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Integrative function of adrenaline receptors for glucagon-like peptide-1 exocytosis in enteroendocrine L cell line GLUTag

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ABSTRACT

Adrenaline reacts with three types of adrenergic receptors, α1, α2 and β-adrenergic receptors (ARs), inducing many physiological events including exocytosis. Although adrenaline has been shown to induce glucagon-like peptide-1 (GLP-1) secretion from intestinal L cells, the precise molecular mechanism by which adrenaline regulates GLP-1 secretion remains unknown. Here we show by live cell imaging that all types of adrenergic receptors are stimulated by adrenaline in enteroendocrine L cell line GLUTag cells and are involved in GLP-1 exocytosis. We performed RT-PCR analysis and found that α1B-, α2A-, α2B-, and β1-ARs were expressed in GLUTag cells. Application of adrenaline induced a significant increase of intracellular Ca^{2+} and cAMP concentration ($[Ca^{2+}]_i$ and $[cAMP]_i$, respectively), and GLP-1 exocytosis in GLUTag cells. Blockade of α1-AR inhibited adrenaline-induced $[Ca^{2+}]_i$ increase and exocytosis but not $[cAMP]_i$ increase, while blockade of β1-AR inhibited adrenaline-induced $[cAMP]_i$ increase and exocytosis but not $[Ca^{2+}]_i$ increase. Furthermore, overexpression of α2A-AR suppressed the adrenaline-induced $[cAMP]_i$ increase and exocytosis. These results suggest that the fine-turning of GLP-1 secretion from enteroendocrine L cells is established by the balance between α1-, α2-, and β-ARs activation.

1. Introduction

Enteroendocrine cells in the gastrointestinal tract secrete a variety of peptide hormones in response to extracellular stimuli including luminal nutrients, circulating hormones, and neurotransmitters. The gut peptide hormones contribute to the regulation of many physiological functions, e. g. gut motility, blood glucose, and appetite [1]. Among these peptide hormones, glucagon-like peptide-1 (GLP-1) is produced by the proglucagon gene in enteroendocrine L cells and secreted from the cells. Secreted GLP-1 enhances glucose-induced insulin secretion from pancreatic β cells. In addition, it is thought to enhance anti-hyperglycemic effect by slowing gastric emptying [2], inhibiting glucagon release [3], promote β cell proliferation [4], and reduce appetite via activation of central and/or vagal nerves [1,5,6]. Thus, modulating GLP-1 secretion and its activity through administration of the GLP-1 mimetics or by inhibiting degradation of GLP-1 has received a lot of attention in recent years for treating type 2 diabetes mellitus (T2DM).

In recent years, various nutrients including glucose, amino acids, and fatty acids have been shown to stimulate GLP-1 secretion from the L cells [7–10]. For example, fatty acid-induced GLP-1 secretion is thought to be dependent on G protein-coupled receptors (GPCRs) [10,11]. Free fatty acid receptor 1 (FFAR1, also known as GPR40) which is expressed in enteroendocrine L cells, is mainly coupled to the Gq protein, which activates phospholipase C (PLC) upon fatty acid binding to the receptor [10]. Thus, the activation of FFAR1 in L cells results in increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and subsequent increased secretion of GLP-1 [10,12]. In contrast with nutrients, the effects of circulating hormones or neurotransmitters on the L cells are less precisely investigated [13–16]. In fact, adrenaline, one of major stress hormone secreted from chromaffin cells of adrenal gland, has been found to stimulate GLP-1 and peptide YY (PYY) secretion from isolated vascularly perfused rat ileum [17]. However, the precise

Abbreviations: ARs, adrenergic receptors; FFAR1, free fatty acid receptor 1; GLP-1, glucagon-like peptide-1; IP_3, inositol triphosphate; PYY, peptide YY; tPA, tissue-type plasminogen activator; TIRF, total internal reflection fluorescence; T2DM, type 2 diabetes mellitus.

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molecular mechanism(s) of adrenaline-induced GLP-1 secretion from enteroendocrine L cells and its physiological significance are poorly understood.

