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Mediation of protein kinase C zeta in μ-opioid receptor activation for increase of glucose uptake into cultured myoblast C_2C_{12} cells

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ABSTRACT

The present study is designed to investigate the role of atypical protein kinase C (PKC) in the signaling of μ-opioid receptors (MOR) for glucose uptake in myoblast C_2C_{12} cells. Loperamide enhanced the uptake of radioactive deoxyglucose into C_2C_{12} cells in a concentration-dependent manner that was abolished in cells pre-incubated with GF109203X at concentrations sufficient to block PKC. Inhibition of the atypical zeta (ζ) isoform of PKC using myristoylated PKC pseudosubstrate resulted in a concentration-dependent decrease of loperamide-stimulated glucose uptake into C_2C_{12} cells. In addition, loperamide elicited the phosphorylation of PKC-ζ in C_2C_{12} cells in a concentration-dependent manner that was abolished by pretreatment with naloxonazine at concentrations sufficient to block MOR. These results suggest the mediation of PKC-ζ in MOR signaling for glucose uptake in C_2C_{12} cells. Activation of PKC-ζ by MOR stimulation is highly relevant to the search for therapeutic targets for glucose transport in insulin-sensitive tissues.

Opioid receptors are coupled to multiple systems and play a role in various biological effects, including analgesia, miosis, bradycardia, general sedation, hypothermia, insensitivity, and depression of flexor reflexes [3]. Opioids have a well-known role in lowering pain [19], in addition to the modulation of immune system and endocrine processes [13]. Currently, we provided new evidence that activation of peripheral μ-opioid receptors (MOR) may modify glucose metabolism-associated genes to improve glucose utilization in order to decrease high plasma glucose in streptozotocin-induced diabetic rats (STZ-diabetic rats), an animal model for type 1 diabetes [2,15]. Activation of MOR in glucose homeostasis in these diabetic rats may in fact be produced by mechanisms other than insulin.

Glucose uptake caused by insulin-stimulated translocation of glucose transporters to the cell membrane is the rate-limiting step in carbohydrate metabolism in skeletal muscle, a major site for glucose disposal [20]. Opioid receptor activation couples a number of intracellular signaling pathways, including the mediation of phospholipase C (PLC) and protein kinase C (PKC) activation [17]. The linkage of MOR and the PLC–PKC pathway in the regulation of glucose uptake can be characterized using the blockade of loperamide-stimulated glucose uptake in mouse skeletal muscle cell line [7]. Thus, activation of MOR may increase glucose uptake in insulin-sensitive tissues via the PKC signaling pathway.

The PKC family has been divided into three groups according to activation mode: conventional isoforms (α, β1, β2, and γ) are dependent on Ca^{2+} and diacylglycerol (DAG) for stimulation of activity; novel isoforms (δ, ε, θ, and η) which are Ca^{2+} independent but DAG dependent; and atypical isoforms (ζ, λ/τ), which are both Ca^{2+} independent and DAG independent [4,9]. Clearly, PKC plays pleiotropic roles in cell polarity, migration, and adhesion [5]. In addition, PKC-ζ acts downstream of insulin receptor and phosphatidylinositol 3-kinase (PI 3-kinase) in the insulin-induced control of glucose uptake [14]. In addition, many findings suggest that glucose and exercise also can activate PKC-ζ through diverse pathways from insulin [10,12]. But, insulin-independent signaling events for increase of glucose transport have not been fully elucidated. Exploring the linkage between peripheral MOR and PKC-ζ in glucose uptake will help delineate the regulation of glucose independent of insulin.

Isolation of skeletal muscle requires intact excision to preserve membrane integrity. Also, the tissue preparation has a relatively short survival time in vitro. Mouse C_2C_{12} cells, derived from the mouse skeletal muscle C_2 cell line, express the morphological,

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biochemical and metabolic properties of isolated skeletal muscle [18]. Thus, we employed this cultured cell to clarify the role of PKC-ζ in MOR-regulated glucose uptake.

