Adrenomedullin inhibits insulin exocytosis via pertussis toxin-sensitive G protein-coupled mechanism

Nobuo Sekine,1 Koji Takano,2 Nako Kimata-Hayashi,2 Takashi Kadowaki,1 and Toshiro Fujita2

Departments of 1Metabolic Diseases and 2Nephrology and Endocrinology, University of Tokyo Graduate School of Medicine, Tokyo, Japan

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Adrenomedullin inhibits insulin exocytosis via pertussis toxin-sensitive G protein-coupled mechanism. Am J Physiol Endocrinol Metab 291: E9–E14, 2006; doi:10.1152/ajpendo.00213.2005.—Direct effects of adrenomedullin on insulin secretion from pancreatic β-cells were investigated using a differentiated insulin-secreting cell line INS-1. Adrenomedullin (1–100 pM) inhibited insulin secretion at both basal (3 mM) and high (15 mM) glucose concentrations, although this inhibitory effect was not observed at higher concentrations of adrenomedullin. The inhibition of glucose-induced insulin secretion by adrenomedullin was restored with 12-h pretreatment with 1 μg/ml pertussis toxin (PTX), suggesting that this effect could be mediated by PTX-sensitive G proteins. Cellular glucose metabolism evaluated by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide colorimetric assay was not affected by adrenomedullin at concentrations that inhibited insulin secretion. Moreover, electrophysiological studies revealed that 10 pM adrenomedullin had no effect on membrane potential, voltage-gated calcium currents, or cytosolic calcium concentration induced by 15 mM glucose. Finally, insulin release induced by cAMP-raising agents, calcium currents, or cytosolic calcium concentration induced by 15 mM glucose, is the case for CGRP and amylin. Moreover, adrenomedullin has been reported to stimulate (16) or inhibit (13) insulin secretion from isolated rat islets. However, these studies were performed using isolated pancreatic islets consisting of various cell types and thus may not be appropriate in evaluating the direct effect of adrenomedullin on β-cell functions per se.

In the present study, we investigated the direct effect of adrenomedullin on insulin secretion from β-cells by using a differentiated rat insulinoma cell line INS-1 (2). This cell line retains various differentiated features of native β-cells (2, 19, 20) and is considered a suitable model for studying the physiological mechanisms of insulin secretion. Here, we show that adrenomedullin inhibits insulin secretion from β-cells, presumably by inhibiting insulin exocytosis, and that this effect may be mediated by pertussis toxin (PTX)-sensitive G proteins.

MATERIALS AND METHODS

Reagents. RPMI 1640 and 2-mercaptoethanol were purchased from GIBCO (Rockville, MD), whereas other components of the culture medium, namely 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide colorimetric assay was not affected by adrenomedullin at concentrations that inhibited insulin secretion. Moreover, electrophysiological studies revealed that 10 pM adrenomedullin had no effect on membrane potential, voltage-gated calcium currents, or cytosolic calcium concentration induced by 15 mM glucose. Finally, insulin release induced by cAMP-raising agents, such as forskolin plus 3-isobutyl-1-methylxanthine or the calcium ionophore ionomycin, was significantly inhibited by 10 and 100 pM adrenomedullin. In conclusion, adrenomedullin at picomolar concentrations directly inhibited insulin secretion from β-cells. This effect is likely due to the inhibition of insulin exocytosis through the activation of PTX-sensitive G proteins.

With respect to the effect of adrenomedullin on insulin secretion, conflicting results have been found. Specifically, adrenomedullin has been reported to stimulate (16) or inhibit (13) insulin secretion from isolated rat islets. However, these studies were performed using isolated pancreatic islets consisting of various cell types and thus may not be appropriate in evaluating the direct effect of adrenomedullin on β-cell functions per se.