In the present study, we used enteroendocrine L cell line GLUTag cells to elucidate the molecular mechanism(s) of adrenaline-induced GLP-1 secretion. We found that α1B-, α2A-, α2B-, and β1-adrenergic receptors (ARs) were expressed in GLUTag cells revealed by RT-PCR analysis. Application of adrenaline induced an increase of $[Ca^{2+}]_i$, intracellular cAMP concentration ($[cAMP]_i$), and GLP-1 exocytotic response. Furthermore, overexpression of α2A-AR significantly suppressed the increase of $[cAMP]_i$ and GLP-1 exocytosis. These findings indicate that adrenaline plays a stimulatory effect on GLP-1 secretion from L cells, and imbalance of α1-, α2-, and β-ARs expression in L cells might be implicated in the development of T2DM.

2. Materials and methods

2.1. Materials

Adrenaline bitartrate and prazosin hydrochloride were purchased from Tokyo Chemical Industry (Tokyo, Japan). 2-aminoethoxyldiphenyl borate (2-APB), poly-L-lysine, penicillin, and streptomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). U-73122 was purchased from Cayman Chemical (Ann Arbor, MI, USA). Propranolol was purchased from WAKO Pure Chemical Industries (Osaka, Japan).

2.2. Plasmid construction

Mouse α2A-adrenergic receptor (AR) clone was purchased from DNAFORM (Kanagawa, Japan). Its cDNA sequence was subcloned into EcoRI/BamHI sites of pmCherry-N1 vector, and was named α2A-AR-mCherry. The other expression plasmids, green fluorescent protein-tagged tissue-type plasminogen activator (tPA-GFP) and yellow fluorescent protein-based cAMP indicator2 (Flamindo2), were constructed as described previously [7,18].

2.3. Cell culture and transfection

GLUTag cells (kindly provided by Dr. Daniel Drucker, University of Toronto) were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 1 g/L glucose, 10% (v/v) heat-inactivated fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin, at 37 °C under 5% CO_2. For gene transfection, the cells were plated onto poly-L-lysine-coated glass coverslips in 35 mm dishes. 2 days after plating, the cells were transfected 2 μg of plasmids using X-tremeGENE HP DNA Transfection Reagent (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instruction. After transfection of Flamindo2 plasmid, the cells were incubated at 37 °C for 8 h, and then at 30 °C for 2–4 days until fluorescence imaging.

2.4. RNA isolation and RT-PCR analysis

Total RNA from GLUTag cells and mouse brain was isolated using RNeasy Mini Kit (QIAGEN, KJ Venlo, Netherlands). After DNase treatment using RNase-Free DNase Set (QIAGEN), cDNA was synthesized using High Capacity RNA-to-cDNA Kit (Life Technologies, Carlsbad, CA, USA). 20 ng of cDNA was amplified using EmeraldAmp PCR Master Mix (TaKaRa, Shiga, Japan). For PCR amplification of α1A-AR (NM_013461), the forward primer 5′-CTAAGGCCATTCTACTTGGGGT-3′ and the reverse primer 5′-CGAGTGCAGATGCCGATGA-3′ were used. For α1B-AR (NM_007416), the forward primer 5′-CGGACGCCAACCAACTACTT-3′ and the reverse primer 5′-AACACAGGACATCAACCGCTG-3′ were used. For α1D-AR (NM_013460), the forward primer 5′-AGTGGGTGTCTTCCTAGCC-3′ and the reverse primer 5′-GCCTAGAACCTCCATAGTGGC-3′ were used. For α2A-AR (NM_007417), the forward primer 5′-GTGACACTGACGCTGGTTTG-3′ and the reverse primer 5′-CCAGTAACCCATAACCTCGTTG-3′ were used. For α2B-AR (NM_009633), the forward primer 5′-TCTTCACCATTTTCGGCAATGC-3′ and the reverse primer 5′-AGAGTAGCCACTAGGATGTCG-3′ were used. For α2C-AR (NM_007418), the forward primer 5′-CTGTGGTGGGTTTCCTCATCG-3′ and the reverse primer 5′-ACTTGCCCGAAGTACCAGTAG-3′ were used. For β1-AR (NM_007419), the forward primer 5′-CTCATCGTGGTGGGTAACGTG-3′ and the reverse primer 5′-ACACACAGCACATCTACCGAA-3′ were used. For β2-AR (NM_007420), the forward primer 5′-GGGAACGACAGCGACTTCTT-3′ and the reverse primer 5′-GCCAGGACGATAACCGACAT-3′ were used. For β3-AR (NM_013462), the forward primer 5′-GGCCCTCTCTAGTTCCCAG-3′ and the reverse primer 5′-TAGCCATCAAACCTGTTGAGC-3′ were used. For histone H2A family member Z (NM_016750), the forward primer 5′-ACAGCGCAGCCATCCTGGAGTA-3′ and the reverse primer 5′-TTCCCGATCAGCGATTTGTGGA-3′ were used.

