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Annexin I Stimulates Insulin Secretion through Regulation of Cytoskeleton and PKC Activity

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Abstract: In previous studies, we found that Annexin I (Anx I) was co-secreted with insulin in response to glucose, and that extracellular Anx I stimulated the release of insulin via the Anx I binding site in rat pancreatic islets and the β-cell line. However, the role that Anx I plays in the insulin secretion was not established. Therefore, in this study, we evaluated the insulin secretion pattern in response to Anx I and the involvement of the cytoskeleton or PKC in Anx I-stimulated insulin secretion in MIN6N8a cells. The peak time of insulin secretion in response to Anx I treatment corresponded with the second phase insulin secretion by glucose in the perifused pseudoislets. In addition, Anx I-stimulated insulin secretion was not affect by readily releasable pool depletion. Taken together, these findings indicate that Anx I treatment was associated with movement of the reserve pool of insulin. Furthermore, Anx I-stimulated insulin secretion was attenuated by treatment with a microfilament inhibitor, cytochalasin B, as well as by PKC down regulation. These results indicate that Anx I may be a regulator of second phase insulin secretion.

Key words: Anx I, insulin secretion, cytoskeleton, perifusion, reserve pool

INTRODUCTION

Glucose-stimulated insulin secretion is characteristically biphasic, consisting of an early transient release (first phase) followed by a sustained secondary release (second phase) (Curry et al., 1968). In mouse pancreatic β-cells, the secretory granules exist in different pools. Specifically, a small fraction of secretory vesicles are stored in the readily releasable pool (RRP) and available for immediate release (Ammala et al., 1993; Wasmeier and Hutton, 2001). The vesicles stored in the RRP are release competent and can undergo exocytosis without any further modification. However, most granules are stored in the reserve pool (RP) and need to be mobilized into the RRP and undergo chemical modifications before they can undergo exocytosis (Parsons et al., 1995). Mobilization of granules may involve physical translocation within the β-cell and/or chemical modification (Rorsman, 1997), as well as docking of the granules near the plasma membrane. For example, cortical actin depolymerization potentiates glucose-stimulated insulin secretion in cultured β-cells. In addition, glucose mediates the interaction between filamentous actin and t-SNARE [target SNAP (soluble N-ethylmaleimide-sensitive factor attachment protein) receptor] proteins (Thurmond et al., 2003). However, the detailed molecular mechanisms controlling the trafficking/mobilization, docking, priming, and fusion of insulin secretory granules have not yet been elucidated (Lang, 1999).

Annexins (Anxs) are Ca^{2+}- and phospholipid binding proteins that form an evolutionary conserved multigene family with members that are expressed throughout the animal and plant kingdoms (Gerke and Moss, 2002). The regulatory roles of extracellular Anxs have been investigated in neutrophils (Perretti and Flower, 1996), anterior pituitary cells (Taylor et al., 1995; Christian et al., 1997), pancreatic β-cells (Srivastava et al., 1999; Iino et al., 2000; Hong et al., 2002), and the β-cell line (Won et al., 2003). In neutrophils, Anx I was found to be secreted during cellular adhesion to the endothelium during the release of gelatinase granules (Perretti and Flower, 1996). Upon release, it is believed that Anx I binds to its receptor, which results in cell detachment, and inhibits transmigration of leukocytes, thereby blocking the inflammatory response (Perretti et al., 1996; Lim et al., 1998). However, although the results of these previous studies provide an explanation for the exocytotic action exerted by Anx I, the mechanisms by which Anx I regulates

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the secretory pathway of insulin have not yet been explained. Therefore, we investigated the pattern of insulin secretion and type of vesicle released in response to treatment with Anx I. In addition, we used cytoskeleton inhibitors to determine whether mobilization of the vesicles was involved in Anx I-stimulated insulin secretion in the insulin-secreting cells.

MATERIALS AND METHODS

Purification of human recombinant Anx I

Human recombinant Anx I was purified as previously described (Huang et al., 1986), with some modifications. All purification steps were performed at 4°C.