In the present study, we investigated the direct effect of adrenomedullin on insulin secretion from β-cells by using a differentiated rat insulinoma cell line INS-1 (2). This cell line retains various differentiated features of native β-cells (2, 19, 20) and is considered a suitable model for studying the physiological mechanisms of insulin secretion. Here, we show that adrenomedullin inhibits insulin secretion from β-cells, presumably by inhibiting insulin exocytosis, and that this effect may be mediated by pertussis toxin (PTX)-sensitive G proteins.
solution that contained the following (in mM): 95 K aspartate, 47.5 KCl, 1 MgCl₂, 0.1 EGTA [tetramethylammonium (TMA) salt], and 10 HEPES (TMA salt, pH 7.2). The standard internal solution comprised the following (in mM): 129 NaCl, 5 KCl, 1 MgCl₂, 1 BaCl₂, and 10 HEPES (Na salt, pH 7.4). To analyze the voltage-gated Ca²⁺ current, Ba²⁺ ion was used as a charge carrier. Voltage-gated Na⁺ channels were blocked by 1 μM tetrodotoxin, and K⁺ currents were blocked by intracellular Cs⁺ and extracellular Ba²⁺. The standard patch electrode solution for the analyses of voltage-gated Ca²⁺ current contained the following (in mM): 95 Cs aspartate, 47.5 CsCl, 1 MgCl₂, 0.1 EGTA [TMA salt], and 10 HEPES (TMA salt, pH 7.2). The standard external solution contained the following (in mM): 129 NaCl, 5 KCl, 1 MgCl₂, and 10 HEPES (Na salt, pH 7.4). During the experiments the extracellular solution was continuously perfused using a peristaltic pump. Agonists were applied by changing the extracellular solution. Approximately 2 min were required to change the bath solution in this perfusion system. The liquid junction potentials between the standard extracellular solution and other solutions used (internal and external) were measured using a 3 M KCl electrode as reference, and all data were corrected for the liquid junction potential. A List EPC-7 amplifier was used to record the membrane current and potential. All experiments were performed at room temperature (22–25°C). Glass capillaries 1.5 mm in diameter and equipped with a filament were used to make patch electrodes. The resistance of patch electrodes was between 5 and 8 MΩ. Current clamp recordings were started after the series resistance fell below 50 MΩ. Voltage clamp recordings were made after the series resistance fell below 20 MΩ. Because the current amplitude was less than 250 pA, errors caused by series resistance were considered negligible.

Intracellular calcium measurement. Cells were loaded with fura 2 by incubating with 2 μM fura 2-AM in Hanks’ balanced salt solution containing 0.1% bovine serum albumin for 40 min at room temperature. Ca²⁺ measurements were performed using a Nikon Diaphot microscope (Nikon, Tokyo, Japan). Each cell was excited at 340 and 380 nm alternately at a frequency of 100 Hz with CAM220 (Nikon-bunko, Tokyo, Japan). A band filter was used to monitor the fluorescence emission at 510 nm. The cytosolic free Ca²⁺ concentration ([Ca²⁺]ᵢ) was determined from the equation [21]:

\[ [Ca^{2+}]_i = K(R - R_{min}/(R_{max} - R)) \]

where \( K \) represents \( K_d(F_{max}/F_{min}) \), where \( K_d \) is the dissociation constant of fura 2 (130 mM at 25°C), and \( F_{min}/F_{max} \) is the ratio of Ca²⁺-free and Ca²⁺-bound fura 2 fluorescence at 380 mM. \( R_{min} \) is the 340/380 fluorescence ratio of Ca²⁺-free fura 2, and \( R_{max} \) is the 340/380 ratio of Ca²⁺-bound fura 2. Calibration was performed on every cell by permeabilizing the cell to Ca²⁺ with 2 μM digitonin. Cells were first permeabilized in Ca²⁺-free saline (5 mM EGTA, 150 mM KCl, and 10 mM HEPES, pH 7.2) for determination of \( R_{min} \) and \( F_{min} \) and then in high Ca²⁺-free saline (2.5 mM CaCl₂, 150 mM KCl, and 10 mM HEPES, pH 7.4) for determination of \( R_{max} \) and \( F_{max} \). [Ca²⁺]ᵢ traces were filtered at a bandwidth of 1 Hz to reduce the noise. Agonists were applied by changing the bath solution with a peristaltic pump. In the [Ca²⁺]ᵢ measurement experiment, ~30 s were required to change the bath solution.

Statistics. Results are presented as means ± SE, and statistical significance was determined by unpaired Student’s t-test. In case of multiple comparisons, data were evaluated by one-way ANOVA followed by post hoc analysis of Scheffé. Differences between experimental and control groups were considered significant at \( P < 0.05 \).

RESULTS

Adrenomedullin inhibits insulin secretion from INS-1 cells. Effects of adrenomedullin on insulin secretion were examined during 30 min of static incubation of INS-1 cells at basal (3 mM) and at stimulatory (15 mM) concentrations of glucose. There was a 2.2-fold stimulation of insulin secretion by 15 mM glucose compared with the basal secretion (Fig. 1). Adrenomedullin (10 and 100 pM) significantly inhibited insulin secretion at both 3 and 15 mM glucose (Fig. 1). Moreover, insulin release at 15 mM glucose was also inhibited by 1 pM adrenomedullin (Fig. 1). This inhibitory effect was, however, no longer observed at even higher doses (10 and 100 nM at 3 mM glucose, 100 nM at 15 mM glucose) of adrenomedullin, although there was still a small but significant decrease in insulin secretion by 10 nM adrenomedullin in the presence of 15 mM glucose (Fig. 1).