C_2C_{12} cells, obtained from Culture Collection and Research Center (CCRC 60083) of the Food Industry Institute (Hsin-Chiu City, Taiwan) were plated at 5×10^4 cells/dish in 35-mm diameter culture dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic solution (penicillin G sodium 10,000 U/ml and streptomycin sulfate 10 μg/ml) and were grown to 70% confluence at 37 °C in humidified atmosphere containing 5% CO_2. To induce fusion, confluent cells were exposed to DMEM supplemented with 10% horse serum instead of FBS. Cells fused into multinucleated myotubes after a further 7–10 days in culture. Medium was changed 24 h prior to experimental manipulations.

The uptake of 2-[^{14}C]-deoxy-D-glucose (2-DG) (New England Nuclear, Boston, MA) into C_2C_{12} cells was determined as described previously [7]. In brief, cells were washed with phosphate-buffered saline (PBS) and incubated for 5 h in serum-free, high glucose (25 mM) DMEM medium. The cells were then transferred to the fresh incubation flasks and incubated with loperamide at indicated concentrations at 37 °C under continuous shaking at 40 cycles/min. According to previous study [7], 2-DG uptake is significantly increased within 3 min in cells exposed to 10 μM loperamide, shows half-maximal stimulation at 15 min, and reaches a plateau after 30 min, which was the optimal time to use in the experiments. The cells were further incubated with 2-DG (1 μCi/ml) for 5 min at 37 °C. Uptake was terminated by an addition of ice-cold PBS. After centrifugation, cells were washed twice with ice-cold PBS. Cell-associated radioactivity was determined by lysing the cells in 1 M NaOH and taking aliquots, after neutralization, to use for radioactivity estimation in a scintillation counter (Beckman LS6000). Nonspecific uptake, obtained by parallel determinations in the presence of 20 μM cytochalasin B (Sigma–Aldrich, Inc., St. Louis, MO, USA) to block transport, was subtracted from the total cell-associated radioactivity. Specific 2-DG uptake was expressed as pmol/mg protein/5 min or as the percentage of the basal level obtained from sample incubated with DMEM only. Protein content was determined by the BioRad protein dye binding assay (Richmond, CA).

Parametric data were expressed as the mean ± SE. The '*n*' in the text refers to the number of separate experiments. Multiple comparisons were analyzed by ANOVA and Dunnett's post hoc test. A *P* value of 0.05 or less was considered as statistically significant.

Consistent with previous observations [7], incubation with loperamide increased 2-DG uptake into C_2C_{12} cells in a concentration-dependent manner (Fig. 1A). Maximal 2-DG uptake obtained in samples incubated with 1 μM of loperamide was 190.3 ± 6.1 pmol/mg protein/5 min, about a 1.3-fold increase over the basal uptake (148.4 ± 5.1 pmol/mg protein/5 min). To assess the contribution of PKC to MOR-stimulated glucose disposal, we examined the effects of GF10923X (BIOMOL, Plymouth Meeting, PA, USA), a broad-range PKC inhibitor [16], on loperamide-induced glucose uptake in C_2C_{12} cells. The cells were incubated with GF10923X for 30 min and exposed to loperamide (1 μM) for another 30 min. As shown in Fig. 1B, although GF10923X (1 μM) had no effect on basal glucose uptake, PKC inhibition markedly decreased loperamide (1 μM)-induced glucose uptake; this result supported the view that PKC is involved in the increase of glucose uptake during MOR activation [7].