Cell Culture

MIN6N8a cells, SV40 T-transformed insulinoma cells derived from the nonobese diabetic mice (kindly provided by Dr. Jun-ichi Miyazaki, Osaka University Medical School, Osaka, Japan) (Yagi et al., 1995), were grown in DMEM (Gibco BRL) containing 25 mM glucose, 15% fetal bovine serum (FBS, Gibco BRL), penicillin (100 U/mL, Sigma) and streptomycin (0.1 mg/mL, Sigma) at 37°C under 5% CO_2. The medium was changed every 3-4 days. The monolayers were trypsinized (0.1% trypsin, 0.02% EDTA, Gibco BRL) after becoming 80-90% confluent. Pseudoislets were prepared as previously described (Hauge-Evans et al., 1999; Squires et al., 2000), after which the cells (5×10^6 cells/well) were cultured under 6-well culture plates that were pre-coated with 1% gelatin (Sigma). The medium was changed every 2-3 days and the experiments were conducted 6-8 days after the cells were sub-cultured.

Perifusion assays

Pseudoislets were washed with modified Krebs-Ringer bicarbonate HEPES buffer (mKRBH; 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 24 mM $NaHCO_3$, 15 mM HEPES, 5 mM glucose, pH 7.4) and then incubated in serum free DMEM for 2 h. The pseudoislets were then washed with mKRBH (pH 7.4), after they were harvested, and transferred to a poly-prep chromatography column (Bio-Rad). Next, the cells were perifused with mKRBH (pH 7.4) at a constant flow rate of 0.3 mL/min for 2 h at 37°C. After a stabilization, the cells were perifused with the same buffer containing 20 mM glucose, 1 μM Anx I or 55 mM KCl. All of the perifusate solution was maintained at 37°C and fractions were collected every 2 or 4 min.

Insulin secretion of MIN6N8a cells

To evaluate the insulin secretion, MIN6N8a cells were cultured at a density of 2×10^5 cells/well in a 48-well culture plate with medium for 48 h. The medium was then discarded, after which the cells were washed with serum-free DMEM containing 5 mM glucose. The cells were then incubated in serum free DMEM containing 5 mM glucose for 2 h. To deplete RRP, the cells were stimulated with 55 mM KCl for 30 min and then washed with mKRBH. Next, the cells were incubated in the presence of 55 mM KCl, 100 nM phorbol 12-myristate-13-acetate (PMA, Calbiochem) or 1 μM Anx I for 30 min. To elucidate the Anx I-related signaling pathway, the cells incubated with varying concentrations of microtubule inhibitor (colchicine, Sigma), microfilament inhibitor (cytochalasin B, Sigma) or PKC inhibitor, bisindolylmaleiamide I (BIM, Sigma) for 30 min. Following pretreatment, the cells were washed and then incubated in the presence of 1 μM Anx I for 1 h. At the end of the incubation period, the medium was harvested and either assayed immediately for the presence of insulin or stored in aliquots at –20°C for subsequent evaluation.

Measurement of insulin secretion

The amount of insulin secreted into medium by MIN6N8a cells was measured by ELISA (enzyme-linked immunosorbent assay) in triplicate as described previously (Kekow et al., 1988).

Cell viability assay

Cell viability was determined by a colorimetric assay (Mosmann, 1983).

RESULT AND DISCUSSION

Insulin secretion in perifused pseudoislets

The integrated secretory response of an intact islet is considered to be greater than the sum of the responses of the individual β-cells in isolation, and dispersed β-cells exhibit reduced glucose responsiveness (Gylfe et al., 1991; Valdeolmillos et al., 1993; Gilon et al., 1994). In the β-cell lines, the formation of pseudoislets is dependent on Ca^{2+}-dependent adhesion molecules. Additionally, cell-to-cell contact improves the functional responsiveness of MIN6 cells (Hauge-Evans et al., 1999). Therefore, in the present study we used pseudoislets to analyze insulin secretion patterns.

When pseudoislets from MIN6N8a cells were incubated with 20 mM glucose, biphasic insulin secretion consisting of a rapid first phase peak (~5 min) followed by a second sustained phase was observed (Fig. 1A). Conversely, when pseudoislets from MIN6N8a cells were treated with Anx I, instead of a rapid first peak in insulin secretion, the level of insulin secretion slowly increased (Fig. 1B). In addition, the time at which insulin secretion in response to Anx I treatment peaked was similar to the time at which the second sustained phase of insulin secretion induced by glucose peaked. However, KCl only stimulated the rapid first phase insulin secretion (Fig. 1C). Glucose-stimulated