2.5. Visualization of intracellular Ca^{2+} and cAMP dynamics

For Ca^{2+} imaging, GLUTag cells plated on coverslips for 2 days were loaded with 2.5 μM of Fluo 3-AM (Dojindo, Kumamoto, Japan) in modified Ringer Buffer (RB: 140 mM NaCl, 3.5 mM KCl, 0.5 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 1.5 mM $CaCl_2$, 10 mM HEPES, 2 mM $NaHCO_3$)-containing 5 mM glucose, and were incubated for 30 min at 37 °C under 5% CO_2. Then the cells were washed twice with RB, mounted in a chamber heated at 37 °C and imaged in RB-containing 0.1 mM glucose. Imaging was performed using an inverted microscope (IX-71, Olympus, Tokyo, Japan) equipped with an oil-immersion objective lens (UApo/340, 40x, NA = 1.35, Olympus) and an EM-CCD camera (Evolve, Photometrics, Tucson, AZ, USA). The cells were excited using a xenon lamp, and images were acquired every 5 s for 20 min. Adrenaline stimulation was performed by perfusion with RB-containing 0.1 mM glucose, at 120 s from the beginning of image acquisition. For cAMP imaging, the cells were washed and imaged as described above after 2 days of Flamindo2 transfection.

2.6. Total internal reflection fluorescence (TIRF) microscopy

To observe the exocytosis of tPA-GFP at the single vesicle level, we used a TIRF microscope essentially as described previously [7]. 2 days after transfection, GLUTag cells were washed and imaged as in *2.5*. Imaging was performed using an inverted microscope (ECLIPSE Ti-E, Nikon, Tokyo, Japan). We used a high numerical aperture objective lens (CFI Apochromat TIRF, 100 ×, NA = 1.49, Nikon), and incident light for total internal reflection illumination was introduced from the objective lens through a single-mode optical fiber and two illumination lenses (TI-TIRF, Nikon). To observe tPA-GFP fluorescence images under total internal fluorescence illumination, an optically pumped semiconductor 488-nm laser (Sapphire 488LP, 30 mW, Coherent, Santa Clara, Canada) was used through a band-pass filter (HQ535/30m, Chroma, Bellows Falls, VT, USA) as an emission filter. The laser beam was passed through an electromagnetically driven shutter (TI-TIRF, Nikon), and the shutter was opened synchronously with an EM-CCD camera (iXon, Andor, Belfast, UK), whose exposure was controlled by MetaMorph software (Molecular Devices, Sunnyvale, CA, USA). Images were acquired every 500 ms for 20 min. Adrenaline stimulation was performed by perfusion with RB containing 0.1 mM glucose, at 120 s from the beginning of image acquisition. The number of exocytotic events during a 20-min period was counted manually.

2.7. Statistics

For statistical analysis, data are shown as mean ± SE. Means were compared by Student's t test or ANOVA followed by Dunnett's test using GraphPad Prism 6 software (GraphPad software, La Jolla, CA, USA).