To define the role of PKC-ζ in loperamide-induced increase in glucose utilization through activation of MOR, the specific inhibitor of PKC-ζ, myristoylated PKC-ζ pseudosubstrate (PKC-ζ–PS; Sigma–Aldrich, St. Louis, MO, USA), was used as described previously [14]. C_2C_{12} cells were incubated with PKC-ζ–PS for 30 min before activation with loperamide (1 μM). As shown in

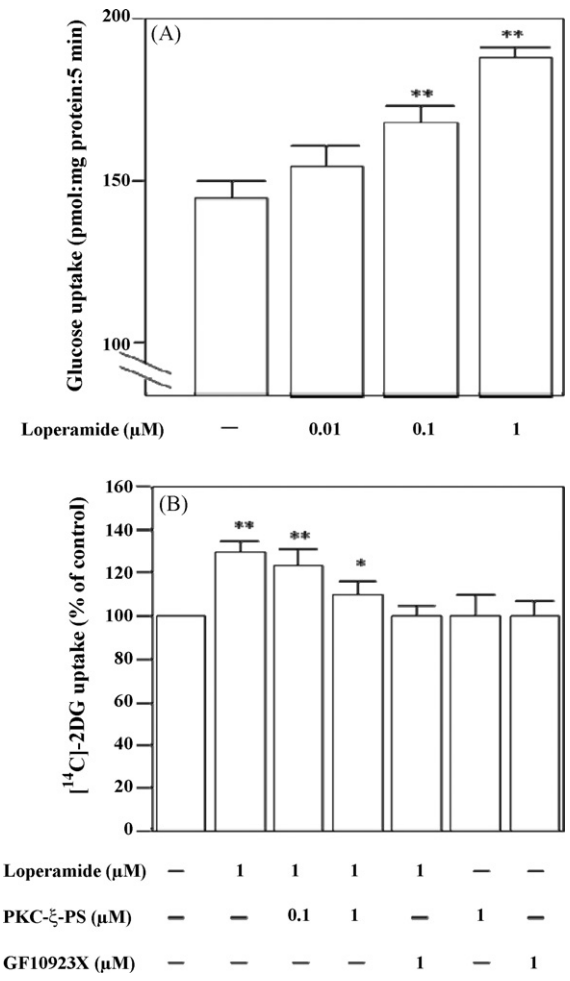

Fig. 1. Effect of loperamide (A) and inhibition of protein kinase C (B) on glucose uptake into C_2C_{12} cells. After pre-incubation with inhibitor or vehicle (the medium (DMEM) used to dissolve the testing reagent) at same volume for 30 min, cells were exposed to loperamide for another 30 min. Results are the mean ± SE of six determinations. The basal level of radioactive glucose uptake was obtained from cells incubated with DMEM only. $^*P < 0.05$ and $^{**}P < 0.01$ compared to basal glucose uptake, respectively.

Fig. 1B, PKC-ζ–PS did not affect basal glucose uptake levels but caused a concentration-dependent inhibition of loperamide-induced glucose uptake, suggesting a positive correlation between PKC-ζ and the stimulatory action of loperamide on glucose uptake. Actually, loperamide-induced glucose uptake was not completely blocked by PKC-ζ–PS, and the inhibitory effect of PKC-ζ–PS was less than that of GF10923X at the same concentration used (1 μM). It showed that not only PKC-ζ but also the other PKC subtypes might be involved in the action of loperamide. Although it has been demonstrated that atypical PKCs play a positive role in the regulation of glucose transport in insulin signaling pathway [10,12], mediation of atypical PKC isoforms in insulin-independent signaling events for increase of glucose transport needs further investigated. Nevertheless, these data not only support the view that PKC is required for glucose uptake during the action of non-insulin-dependent stimuli, but the clear role of PKC-ζ in MOR activation of glucose uptake also demonstrate.

It has been documented that glucose transport by insulin is mediated by PKC-ζ at a location downstream from PI 3-kinase because PKC-ζ expression and activity are positively related to glucose uptake [14]. However, mechanism(s) responsible for the activation of PKC-ζ have remained unclear. Some research suggests that the phosphorylation of PKC-ζ on threonine (Thr) 410, a PI 3-kinase-dependent protein kinase (PDK)-1-dependent phos-