The metabolic evidence of synergistic interaction between DAMGO and DPDPE on undifferentiated SH-SY5Y cells

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Recent studies have demonstrated the analgesic synergy between μ- and δ-opioid receptor, but evidence obtained at the cellular level is scanty. This work was designed to find the evidence of synergy between the actions of D-Ala^2-Mephe^4-gly-ol^5 enkephalin (DAMGO) and D-Phe^2, D-Phe^5 enkephalin (DPDPE) on undifferentiated SH-SY5Y cells. Microphysiometer was used to detect the functional activity of cells by measuring the real-time extracellular acidification rate (ECAR). The results demonstrate the unequivocal synergy between DAMGO and DPDPE at least within certain ratios. In addition, combined administration of the two drugs in the synergistic ratios attenuates receptor desensitization. These data indicate that DAMGO and DPDPE have a synergistic effect at cellular level. *NeuroReport* 12:845–849 © 2001 Lippincott Williams & Wilkins.

Key words: DAMGO; DPDPE; Microphysiometer; SH-SY5Y cells; Synergy; μ-opioid receptor; δ-opioid receptor

INTRODUCTION

Opioids modulate several physiological and pathophysiological functions and are used extensively in the management of pain. However, chronic use of opioid agonists results in tolerance and dependence, which limit their clinical application. Recently, several lines of evidence have suggested the analgesic synergy between μ- and δ-opioid receptors. Accordingly, there has been considerable interest in the possibility of combined use of opioids as an alternative to a single drug therapy, in an attempt to use lower doses of individual agents and limit adverse side effects [1]. However, it is uncertain whether the synergy is due to the modulation of neuronal circuit or the allosteric regulation between receptors at cellular level, and very few studies have been performed focusing on the synergistic interaction at the cellular level, especially in real-time. In the present study, we have used microphysiometer and undifferentiated SH-SY5Y cell to explore the metabolic evidence of the interaction between the μ- and δ-opioid receptor. The results point to an unequivocal synergy between DPDPE and DAMGO on undifferentiated SH-SY5Y cells.

Receptor activation stimulates a cascade of signal transduction pathway, such as second messenger production, receptor phosphorylation etc. All these processes need ATP as the direct energy provider, followed by accumulation of acidic metabolites, and an increase of extracelluar acidification rate. This silicon-based microphysiometer provides a measure of metabolic activity by detecting small changes of pH in the cell perfusate [2,3]. As a means to detect activation of G-protein coupled receptors, the microphysiometer displays greater sensitivity compared with measuring second messenger formation [4]. The microphysiometer is also suitable for detecting the interaction between two receptors. Richard *et al*. chose HT-29 cells that express both NT receptors and purinoceptors endogenously; they found that desensitization of the NT receptor did not influence the activation of purinoceptor [5].

MATERIALS AND METHODS

Cell culture: 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 a 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. Cells were plated into cell capsule cups supplied by Molecular Devices Corp. at a density of $\sim 3.5 \times 10^5$ cells/ml per cup the day prior to study.

Measurement of extracellular acidification rates: The microphysiometer (Molecular Devices Corp.) contains a micro-volume flow chamber in which cells adhere to polycarbonate membrane in a three-part disposable assembly. DMEM (serum-free and bicarbonate, HEPES-free) flows at ~100μl/min through the chambers and the disposable assembly. For determination of acidification rates (as μV/s), DMEM flow is halted every 2 min, allowing accumulation of extracellular acidic metabolites as a result

of cellular metabolism. Numerically, 1 μV/s is approximately equal to 1×10^{-3} pH units/min. The percentage change in ECAR is calculated by subtracting the baseline immediately prior to exposure to drugs.