3. Results

3.1. Adrenaline induces an increase of $[Ca^{2+}]_i$ and $[cAMP]_i$

There are nine adrenergic receptors (ARs) classified into three different types (i.e., α1, α2, and β) in the cells. To clarify which adrenergic receptors are expressed in GLUTag cells, RT-PCR analysis was performed. We found that α1B-, α2A-, α2B-, and β1-AR were expressed in GLUTag cells (Fig. 1A), suggesting that GLUTag cells sense adrenaline via three different G protein signaling pathways (i.e., α1, α2, and β1 for Gq, Gi, and Gs pathways, respectively). We next examined whether adrenaline induces the intracellular Ca^{2+} and cAMP concentration ($[Ca^{2+}]_i$ and $[cAMP]_i$, respectively) changes in GLUTag cells. To monitor $[Ca^{2+}]_i$ and $[cAMP]_i$, we used Ca^{2+}-sensing dye Fluo3 and genetically encoded yellow fluorescent cAMP indicator Flamindo2 [18], respectively. Fluo3 shows increase of its fluorescence intensity in response to Ca^{2+}, and Flamindo2 shows decrease of its fluorescence intensity in response to cAMP [18]. Application of 100 nM adrenaline significantly increased fluorescence intensity of Fluo3, implying $[Ca^{2+}]_i$ increase (Fig. 1B and C). On the other hand, application of 100 nM adrenaline decreased fluorescence intensity of Flamindo2, implying $[cAMP]_i$ increase (Fig. 1D and E). To confirm whether adrenaline causes GLP-1 exocytosis in GLUTag cells, we visualized the single exocytotic events in live GLUTag cells by total internal reflection fluorescence (TIRF) microscopy [7,19,20]. To monitor GLP-1 exocytotic response, we used a green fluorescent protein (GFP)-tagged tissue-type plasminogen activator (tPA) which functioned as a surrogate maker for GLP-1-containing vesicles and exocytotic response [7]. Overexpression of tPA-GFP in GLUTag cells showed a highly punctate pattern of fluorescence under the TIRF microscope (Fig. 1F, top). After application of 100 nM adrenaline, the fluorescent spots suddenly brightened and then dimmed (Fig. 1F, bottom in 20 s panel), reflecting GLP-1 was secreted into extracellular space. During application of adrenaline, the total number of the exocytotic events in GLUTag cells increased significantly, compared with unstimulated control cells (Fig. 1G). These results suggest that adrenaline stimulates both Gq and Gs pathways and induces the subsequent GLP-1 secretion.