PTX abolishes the inhibition of insulin secretion by adrenomedullin. Because adrenomedullin receptors are known to couple to G proteins (9, 15), the inhibitory effect of adrenomedullin on insulin secretion was believed to be mediated by a PTX-sensitive G protein (22). We therefore examined the effect of PTX on the inhibition of glucose-induced insulin secretion by adrenomedullin. Pretreatment of INS-1 cells with 1 μg/ml PTX for 12 h resulted in an enhancement of insulin-secretory response to 15 mM glucose (Fig. 2). On the other hand, the inhibitory effect of 10 pM adrenomedullin on glucose-induced insulin secretion was abolished by the pretreatment with PTX (Fig. 2), indicating that this inhibitory effect involved a PTX-sensitive G protein.

Effect of adrenomedullin on glucose metabolism in INS-1 cells. In the next series of experiments, we investigated the effect of adrenomedullin on the factors implicated in glucose-induced insulin secretion. To examine whether adrenomedullin inhibits glucose-induced insulin secretion by affecting glucose metabolism, we evaluated cellular glucose metabolism in parallel with insulin secretion using the MTT colorimetric assay. The stimulatory glucose concentration (15 mM) demonstrated a 60% increase in MTT reduction compared with 3 mM glucose (Fig. 3). Adrenomedullin (10 pM), which inhibited glucose-induced insulin secretion (Fig. 1), did not significantly affect MTT reduction that had been stimulated by 15 mM glucose (Fig. 3).

Effects of adrenomedullin on membrane potential and membrane currents. Another possible mechanism underlying the adrenomedullin-induced inhibition of insulin secretion is the inhibition of membrane excitability by way of changing membrane potential or inhibiting voltage-gated calcium channels.
was not observed, but a sustained current remained (Fig. 4B, middle). Induction of T-type and L-type components with a minor residual current. To examine which type of VGCC was activated, the current was compared before and after adrenomedullin application to the cell.

To investigate the effect of adrenomedullin on the membrane current, a ramp pulse was used to evoke a membrane current, which was compared before and after adrenomedullin application in 15 mM glucose. The membrane current evoked thus did not alter significantly upon the application of 10 pM adrenomedullin (Fig. 4A). Similar results were obtained in seven other cells.

Because the VGCC are key components in the regulation of insulin secretion, the effect of adrenomedullin on these channels was examined in 15 mM glucose. At the holding potential of −70 mV, there were two components to the voltage-gated calcium current (Fig. 4B, left). One was a transient one whose rapid inactivation was followed by a sustained inward current. To examine which type of VGCC was activated, the current was observed at the holding potential of −30 mV. This depolarized holding potential, the initial transient component was not observed, but a sustained current remained (Fig. 4B, middle), indicating that the initial transient component was a T-type VGCC. The sustained current was remarkably inhibited by application of nitrendipine, an L-type VGCC blocker, leaving only a minor current (Fig. 4B, right). These data indicate that the VGCC of INS-1 cells are mainly comprised of the T-type and L-type components with a minor residual current. When 10 pM adrenomedullin were applied to cells with 15 mM glucose, neither of the two components was affected (Fig. 4C, left and right). Similar results were observed when 100 pM and 1 nM adrenomedullin were applied (n = 10 for each concentration; Fig. 4D).

Effect of adrenomedullin on [Ca2+]i in INS-1 cells. In addition to the effect of adrenomedullin on calcium currents as described above, we also measured [Ca2+]i using fura 2 as the calcium indicator. When glucose concentration of the medium was changed from 3 to 15 mM, [Ca2+]i markedly increased from the basal level (~60 nM) to ~250 nM. Application of 10 pM adrenomedullin, however, did not change [Ca2+]i (Fig. 5A). Application of 100 pM adrenomedullin also did not change [Ca2+]i. Figure 5B summarizes the results of these experiments (n = 7).

