1-13}$. Lithium was found to affect the kinetics of both these signals and its effect could be counteracted by the presence extracellular inositol. The implications of these results are discussed in the present communication.

METHODS

Isolation of rat ventricular myocytes

Rat ventricular myocytes were prepared according to Tai *et al* (1992). Briefly, a male Sprague-Dawley rat was decapitated and the heart was rapidly removed and perfused retrogradely for 5 min with Joklik modified Eagle's medium (MEM) supplemented with 10 mM $NaHCO_3$, 10mM Hepes and 1.25mM Ca^{2+}, followed by calcium-free MEM for the next 5 min. Then the heart was perfused with TYPE I collagenase (125 U/ml) in calcium- free MEM for 40 min. Ventricles myocytes were dissociated by repeated pipetting in the collagenase MEM. The myocytes were washed three times with calcium-free MEM and finally resuspended in MEM containing calcium (30uM). Seventy to eighty percent of the myocytes were rod-shaped and were not trypan-blue permeable. The calcium concentration of the MEM was increased gradually to 1 mM in 30 min. The preparation remained viable at ambient temparature for more than two hours.

Extraction of InsP_3 in isolated rat ventricular myocytes and InsP_3 assays

Ventricular myocytes were incubated with or without dynorphin $_{1-13}$ for appropriate periods of time at 37C. Ins(1,4,5)P_3 was extracted from isolated rat ventricular myocytes according to the method of Ventura *et al* (1992) with slight modifications. Approximatley 1 - 1.25 x 10^4 ventricular myocytes in 0.25 ml MEM containing 1.25mM calcium were quenched with 0.2 volume of ice-cold 30% perchloric acid for 20 min. The precipitated protein was sonicated with a Fisher Sonic Dismembrator Model 300 at a setting of 40% for 40 seconds twice with a pause of 15 seconds in between. Proteins were sedimented by centrifugation at 3000g for 15 min at 4 C. Supernatants were neutralised with 1.5M KOH containing 60mM HEPES buffer. The mixture was centrifuged at 3000g for 10 min at 4C. The supernatants were collected and lyophilised until assay for Ins(1,4,5)P_3 content. Before the start of the assay the residue was resuspended in 240uL distilled water. An aliquot of 100ul of extract was taken for the determination of InsP_3 level. The InsP_3 was assayed in duplicate with a commercial *D*-myo-inositol 1,4,5-trisphosphate assay system from Amersham. The protein was determined by the method of Lowry et al (1952) using bovine serum albumin (fraction V) as a standard.

Measurment of intracellular calcium

Myocytes were of a rod shape with clear striation and were loaded with 2uM Fura-2 AM at room temperature for 20 min in HEPES buffered-Joklik medium with 1% BSA and 1mM Ca^{2+}. The

cells loaded with Fura-2 were resuspended in Krebs-Henseleit buffer ($NaHCO_3$ replaced with 25mM HEPES) containing 1mM Ca^{2+} and stored at room temperature for at least 1h before use. For cell suspension studies, a Hitachi F-400 was used. For single cell memasurement, fluorescence measurements were performed with a Nikon inverted microscope equipped with a fluorometer system (PTI) according to Sheng and Wong (Anesthesia, same issue).

Paired Student's T-test was used to determine the difference before and during drug administration and one way of analysis of variance was used to determine the difference among groups. Significance level was set at p 0.05.

RESULTS

When dynorphin $_{1-13}$ at a dose of 50uM was added to isolated rat cardiomyocyte and incubated for two minutes, the cellular Ins(1,4,5)P_3 content increased from 0.12 +/-0.02 pmole/mg protein in the control to 0.37+/-0.08 pmole/mg protein in the stimulated samples. At a dose of 100uM, the amount of Ins(1,4,5)P_3 generated by the cardiomyocytes was 0.5+/-0.02 pmole/mg protein. The *k*-antagonist, Mr2266 at a concentration of 100uM was able to suppress the increase in Ins(1,4,5)P_3 by greater than 90%, demonstrating that the Ins(1,4,5)P_3 response was due to dynorphin $_{1-13}$. Preincubation of the cardiomyocytes with 10mM LiCl also abolished the Ins(1,4,5)P_3 response towards dynorphin (Control: 0.1+/-0.01 pmole/mg protein; with dynorphin (50uM): 0.62+/-0.16pmole/mg protein; Li^+ and dynorphin: 0.05+/-0.01 pmole/mg protein). However, in the presence of inositol (10mM), preincubation of cardiomyocytes with LiCl has no effect on the Ins(1,4,5)P_3 response (Li^+ and dynorphin and inositol: 0.60+/-0.2 pmole/mg protein.)

Incubation of cardiomyocytes with 50uM dynorphin for variuos time intervals showed that the change in the Ins(1,4,5)P_3 level was consisted of two phases: a rapid initial rising phase during which Ins(1,4,5)P_3 attained a maximum value (3.00+/-1.43 pmole/mg protein) in thirty seconds. This was followed by a steady phase in which Ins(1,4,5)P_3 remained at a value intermediate between the maximum and the basal value. In the presence of Li^+, the initial rise in the accumulation of Ins(1,4,5)P_3 was not statistically different from the control (i.e. in the absence of lithium). However, the Ins(1,4,5)P_3 level in the steady phase was not different from that of the basal level. Such a supression of Ins(1,4,5)P_3 in the steady phase was relieved by co-incubation of 5 or 10 mM inositol together with Li^+. In this case, both the initial and the steady phases of Ins(1,4,5)P_3 were not statistically different from the control i.e. without both Li^+ and inositol.

Isolated cardiomyocytes also responded to dynorphin$_{1-13}$ with an increase in intracellular calcium. This response was registrable with 50uM of dynorphin. Single cell intracellular calcium measurments showed that the increase in intracellular calcium level is small but significant and lasted as long as the agonist is present. In the presence of LiCl, the elevation in calcium level only lasted for two minutes. However, the presence of inositol together with LiCl again enabled the calcium level to remain elevated for as long as the agonist was present.

DISCUSSION

The present study demonstrated that the *k*-opioid stimulated metabolism of inositol lipids shares with other hormones or agonists in its sensitivity towards the presence of lithium ions. Lithium's ability to suppress the steady phase level of Ins(1,4,5)P_3 was most probably due to the inhibition of the inositol monophosphatase within the cardiomyocytes, leading to a depletion of intracellular inositol. The synthesis of inositol lipids was therefore impaired. Eventually very little phosphatidylinositol 4,5-bisphosphate would be available for the formation of Ins(1,4,5)P_3. This explanation is supported in the present study by the observation that addition of extracellular inositol to the medium during stimulation can fully eliminate the inhibitory effect of lithium. In the presence of added inositol, the inhibition of inositol monophosphatase by Li^+ will not block the supply of free inositol for the synthesis of inositol lipids.

Lithium also produced an inhibitory effect on the mobilisation of intracellular calcium. This effect was only apparent after two minutes of stimulation with the k-agonist. Interestingly, addition of extracellular inositol also alleviate lithium's effect on calcium mobilisation. The addition of inositol, which ensures the continuous production of Ins(1,4,5)P_3, could relieve the lithium's effect on caclium mobilisation, strongly suggests that the idea inositol 1,4,5-trisphosphate serves as the cause for the release of intracellular calcium seen during prolonged stimulation of the cardiomyocytes with the k-agonist.

For its ability to interfere with the signalling mechanism of *k*-opioids, lithium might have the potential as an important tool for the investigation of the neurological action of these endogenous opioid peptide.

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