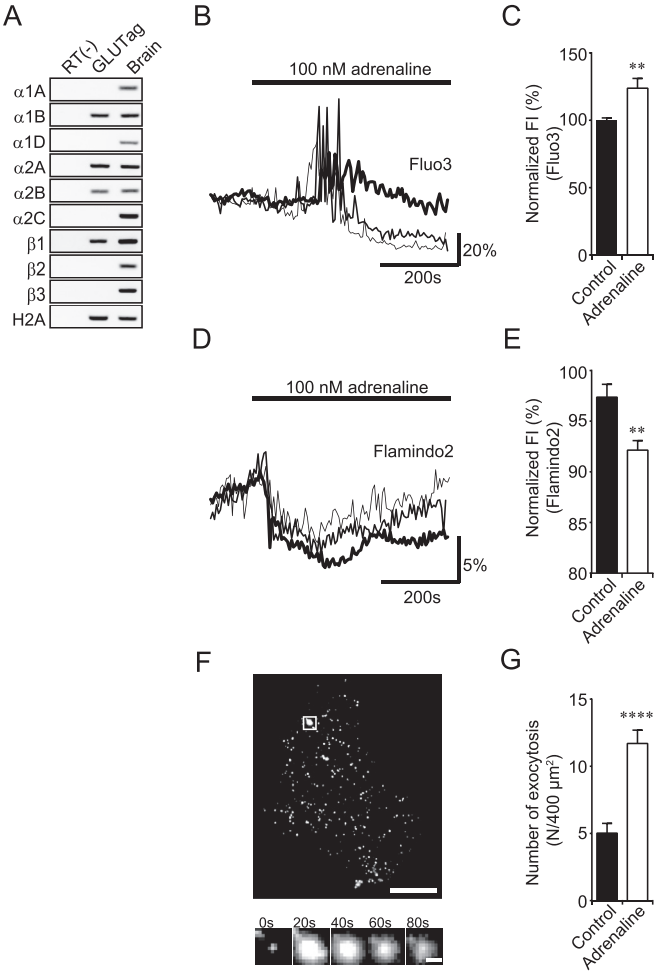

Fig. 1. Expression of adrenergic receptors in GLUTag cells and effects of adrenaline on $[Ca^{2+}]_i$, $[cAMP]_i$, and exocytosis. (A) Expression of adrenergic receptors in GLUTag cells by RT-PCR analysis. (B and D) Typical time course of $[Ca^{2+}]_i$ (B) and $[cAMP]_i$ (D) changes during the application of 100 nM adrenaline in GLUTag cells. Three traces from independent experiments are shown in all graphs. (C and E) Normalized amplitude of $[Ca^{2+}]_i$ (C) and $[cAMP]_i$ (E) calculated from the peak amplitude of fluorescence intensity induced by adrenaline. (F) Typical total internal reflection fluorescence microscopic image of a tPA-GFP transfected GLUTag cell. Scale bar: 10 μm (top). Sequential images during the exocytosis of vesicle outlined by square in (top). Scale bar: 1 μm (bottom). (G) The number of exocytosis during the application of adrenaline. Data are shown as mean ± SE. $n > 19$ cells in (C), $n > 20$ cells in (E), and $n > 12$ cells in (G). $^{**}p < 0.01$, $^{****}p < 0.0001$.

3.2. α1-and β-ARs are involved in adrenaline-induced $[Ca^{2+}]_i$ and $[cAMP]_i$ increase

The adrenaline-induced $[Ca^{2+}]_i$ and $[cAMP]_i$ increase would be due to the activation of Gq-coupled α1B-AR and Gs-coupled β1-AR, respectively. To elucidate a role for α1B- or β1-AR on $[Ca^{2+}]_i$ and $[cAMP]_i$ increase, we employed prazosin and propranolol to block α1-and β-ARs signal transduction pathways, respectively. Blockade of α1-AR with prazosin significantly reduced the adrenaline-induced $[Ca^{2+}]_i$ increase (Fig. 2A and B), whereas application of prazosin had little effect on the adrenaline-induced $[cAMP]_i$ increase (Fig. 2C and D). The α1-ARs are coupled to the Gq protein, which activates phospholipase C (PLC) and increases $[Ca^{2+}]_i$ via inositol trisphosphate (IP_3)-mediated release from the endoplasmic reticulum. We thus examined whether PLC and IP_3 receptor inhibitors (U-73122 and 2-aminoethoxydiphenyl borate (2-APB), respectively) suppress the $[Ca^{2+}]_i$ responses induced by adrenaline. Co-administration of either U-73122 or 2-APB with 100 nM adrenaline significantly suppressed the adrenaline-induced $[Ca^{2+}]_i$ increase (Fig. 2A and B). Furthermore, the total number of exocytotic events in GLUTag cells was significantly decreased by application of prazosin, U-73122, or 2-APB (Fig. 2E).

We next investigated whether β-ARs blocker propranolol inhibits $[Ca^{2+}]_i$ and $[cAMP]_i$ increase induced by adrenaline. As we expected, application of propranolol had little effect on adrenaline-induced $[Ca^{2+}]_i$ increase (Fig. 3A and B), while adrenaline-induced $[cAMP]_i$ increase and exocytotic events were inhibited by propranolol (Fig. 3C–E). Since α1B- and β1-ARs are expressed in GLUTag cells (Fig. 1A), these results suggest both Gq and Gs pathways play an important role in adrenaline-induced GLP-1 secretion from intestinal L cells.

