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The metabolic evidence of synergistic effect between ohmefentanyl and [D-Pen^2, D-Pen^5] enkephalin on differentiated SH-SY5Y cells in humans

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Abstract

Interactions between selective opioid agonists acting at μ- and δ-opioid receptors were evaluated by co-administering a low-effective dose of the selective μ-opioid receptor agonist ohmefentanyl (OMF) with sequentially increasing doses of the selective δ-opioid receptor agonist [D-Pen^2, D-Pen^5] enkephalin (DPDPE). Microphysiometer was used to measure the extracellular acidification rate (ECAR) of living cells in real-time, which reflected the functional activity after agonist-receptor binding. The synergy (i.e. a more than additive effect) was observed with combinations of these two opioid agonists on differentiated SH-SY5Y cells functionally expressing both μ- and δ-opioid receptors. The demonstration of the synergy suggests that the agonists of the subtypes of opioid receptors can interact at cellular level.

Keywords: Ohmefentanyl; [D-Pen^2; D-Pen^5] Enkephalin; Synergy; Microphysiometer; Extracellular acidification rate; SH-SY5Y cells

Several lines of evidence have suggested the analgesic synergy between μ- and δ- opioid receptor. There has been considerable interest in the possibility of synergistic interaction between drugs as an alternative to a single drug therapy, since this may provide the advantage of using lower dose of individual agents to reduce the adverse side effects [13]. However, very few studies about the interaction have been performed at cellular level, especially in real-time detection. The microphysiometer (Molecular Devices Corp., USA) provided the possibility of continuous observation of real-time acidification rate in the external microenvironment of cells as an indicator of functional activity. Since the main products of cellular metabolism are lactic acid and carbon dioxide, the amount of metabolites extruded into extracellular environment can be used as a measurement of cellular metabolism [7,8]. As a means to detect activation of G-protein coupled receptors, the microphysiometer displays greater sensitivity compared with measuring second messenger formation [1]. Other advantages include measurements of cellular responses in real time, absence of radioactivity, etc. In addition, the microphysiometer is also suitable for detecting the interaction between two receptors [10]. In the present study, we use the microphysiometer to explore the metabolic changes associated with agonist-receptor binding on differentiated SH-SY5Y cell, a human neuroblastoma cell line functionally expressing both μ- and δ-opioid receptors. The results indicate that there is a synergy between μ- opioid receptors agonist ohmefentanyl (OMF) and δ-opioid receptor agonist [D-Pen^2, D-Pen^5] enkephalin (DPDPE) on differentiated SH-SY5Y cells.

SH-SY5Y, NG108-15 and CHO cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL) containing 10% heat-inactive fetal bovine serum (Gibco BRL) and maintained in 5% CO_2 humidified atmosphere at 37°C until the cell monolayer reached 70–80% confluence. The medium for NG108-15 cells was supplied with hypoxanthine, aminopterin and thymidine. SH-SY5Y cells were differentiated toward neuronal phenotype by addition of 1×10^{-5} M retinoic acid (RA, sigma) for 6 days, and the RA-containing medium was changed every 2 days. Cells were plated into cell capsule cups supplied by Molecular Devices Corp. at a density of approximately 3.5×10^5 cells/ml/cup the day prior to study.

The microphysiometer contains a micro-volume flow chamber in which cells adhere to polycarbonate membrane in a three-part disposable assembly. The flow chamber and

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disposable assembly are above and adjacent to a silicon-based light addressable potentiometric sensor that can measure small changes in the local acidification rate. DMEM (serum-free and bicarbonate, HEPES-free) flows at a rate of approximately 100 μl/min through the chambers and disposable assembly. A voltage signal proportional to pH is measured and recorded every second. For determination of extracellular acidification rates (as μV/s), DMEM media flow is halted every two minutes, allowing the accumulation of extracellular acidic metabolites produced as a result of cellular metabolism. Numerically, 1 μV/s is approximately equal to 1×10^{-3} pH units/min. The percent change of extracellular acidification rate (ECAR) is calculated by subtracting the baseline immediately prior to exposure to drugs.