Adrenomedullin inhibits insulin release induced by forskolin plus IBMX or by ionomycin in INS-1 cells. Finally, to investigate the effect of adrenomedullin on insulin exocytosis, we examined whether adrenomedullin could inhibit insulin release induced by a rise in either cAMP or [Ca2+]i, both of which directly induce insulin exocytosis of insulin granules (24). The cAMP-raising agent forskolin in combination with IBMX increased insulin release from INS-1 cells 3.3-fold, whereas a much smaller but significant increase was observed with the Ca2+ ionophore ionomycin (Fig. 6). Adrenomedullin at both 10 and 100 pM significantly inhibited insulin release induced by these agents (Fig. 6).

DISCUSSION

The present study demonstrated that adrenomedullin directly inhibits insulin secretion from β-cells and that the inhibitory effect of adrenomedullin can be ascribed to the inhibition of insulin exocytosis through a PTX-sensitive G protein. We also found that, in INS-1 cells, adrenomedullin inhibited insulin secretion from INS-1 cells at low concentrations (picomolar range of adrenomedullin) but was ineffective at higher concentrations.

The effect of adrenomedullin on insulin secretion from isolated rat pancreatic islets thus far has yielded conflicting results. Several studies have shown that adrenomedullin inhibits insulin release, whereas others have found no effect or even a stimulatory effect (4, 24). Thus, it remains to be determined whether these discrepancies are due to differences in the species of origin of the pancreatic β-cells or the specific tissues they were isolated from. These observations are consistent with the notion that adrenomedullin acts through a PTX-sensitive G protein.

![Graph](https://example.com/graph.png)

**Fig. 2.** Effect of pertussis toxin (PTX) on the inhibition of insulin secretion by adrenomedullin on Glc-induced insulin secretion. For the PTX-treated group, INS-1 cells were incubated for 12 h in culture medium containing 1 μg/ml PTX. After culture medium was removed, cells were preincubated for 30 min in KRBH containing 3 mM Glc and then incubated for another 30 min at 3 or 15 mM Glc in the presence or absence of 10 pM adrenomedullin as indicated. The resting potential was defined as the potential needed to set the whole cell current at zero under the voltage clamp. In the absence of PTX treatment, a sustained current, which was compared before and after adrenomedullin application to the cell. The presence or absence of adrenomedullin was examined in 15 mM glucose. At the holding potential of ~70 mV, there were two components to the voltage-gated calcium current (Fig. 4B, left). One was a transient one whose rapid inactivation was followed by a sustained inward current. To examine which type of VGCC was activated, the current was observed at the holding potential of ~30 mV. This depolarized holding potential, the initial transient component was not observed, but a sustained current remained (Fig. 4B, middle), indicating that the initial transient component was a T-type VGCC. The sustained current was remarkably inhibited by application of nitrendipine, an L-type VGCC blocker, leaving only a minor current (Fig. 4B, right). These data indicate that the VGCC of INS-1 cells are mainly comprised of the T-type and L-type components with a minor residual current. When 10 pM adrenomedullin were applied to cells with 15 mM glucose, neither of the two components was affected (Fig. 4C, left and right). Similar results were observed when 100 pM and 1 nM adrenomedullin were applied (n = 10 for each concentration; Fig. 4D).

**Fig. 3.** Effect of adrenomedullin on 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction in INS-1 cells. INS-1 cells were incubated as described for the determination of insulin secretion followed by another 30 min incubation in the presence of 0.5 mg/ml MTT. Reduction of MTT was colorimetrically determined. There was no significant difference between 3 mM Glc alone and 3 mM Glc + 10 pM adrenomedullin, nor between 15 mM Glc alone and 15 mM Glc + adrenomedullin (10 pM, 100 pM, and 100 nM). Values are means ± SE from 3 independent experiments performed in quadruplicate. Statistical analysis by ANOVA: *P < 0.05 vs. 3 mM Glc.
Adrenomedullin inhibits insulin secretion at 10 nM to 1 nM concentrations (10 pM, 100 pM, and 1 nM) on the peak amplitude of voltage-gated calcium channels (VGCC) is summarized (n = 10). Peak amplitude is expressed as %control at 15 mM Glc being 100%. There were no significant differences among currents (one-way ANOVA).