3.3. Overexpression of α2A-AR suppressed adrenaline-induced $[cAMP]_i$ increase and exocytosis

Overexpression of α2A-AR caused by a single-nucleotide polymorphism in the human α2A-AR gene induced decrease of insulin

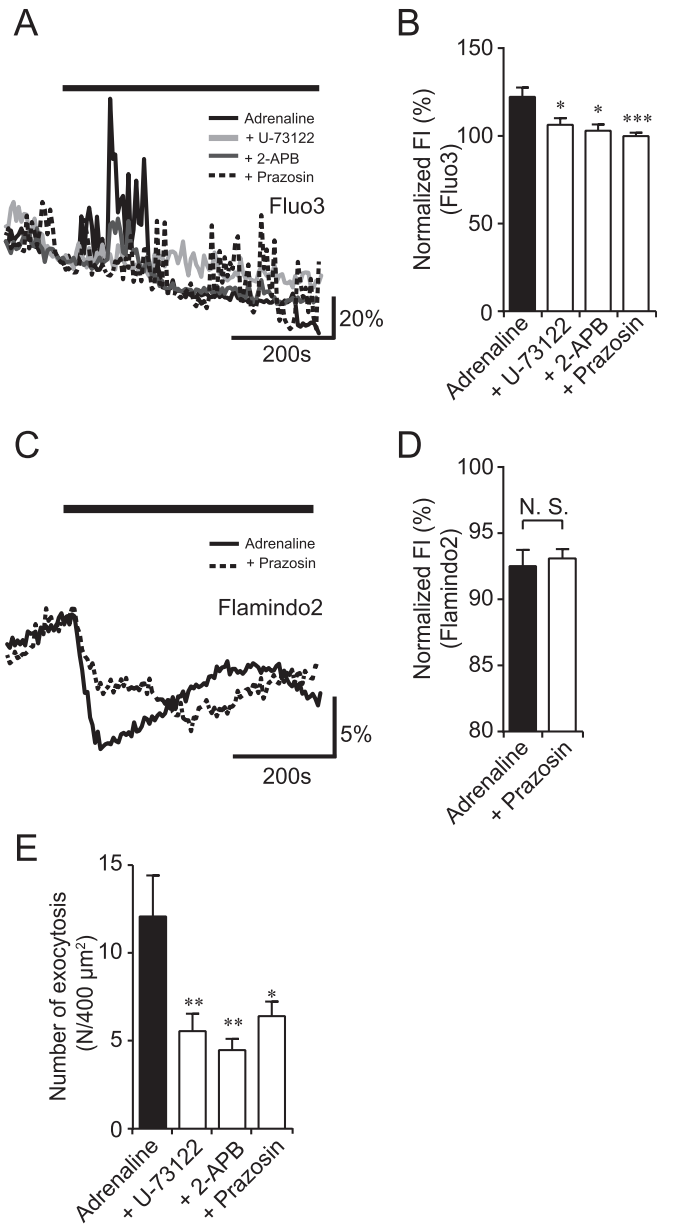

Fig. 2. Effects of α1 adrenergic receptor antagonists on $[Ca^{2+}]_i$, $[cAMP]_i$, and exocytosis. (A) Typical time course of $[Ca^{2+}]_i$ during the application of α1 adrenergic receptor antagonist prazosin, PLC inhibitor (U-73122), and IP3 receptor inhibitor (2-APB) together with 100 nM adrenaline. (B) Normalized amplitude of $[Ca^{2+}]_i$ calculated from the peak amplitude of fluorescence intensity. (C) Typical time course of $[cAMP]_i$ during the application of prazosin together with 100 nM adrenaline. (D) Normalized amplitude of $[cAMP]_i$ calculated from the peak amplitude of fluorescence intensity. (E) The number of exocytosis. Data are shown as mean ± SE. $n > 12$ cells in (B), $n > 19$ cells in (D), and $n > 9$ cells in (E). N.S., no significant, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