While there are several methods of analysis currently available to analyze the interactions between biologically active agents, the method used here was a two-factor, repeated measure ANOVA. According to the method, we chose the increasing doses of DPDPE and a combination of the low dose of OMF with sequentially increasing doses of DPDPE. If the two dose-response curves displayed parallel pattern, it indicated that combination of the two drugs produced an additive effect. Whereas, if the curves were divergent, the two drugs may have a synergistic interaction [6].

SH-SY5Y cells were exposed to OMF (a generous gift from professor Zhi-Qiang Chi of Shanghai Institute of Materia Medica, Chinese Academy of Sciences) and DPDPE (Sigma), respectively for 30 s, 2, 4 and 6 min. It was found that 30 s was high enough to induce an effective response. Considering that long-time (more than 30 s) exposure may induce receptor desensitization that may affect the subsequent response, we chose 30 s as the exposure time in the following experiments.

Exposure of resting SH-SY5Y cells to different doses (1, 2, 4, 8, 16, 32 and 64 $\times 10^{-6}$ M) of OMF produced a dose-dependent increase in ECAR (Fig. 1A), and it began to show significant effect at the dose of 8×10^{-6} M ($P < 0.01$). Each curve was repeated at least five times. In order to exclude the unspecific reaction, high selectively opioid receptor antagonists were used. Cells were challenged by 5×10^{-6} M naloxone for 5 min prior to exposure to OMF. The response of OMF at the doses of 8, 16 and 32 $\times 10^{-6}$ M could be blocked by naloxone completely, but it only blocked the response of OMF at the doses of 64 and 100×10^{-6} M partly (Fig. 1B). OMF used in this experiment is injection with the highest concentration of 125 μM (only this form can be get). The incomplete block may be due to the unspecific acidity of the solution. Then the doses higher than 64 μM were excluded from the dose-response curve.

Exposure of cells to different doses (10^{-4}–10^{-9} M) of DPDPE produced a bell-shaped dose-response relationship. The ECAR reached a maximum at 10^{-5} M. Cells were challenged by 10^{-6} M δ-opioid antagonist ICI174, 864 for 5 min before exposure to DPDPE. ICI174,864 could block the response of DPDPE at the doses of 0.5, 1 and 2×10^{-6} M completely (Fig. 2).

Furthermore, CHO cells, CHO cells transfected with μ-opioid receptors and NG108-15 cells expressing δ-opioid receptors were used to test the specificity. As shown in Fig. 3, 8×10^{-6} M OMF induced the increase of ECAR in CHO-μ-opioid receptor cells, but no responses in CHO and NG108-15 cells. Similarly, 2×10^{-6} M DPDPE could enhance the ECAR in NG108-15 cells, but not in CHO and CHO-μ-opioid receptor cells.

The effect of combined exposure to these two drugs was designed according to a two-factor, repeated measure ANOVA. Since 8×10^{-6} M OMF is the lowest effective dose, it was chosen as the fixed dose and combined with sequentially increasing doses of DPDPE. The doses of DPDPE were inside the linear part of the dose-response curve. They were 0.5, 1 and 2×10^{-6} M. We compared the difference among doses as well as between the group of increasing doses of DPDPE and the combined group, respectively. The difference was significant ($P < 0.05$). Furthermore, we analyzed the interaction between group