Determination of synergy between DPDPE and DAMGO: Briefly, the experimental design used to evaluate the interaction between two drugs with isobolographic analysis requires determination of the potency of the combination. The potency of an agent or of a mixture is measured as a dose that produces a response in 50% of the subjects (ED_{50}). The potency of mixtures was then compared with a theoretically additive dose, denoted Z*add. The calculation of Z*add and its 95% confidence interval (CI) has been described in detail by Tallarida [6]. If the 95% CI of Z*add and that of Z*mix do not overlap, then we conclude that the mixture's effect departs from simple additivity. If Z*mix < Z*add, the mixture is synergistic under this ratio; whereas the relation Z*mix > Z*add means antagonism. Of course, equality means that the mixture is simply additive [6]. In this study, DAMGO and DPDPE were mixed in fixed proportions (i.e. 1:10, 5:1 and 1:25) according to their ED_{50} used alone. ED_{50} values of DPDPE and DAMGO and their 95% confidence interval (CI) were calculated by non-linear regression with Prism. The isobologram was constructed by plotting the ED_{50} value of DPDPE and DAMGO alone. The line connecting the two points was called the isobological line or theoretical additive line, consisting of all the points for the additive effect at all the ratios between DAMGO and DPDPE. The points lying below this line represent synergism, whereas the points above the line suggest antagonism.

Determination of desensitization induced by the drugs at ED_{50}: To determine whether a combination of the two drugs in certain ratios attenuates desensitization, SH-SY5Y cells were pretreated with the drugs in the dose of each ED_{50} for 7 min and allowed to recover for 7 min. Cells were subsequently restimulated with the same dose as the last time for another 7 min. ECAR were recorded as mentioned above. To determine receptor desensitization, we used one-way ANOVA to compare the difference between the first and the second application of the drugs.

RESULTS

The time-response and dose-response relationship of DAMGO and DPDPE and their ED_{50}: Exposure of SH-SY5Y cells to both DPDPE and DAMGO produced a time- and concentration-dependent increase in the extracellular acidification rate (ECAR). Concentration-response curves were obtained with various concentrations of DPDPE and DAMGO ranged from 10^{-11} mol/l to 10^{-5} mol/l. We chose the duration of drug exposure as 30 s or 3 min. Figure 1a,b shows that ECAR enhancement was significantly higher in the 3 min regimen than that in 30 s. Preliminary studies revealed that longer exposure over 3 min produced no further activation of the cell metabolism, therefore 3 min was chosen as the exposure time in the following experiments. The ED_{50} for DPDPE and DAMGO in 3 min was 1.62×10^{-8} mol/l and 1.67×10^{-7} mol/l, respectively. To test the specificity of the responses, antagonists of each receptor were used. Figure 1c, shows that the μ-opiopid antagonist naloxone at 5×10^{-6} mol/l can block the response of DAMGO; while the δ-opioid antagonist ICI174,864 at a concentration of 1×10^{-6} mol/l can block the response of DPDPE completely. 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. 2, 1×10^{-6} mol/l DAMGO induced an increase of ECAR in CHO-μ-opioid receptor cells, but no responses in CHO and NG108-15 cells. Similarly, 1×10^{-6} mol/l DPDPE could enhance the ECAR in NG108-15 cells, but not in CHO and CHO-μ-opioid receptor cells. Each curve was repeated at least five times.

The effect of the combined drugs in different ratios: According to the ED_{50} of DPDPE and DAMGO, we mixed the two drugs in the ratios of 1:10 and 5:1. The curves of the mixtures showed a marked shift to the left and upward (Fig. 3a). The 95% confidence interval of the experimental value and the theoretical value did not overlap. In addition, the ED_{50} of the experimental value is significantly less than that of the theoretical value (Table 1). This suggests that the ED_{50} of the mixtures is significantly less than expected from simple additivity, i.e. the mixture displayed obvious synergy at least in the ratios of 1:10 and 5:1. Each curve was repeated at least five times.