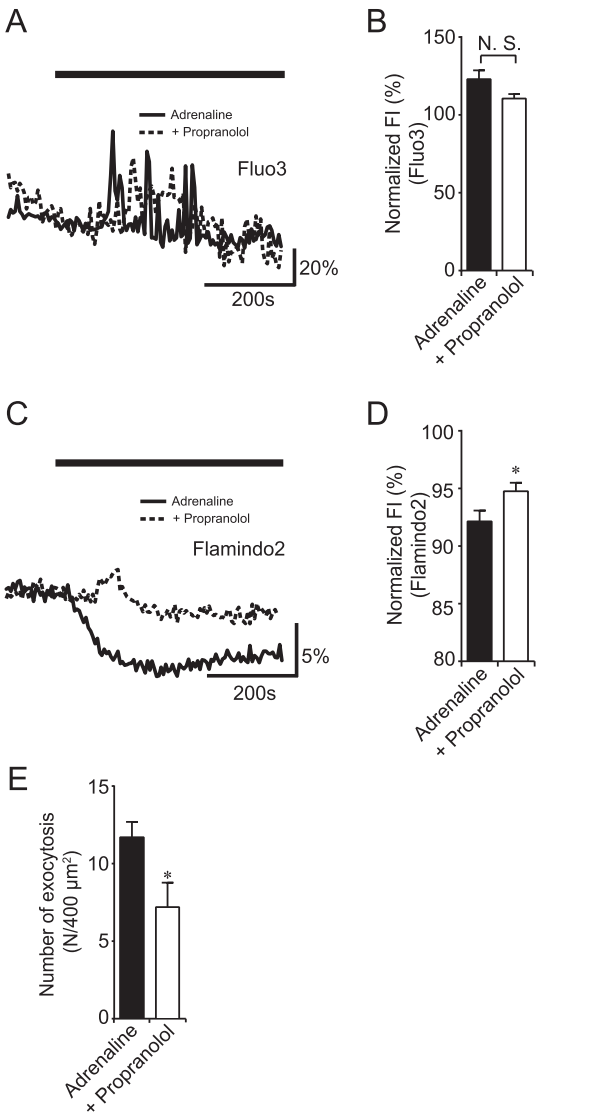

Fig. 3. Effects of β1 adrenergic receptor antagonist on $[Ca^{2+}]_i$, $[cAMP]_i$, and exocytosis. (A) Typical time course of $[Ca^{2+}]_i$ during the application of β1 adrenergic receptor antagonist propranolol together with 100 nM adrenaline. (B) Normalized amplitude of $[Ca^{2+}]_i$ calculated from the peak amplitude of fluorescence intensity. Data of adrenaline are from Fig. 1C. (C) Typical time course of $[cAMP]_i$ during the application of propranolol together with 100 nM adrenaline. (D) Normalized amplitude of $[cAMP]_i$ calculated from the peak amplitude of fluorescence intensity. Data of adrenaline are from Fig. 1E. (E) The number of exocytosis. Data of adrenaline are from Fig. 1G. Data are shown as mean ± SE. $n > 24$ cells in (B), $n > 20$ cells in (D), and $n > 8$ cells in (E). N.S., no significant, $^*p < 0.05$.

secretion, and increased type2 diabetes mellitus (T2DM) risk [21]. Since α2A-AR is also expressed in GLUTag cells (Fig. 1A), we examined whether overexpression of α2A-AR suppresses the adrenaline-induced $[cAMP]_i$ increase and GLP-1 exocytosis. We found that application of 100 nM adrenaline did not induce a significant $[cAMP]_i$ increase in α2A-AR-overexpressing GLUTag cells (Fig. 4A and B). Moreover, the number of adrenaline-induced exocytosis on the α2A-AR-overexpressing cells was indistinguishable from that on control cells (Fig. 4C), suggesting that α2A-AR has a strong inhibitory effect on the adrenaline-induced GLP-1 exocytosis from GLUTag cells.

4. Discussion

In the present study, we analyzed the effect of adrenaline on GLP-1 exocytosis and its molecular mechanisms in the GLUTag cells. We found that all three types of adrenergic receptors (i.e., α1B-, α2A-, α2B-, and β1-adrenergic receptors (ARs)) were expressed in GLUTag cells, and application of adrenaline significantly increases $[Ca^{2+}]_i$, $[cAMP]_i$, and GLP-1 exocytosis (Fig. 1). We also showed that the $[Ca^{2+}]_i$ and $[cAMP]_i$ increase is triggered via α1-and β-AR, respectively, and both $[Ca^{2+}]_i$ and $[cAMP]_i$ increases are important for GLP-1 exocytosis (Figs. 2 and 3). Despite of the fact that application of adrenaline increased $[cAMP]_i$, Gi-coupled α2A- and α2B-AR mRNAs were expressed in the GLUTag cells. This complex phenomenon would result from the difference