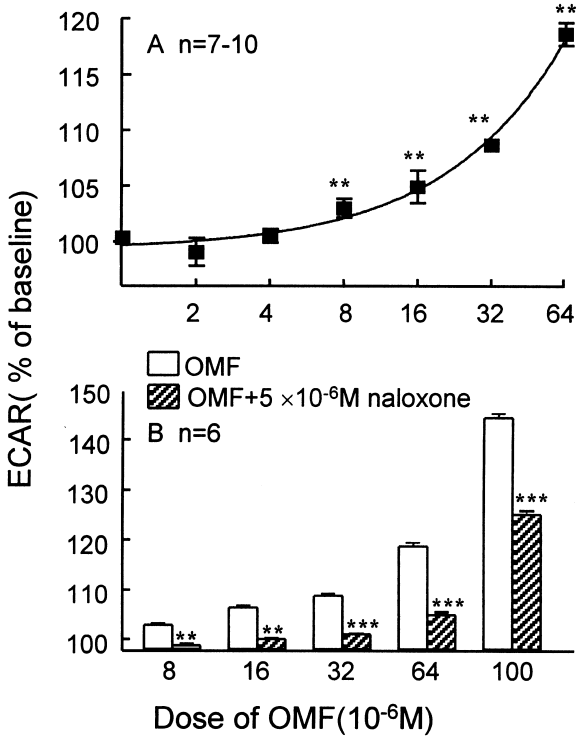

Fig. 1. Dose-response relationship of OMF in inducing the enhancement of extracellular acidification rate (ECAR) on differentiated SH-SY5Y cells in 30 s. (A) SH-SY5Y cells were challenged by OMF for 30 s with different doses. 8×10^{-6} M is the lowest effective dose compared with NS. $^{**}P < 0.01$ compared with NS group. (B) Cells were challenged by 5×10^{-6} M naloxone for 5 min prior to exposure to different doses of OMF for 30 s. The responses induced by 8, 16 and 32 $\times 10^{-6}$ M OMF were blocked by naloxone completely. However, the responses of 64 and 100 $\times 10^{-6}$ M OMF were only partly blocked due to the unspecific effect. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with OMF group.

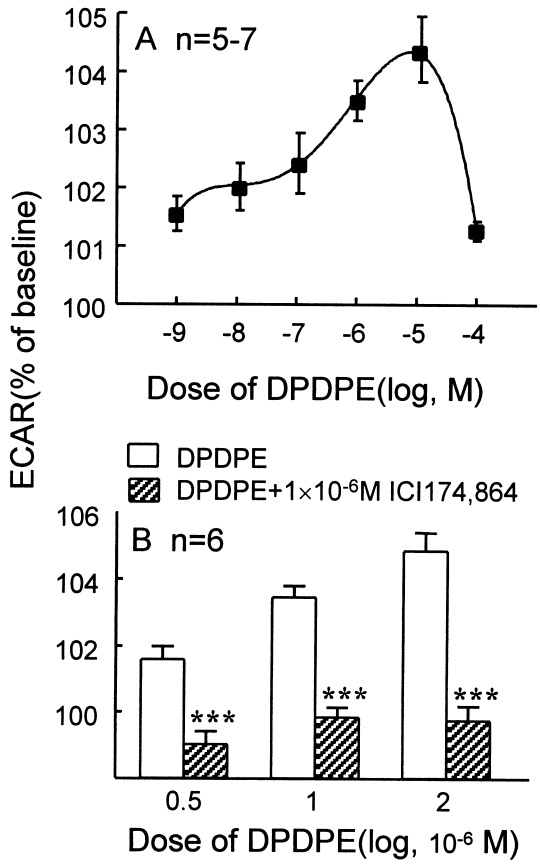

Fig. 2. Dose-response relationship of DPDPE in inducing the enhancement of extracellular acidification rate (ECAR) on differentiated SH-SY5Y cells in 30 s. (A) SH-SY5Y cells were challenged by DPDPE with different doses for 30 s. (B) Cells were challenged by 1×10^{-6} M ICI174,864 for 5 min prior to exposure to different doses of DPDPE for 30 s. The responses induced by DPDPE were blocked by ICI174,864 completely. $^{***}P < 0.001$ compared with OMF group.

and dose. The interaction was significant ($P < 0.05$). In addition, we compared the slopes of each curve. The difference was also significant ($P < 0.05$). The results indicated that the combination regimen produced synergistic effect on inducing the enhancement of ECAR (Fig. 4).

Our results clearly indicate that combination of δ-opioid receptor agonist DPDPE with μ-opioid receptor agonist OMF could synergistically enhance ECAR, which implied the increase of metabolic function. Activation of both μ- and δ-opioid receptor can promote a cascade of signal transduction including inhibition of adenylyl cyclase and regulation of cation channels via heterotrimeric G-protein [4], etc. These processes need ATP as the energy, followed by accumulation of the acidic metabolites that are extruded into the extracellular environment. As a result, ECAR increases in a concentration-dependent manner after receptors activation [5].