Results (13, 16). Martínez et al. (13) have reported that adrenomedullin inhibits insulin secretion at 10 nM to 1 μM in a concentration-dependent manner, although Mulder et al. (16) have shown that adrenomedullin at 10 and 100 nM stimulates insulin secretion from isolated rat islets. Interestingly, the report by Martínez et al. (13) further revealed that adrenomedullin inhibited insulin secretion while increasing cAMP concentrations in the islet cells. However, adrenomedullin-induced cAMP increase is incongruous with the inhibition of insulin secretion because the elevation of intracellular cAMP concentration by agents such as those by glucagon, glucagon-like peptide-1, or forskolin results in the potentiation of glucose-stimulated insulin secretion (24). In this context, the results of these two previous reports may give insights into the mechanisms involved in the inhibitory effect of adrenomedullin on insulin secretion. Glucose is known to stimulate insulin secretion through its metabolic action on β-cells by generating metabolic coupling factors that promote exocytosis of insulin granules (18). Increase in ATP levels or in ATP-to-ADP ratio through the mitochondrial metabolism of glucose leads to closure of the ATP-sensitive K⁺ channel, thereby causing membrane depolarization. Membrane depolarization facilitates the opening of the VGCC, inducing Ca²⁺ influx into the β-cell and thus promoting insulin exocytosis. Several cellular properties may be attributed to this inhibitory effect: 1) glucose metabolism within the cell that is coupled to cellular excitability of the β-cell; 2) cellular excitability itself, specifically the membrane potential or the voltage-gated calcium currents; 3) intracellular cAMP concentration; and 4) the exocytosis ma-
chinery of insulin granules. These intracellular events mediating glucose-induced insulin secretion are depicted in Fig. 7. Our results revealed that the former three components were not influenced by adrenomedullin, leaving its effects on the exocytosis machinery as the only possibility. It has been known that exocytosis of insulin granules is stimulated by IBMX plus forskolin through the elevation of cAMP and by ionomycin through the rise in [Ca^{2+}], (24). Therefore, the finding that adrenomedullin inhibited insulin release induced by IBMX plus forskolin or by ionomycin supports the notion that the action of adrenomedullin on insulin secretion induced by these agents is due to the inhibition of insulin exocytosis. The small increase in insulin secretion by ionomycin may be due to the low concentration (3 mM) of glucose, leaving the possibility that the effect might better be demonstrated at higher concentrations of glucose.

With respect to the mechanisms underlying the effect of adrenomedullin on insulin exocytosis, the inhibition of glucose-induced insulin secretion by adrenomedullin was found to be reversed by pretreatment with PTX, suggesting that the action of adrenomedullin is mediated by PTX-sensitive G proteins such as Gi or Gq. As has been shown in other experimental systems (11), pretreatment with PTX resulted in an enhancement of glucose-induced insulin secretion in INS-1 cells. This finding thus supports the idea that PTX-sensitive G proteins are implicated in the regulation of insulin secretion (18) and indicates that PTX treatment leads to simulation of insulin release by blocking constitutive Gi/Gq activity in β-cells. Similar results have been obtained by Tsuchida et al. (22) on the inhibitory effect of adrenomedullin on amylase secretion from pancreatic acini. In their report, adrenomedullin inhibited CCK-induced amylase secretion at low concentrations (1 pM to 1 nM) comparable with those of adrenomedullin in inhibiting insulin secretion in INS-1 cells, an effect that was also abolished by PTX-pretreatment (22). In pancreatic acinar cells, amylase secretion is stimulated by an increase in intracellular Ca^{2+} concentration. Tsuchida et al. (22) have shown that this inhibitory action of adrenomedullin did not result from the modulation of CCK-induced calcium response but rather from inhibiting the calcium-induced amylase secretion, suggesting that adrenomedullin inhibits amylase secretion directly by acting on the exocytosis machinery.

It should be noted that insulin exocytosis could be directly regulated by receptor-mediated activation of the heterotrimeric G proteins (12). With the use of streptolysin O-permeabilized insulin-secreting cells, Lang et al. (12) have shown that the activation of both the α2-adrenergic receptors and Gi/Gq directly inhibit calcium-induced insulin exocytosis in a PTX-sensitive manner. We speculate that a similar mechanism is involved in the inhibitory action of adrenomedullin on calcium-induced insulin exocytosis, as was demonstrated by ionomycin in the present study.

Finally, from a clinical perspective, results of the present study provide the evidence that adrenomedullin could be involved in both the physiological regulation of insulin secretion as well as the pathogenesis of diabetes as a causative factor of impaired insulin secretion. It has been reported (5) that diabetic patients show increased plasma adrenomedullin concentrations reaching the picomolar range. In vitro studies (6) have indicated that hyperglycemia can lead to an increased vascular expression of adrenomedullin. It could therefore be speculated that adrenomedullin in the serum of diabetic patients may play a role in further impairing the insulin secretion.

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REFERENCES