As shown in Fig. 3b, an isobologram for the evaluation of interaction between DPDPE and DAMGO was constructed. Doses of DPDPE were arranged on the Y axis and doses of DAMGO were on the X axis. The ED_{50} of these two drugs were represented by Z1 and Z2 respectively. Points that produce equal effect on ECAR were connected as the isobolograph. The theoretical additive relationship between DPDPE and DAMGO was described by the equation of a straight line joining the Z1 and Z2. All points on this isobole of additivity represent dose pairs that are additive, whereas the points lie below or above this line represent synergism or antagonism, respectively. Here P1 is the position for Z*mix when DPDPE/DAMGO equals 1:10, and P2 is the position for Z*mix when DPDPE/DAMGO equals 5:1. Both P1 and P2 lay below the line of additivity, indicating that the combination of DAMGO and DPDPE at these two ratios display synergistic effect on inducing ECAR.

When we mixed the two drugs in the ratio 1:25, the dose–effect curve of the mixture was between the curves of each drug alone. The 95% CI of the ED_{50} of experimental value was found to overlap with that of the theoretical one, suggesting an additive effect under this ratio (data not shown).

Desensitization induced by the drugs at ED_{50}: SH-SY5Y cells were divided into four groups, and exposed to DPDPE, DAMGO and the mixture of the two drugs in the ratios of both 1:10 and 5:1 with the dose of each ED_{50} respectively for 7 min. The enhancement of ECAR showed no significant difference among all these groups, confirming the existence of synergistic effect in these two ratios. After recovery for 7 min, each group was restimulated by the corresponding doses as the last time for another 7 min. In DPDPE and DAMGO alone group, the ECAR of the second stimulation was lower than that of the previous stimulation, indicating that the receptor desensitized after