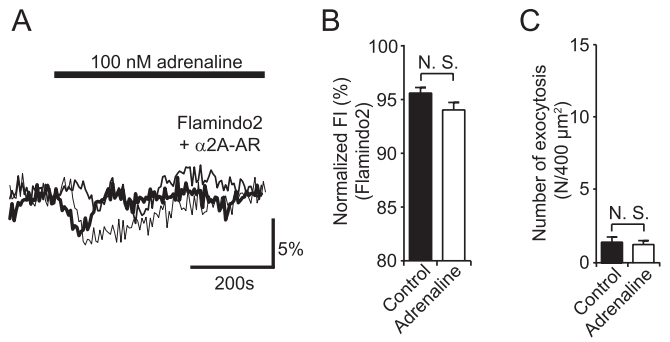

Fig. 4. Effects of overexpression of α2A adrenergic receptor on $[cAMP]_i$ change and exocytosis. (A) Typical time course of $[cAMP]_i$ in α2A adrenergic receptor-overexpressing GLUTag cells following the application of adrenaline. (B) Normalized amplitude of $[cAMP]_i$ calculated from the peak amplitude of fluorescence intensity. (C) The number of exocytosis. Data are shown as mean ± SE. $n > 18$ cells in (B) and $n > 9$ cells in (D).

between amounts of transcribed mRNAs and translated proteins. The amount of Gs, Gi, and Gq proteins would also be different and affect the signaling pathways. Therefore, these factors might be contributed to the Gs-coupled β-AR signaling pathway rather than α2A- and α2B-ARs, and thereby the $[cAMP]_i$ increase.

We showed that overexpression of α2A-AR suppressed adrenaline-induced $[cAMP]_i$ increase and GLP-1 exocytosis in GLUTag cells. Since α2A-AR generally couples to Gi, overexpression of α2A-AR promotes Gi function, leading to inhibition of cAMP production and GLP-1 exocytosis. Interestingly, we found that the decrease of basal GLP-1 exocytosis in α2A-AR overexpressing GLUTag cells (Fig. 4C) compared to those in control cells (Fig. 1G). This might be ascribed to the tonic activation of the Gi signaling pathway downstream of α2A-AR. Previous studies have shown that overexpression of α2A-AR contributes to the development of T2DM through suppression of insulin secretion from individual pancreatic β cells [21] and inhibition of β cell proliferation [22]. Since GLP-1 promotes β cell proliferation [5] and inhibit apoptosis [23], impairment of GLP-1 secretion can be seen as an additional cause for the development of T2DM symptoms. Furthermore, several studies report that various chemical substances such as ethanol, GCs, cytokines, and adrenaline itself, alter expression of α2A-AR in β cells, intestinal cells, or glial cells [24–26]. Therefore, controlling of α2A-AR expression in L cells or β cells can be a promising treatment target for T2DM.

Adrenaline has been previously shown to stimulate glucagon secretion from pancreatic α cells and inhibit insulin secretion from β cells. Study by Claustre and colleagues [17] and our present study on enteroendocrine L cells, however, indicate a stimulatory role of adrenaline on GLP-1 secretion, which may contradict with the effects on insulin because released GLP-1 enhances glucose-induced insulin release. This inconsistency enables us to create the complex response for nutrient intake in any environmental conditions. The expression level of α2A-AR is modulated by several stress hormones including adrenaline and other chemical molecules [24–27]. In other words, chronic exposure of these stress molecules to cells changes the responsivity of adrenaline. Further study will be required to elucidate the mechanism of GLP-1 secretion with chronic exposure of stress hormones, thereby understanding the role of GLP-1 secretion for the metabolic and behavioral regulation under stress *in vivo*.

In conclusion, we showed that GLP-1 exocytosis in intestinal L cells was modulated by all three types of adrenergic receptors, and proposed that the expression balance of three types of receptors produces different responsiveness on adrenaline to adjust for environmental changes.

Author disclosure statement

The authors have no financial interest to disclose.

Conflict of interest

None.

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.151.

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