Accumulated evidence has been shown that μ- and δ- opioid receptor may interact at either physical and/or

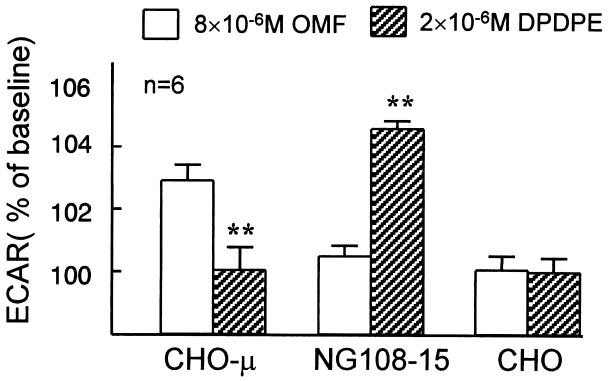

Fig. 3. Effect of OMF and DPDPE in inducing the enhancement of extracellular acidification rate (ECAR) on CHO-μ, NG108-15 and CHO cells in 30 s. Cells were exposed to 8×10^{-6} M OMF and 2×10^{-6} M DPDPE, respectively. $^{**}P < 0.01$ compared with the OMF groups.

functional level. For example, simultaneous activation of μ- and δ-opioid receptors generates a synergistic release of adenosine from spinal cord synaptosomes [2]; DPDPE (i.t.) enhanced DAMGO (i.t.) induced antinociception in spinal cord [3]; antinociception of morphine (i.c.v.) positively modulated by DPDPE and [D-Ala^2, Glu^4] deltorphin given by the same route [9]. This work is the first real-time detection indicating that the synergy exists between μ- and δ-opioid receptors agonists at the cellular level.

The mechanism underlying the interaction remained unclear, but pharmacological and biochemical evidence has suggested the close association between μ- and δ-opioid

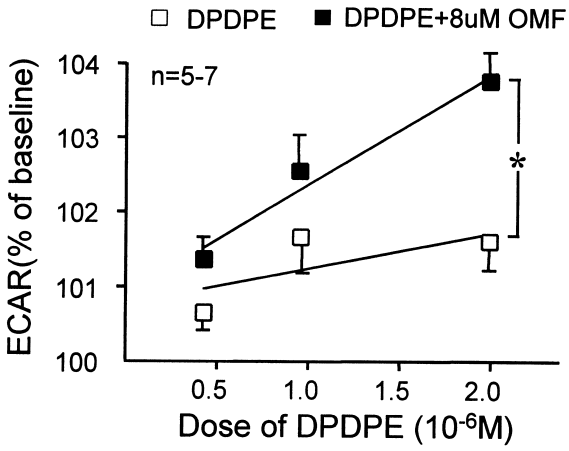

Fig. 4. The synergistic effect of DPDPE and OMF in inducing the enhancement of extracellular acidification rate (ECAR) on differentiated SH-SY5Y cells in 30 s. OMF in the dose of 8×10^{-6} M was combined with sequentially increasing dose of DPDPE, which was inside the linear part of the dose-response curve. The doses were 0.5×10^{-6} M, 1×10^{-6} M and 2×10^{-6} M. Two-factor repeated measures ANOVA was used to compare the interaction among different groups and different doses, respectively, as well as the interaction between group and dose. All the comparisons are significant ($P < 0.05$). In addition, the slopes of each dose-response curve was also significantly different ($^{*}P < 0.05$).

receptors. It has known that μ- and δ-opioid receptors colocalize in the same neuron and share the same G-protein, for example, in the mouse hypogastric ganglion [11] and spinal dorsal root ganglion [14]. The proposed overlap of cellular distribution and signal transduction pathway of these receptors suggest potentially specific functional interaction between μ- and δ-opioid receptors at cellular level. Rothman and his colleague presented a hypothesis suggesting that some μ- and δ-opioid receptors may exist in a functionally associated state [12]. The opioid receptors that functionally interact in the form of μ-δ complex have been termed $\mu_{complex}$ (μ_{cx}) and $\delta_{complex}$ (δ_{cx}) opioid receptors, while those μ- and δ-opioid receptors not interacting with each other were termed $\mu_{non\text{-}complex}$ (μ_{ncx}) and $\delta_{non\text{-}complex}$ (δ_{ncx}). The interaction receptors are under allosteric regulation. Maybe it is this physical association that enables mutually interaction.

In summary, using microphysiometer, synergy (i.e. a more than additive effect) was observed with combinations of μ-opioid receptor agonist OMF with δ-opioid receptor agonist DPDPE on differentiated SH-SY5Y cells, which suggested that the agonists of subtypes of opioid receptors can interact at cellular level, but the mechanism of the interaction remains to be elucidated.

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