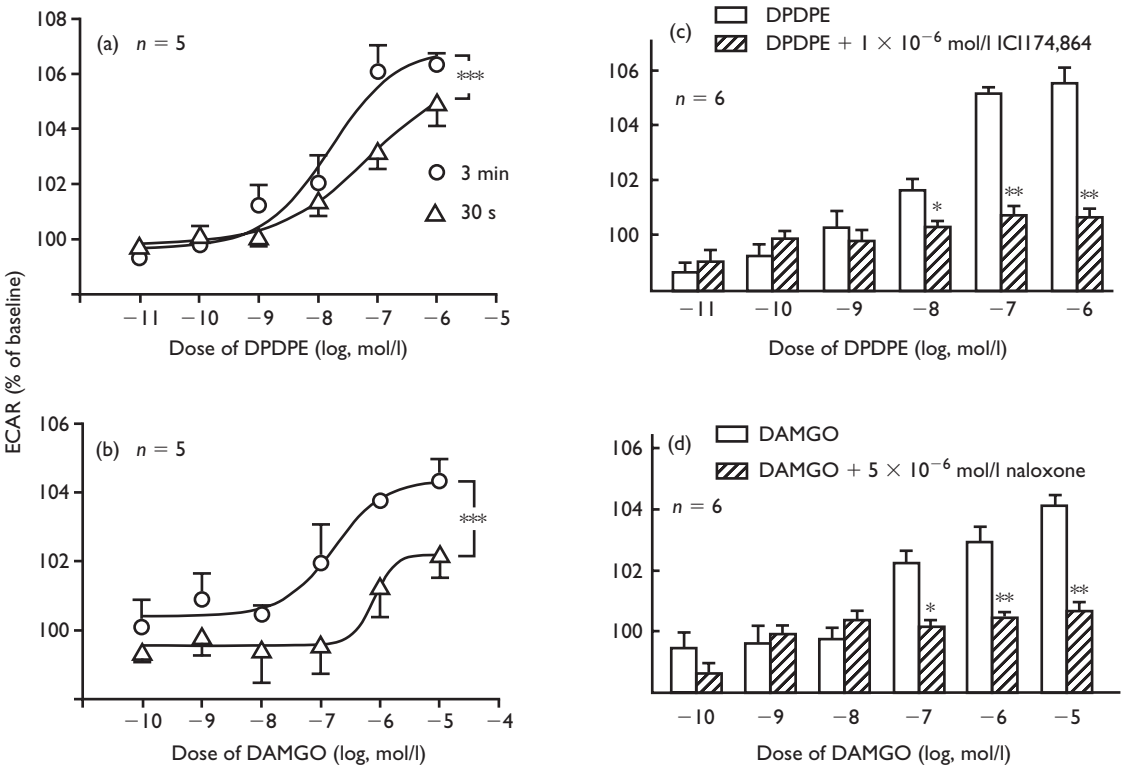

Fig. 1. Dose–response relationship of DPDPE and DAMGO on undifferentiated SH-SY5Y cells. SH-SY5Y cells were challenged with DPDPE (**a**) and DAMGO (**b**) for 30 s and 3 min with different doses. The ED_{50} value of DPDPE and DAMGO is 1.62×10^{-8} mol/l and 1.62×10^{-7} mol/l, respectively. *** $p < 0.001$ *vs* 30 s group. (**c**,**d**) The effect of antagonists on cells. Cells were challenged with 1×10^{-6} mol/l ICI174,864 or 5×10^{-6} mol/l naloxone for 5 min prior to exposure to different doses of DPDPE or DAMGO respectively. The responses of the agonists were blocked completely. * $p < 0.05$ and ** $p < 0.001$ *vs* agonists alone groups.

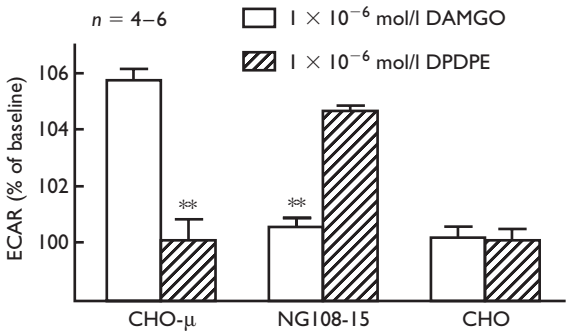

Fig. 2. Effect of DAMGO and DPDPE in inducing the enhancement of extracellular acidification rate (ECAR) on CHO-μ, NG108-15 and CHO cells in 3 min. Cells were exposed to 1×10^{-6} mol/l DAMGO and 1×10^{-6} mol/l DPDPE, respectively. ** $p < 0.01$ *vs* the DAMGO groups.

repeated exposure of the two agonists. Whereas in the mixed exposure group, ECAR showed no significant difference between the first and the second stimulation, suggesting a reduction of the receptor desensitization (Fig. 4a). The same conclusion could be obtained by calculating the difference of ECAR between the first and second application, as is shown in Fig. 4b.

DISCUSSION

Our results clearly indicate that combination of μ-opioid receptors agonist DAMGO and δ-opioid receptors agonist DPDPE in certain ratios can enhance the ECAR synergistically in SH-SY5Y cells, resulting from a significant increase of metabolic function. This is coincident with the results of our previous experiment, which shows that the synergistic effect also exists between μ-opioid receptors agonist ohmefentanyl (OMF) and DPDPE in inducing the increase of ECAR. Accumulating evidences have suggested that μ- and δ- opioid receptor may interact at either physical and/or functional level. For example, simultaneous activation of μ- and δ-opioid receptors generates a synergistic release of adenosine from spinal cord synaptosomes [7]; DPDPE (i.t.) enhanced DAMGO (i.t.)-induced antinociception in spinal cord [8]; antinociception of morphine (i.c.v.) positively modulated by DPDPE and [D-Ala^2,Glu^4]deltorphin given by the same route [9]. Recent knockout studies indicate that the presence of μ-opioid receptor is essential for the full function of δ-opioid receptors, spinal and supra-spinal antinociception of DPDPE and deltrophin II decreased in μ- opioid receptors knockout mice [10]. The autoradiographic mapping of δ-receptor distribution in the μ-opioid receptor knockout mice showed that there is 20–60% decrease of δ-opioid receptor in some regions of brain, although no major changes occur [11]. There are also some conflicting results. Palazzi demonstrated that long-term

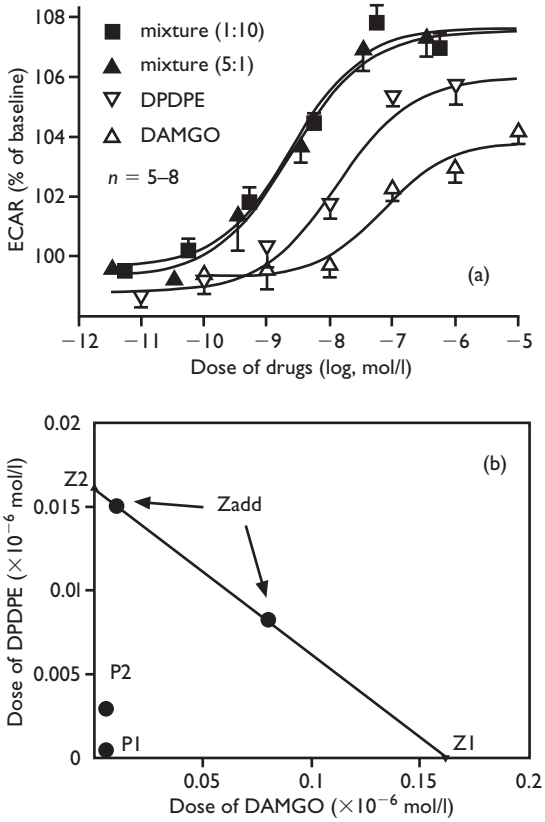

Fig. 3. The synergy between DPDPE and DAMGO in the ratios of 1:10 and 5:1 on undifferentiated SH-SY5Y cells in 3 min. (**a**). The dose–response relationship of the mixture. The ED_{50} value of the mixture is 2.39×10^{-9} mol/l and 2.51×10^{-9} mol/l, respectively. (**b**) Doses of DAMGO are shown on the X axis and doses of DPDPE on the Y axis. Z1 and Z2 represent ED_{50} of DAMGO and DPDPE for inducing the extracellular acidification rate, respectively. The additive relationship between DAMGO and DPDPE is described by a straight line joining Z1 and Z2. Zadd represents ED_{50} of the theoretical additive dose for the given ratios. All points on this isobole of additivity represent dose pairs that are additive. P1 and P2 represent the position of actuate ED_{50} of the mixture in the ratios of 1:10 and 5:1. Both P1 and P2 lie well below the additive line, indicating that the combination of DAMGO and DPDPE in these ratios produces synergy in inducing extracellular acidification rate.

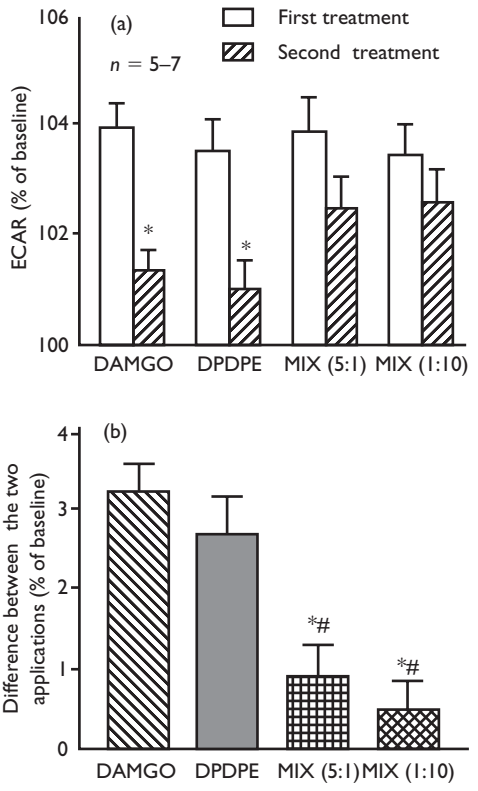

Fig. 4. Desensitization of ECAR response induced by two applications of the same drugs at the ED_{50}. (**a**) SH-SY5Y cells were challenged with 2×10^{-7} mol/l DAMGO, 2×10^{-8} mol/l DPDPE and 3×10^{-9} mol/l mixture in which DPDPE and DAMGO were combined in the ratios of 1:10 and 5:1 respectively. The cells were exposed to the drugs for two sessions of 7 min each, separated by a recovery period of 7 min. $^{*}p < 0.05$ *vs* the first treatment. (**b**) Difference of ECAR response between the two applications of the same dose of drugs. $^{*}p < 0.05$ *vs* DAMGO alone; $^{\#}p < 0.05$ *vs* DPDPE alone.

exposure of DAMGO and DPDPE had synergistic effect on SK-N-BE cells, but acute administration of the two drugs had the opposite effect [12].

The interaction between two G protein coupled receptors is a very popular phenomenon. *In vivo*, neurotransmitters are often released from a neuron with other co-existing neurotransmitters that can act as neuromodulators, amplifying the response of a co-released neurotransmitter. Activation of a number of G protein-coupled receptors does not always lead to direct effects on particular signaling pathways separately, but rather to an amplification of the response produced by a separate coincident signal within the same cell. This amplification maybe depends on the stoichiometry of the intracellular signaling proteins, the threshold for activation, and the level of the signaling pathway at which measurements are determined [13]. It has been shown that μ- and δ-opioid receptors colocalize in the same neuron and share the same G-protein, e.g. in the mouse hypogastric ganglion and spinal dorsal root ganglion etc. The proposed overlap in cellular distribution and signal transduction pathway of these receptors suggests potential functional interaction between μ-opioid and δ-opioid receptors at cellular level. The work of Demoliou-

Table 1. ED_{50} and 95% confidence interval of the mixtures in different ratios.

Ratio of DPDPE:DAMGO	ED_{50} (mol/l) (95% confidence interval)	
	Experimental value	Theoretical additive value
1:10	2.39×10^{-9} (1.075×10^{-9}, 5.31×10^{-9})	7.44×10^{-8} (7.39×10^{-8}, 1.99×10^{-7})
5:1	2.51×10^{-9} (1.08×10^{-9}, 8.41×10^{-9})	1.44×10^{-8} (2.44×10^{-8}, 1.33×10^{-7})

Mason suggested that the heterologous κ–δ receptor complex could enhance the binding affinity of these two receptors [14]. Rothman and his colleagues presented a hypothesis suggesting that some μ-opioid and δ-opioid receptors may exist in a functionally associated state [15]. The opioid receptors that functionally interact in the form of a μ–δ complex have been termed $\mu_{complex}$ (μ_{cx}) and $\delta_{complex}$ (δ_{cx}) opioid receptors, while those μ-opioid 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. Very recently, Jordan [16] presented the direct biochemical and pharmacological evidence supporting heterodimerization of two fully functional κ- and δ-opioid receptors, the κ–δ heterodimer could synergistically bind to highly selective agonists and potentiate signal transduction. It seems that the heterodimerization is the popular mechanism modulating the receptor interaction, but whether there is μ–δ heterodimer is unknown.

Receptor desensitization is the main molecular basis for opioid tolerance and dependence [17]. Can combined administration of DAMGO and DPDPE in the synergistic ratios attenuate the receptor desensitization? From Fig. 3, one can see that the degree of receptor desensitization was much less when a mixture of DAMGO and DPDPE was used, compared with the single application of DAMGO or DPDPE, although the degree of receptor activation was the same as manifested by the change of ECAR. It is possible that in the case of combined use of opioid agonists, fewer opioid receptors are activated that is enough for the activation of the post-receptor signal transduction pathways to produce an identified pharmacological effect. As a result, with the fewer activated receptor, the degree of desensitization were attenuated while the two drugs combined in these ratios.

CONCLUSION

Using a microphysiometer, we studied the synergy between the agonist of μ- and δ-opioid receptor on undifferentiated SH-SY5Y cells in real-time. The results suggested that the combination of DPDPE and DAMGO in some ratios produced synergistic interaction and attenuated the receptor desensitization on undifferentiated SH-SY5Y cells.

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