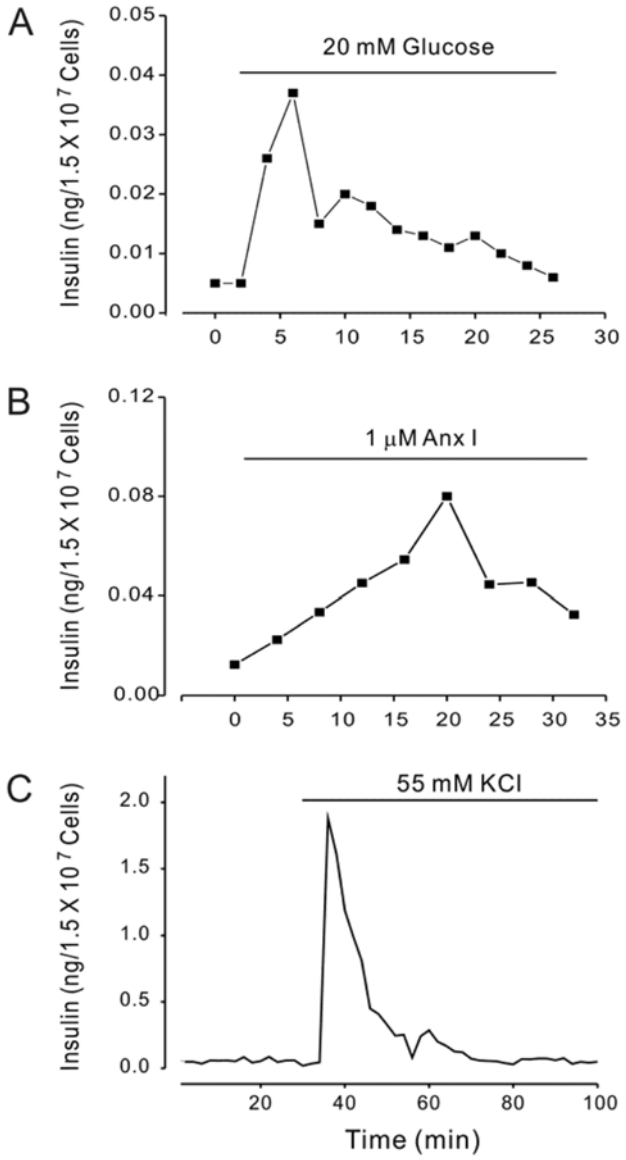

Fig. 1. Perifusion of pseudoislets by glucose, Anx I or KCl. Pseudoislets were stabilized with mKRBH and then, the pseudoislets were perifused with 20 mM glucose (A), 1 μM Anx I (B) and 55 mM KCl (C) at 37°C for 30 min. The amount of insulin secretion in the fraction was then measured by ELISA. Data are representative trace from three independent experiments.

biphasic insulin secretion involves at least two signaling pathways, the K_{ATP} channel-dependent and K_{ATP} channel-independent pathways (Rorsman and Renstrom, 2003; Straub and Sharp, 2004). Activation of K_{ATP} channel-dependent pathway results in an exocytosis of the RRP which induces the first phase of the glucose-stimulated insulin release by depolarizing the membrane following Ca^{2+} entry (Henquin, 2000; Bratanova-Tochkova et al., 2002). However, both pathways are involved in the second sustained phase of the glucose insulin release, with the K_{ATP} channel-dependent pathway inducing increased $[Ca^{2+}]_i$ levels, and the K_{ATP} channel-independent pathways providing additional signals (Bratanova-Tochkova et al., 2002). In addition to augmenting the response to the increased $[Ca^{2+}]_i$, the K_{ATP} channel-independent pathway is activated by hormones such as acetylcholine, vasoactive intestinal peptide (VIP) and diacylglycerol (DAG). Upon activation, the K_{ATP} channel-independent pathway activates PKC or stimulates adenylyl cyclase, thereby inducing a rise in cAMP and activating PKA (Sharp, 1979; Prentki and Matschinsky, 1987). Therefore, these results suggest that Anx I-stimulated insulin secretions may involve the K_{ATP} channel-independent pathways.

Anx I-stimulated insulin secretion in pseudoislets

We evaluated the insulin secretion that was induced by Anx I during the second sustained phase insulin secretion. Only fuel secretagogues are capable of eliciting the first rapid phase and the second sustained phase insulin secretion. However, non-nutrient secretagogues, such as sulfonylureas and high external K^+, generally stimulate only first-phase insulin secretion (Curry et al., 1968; Grodsky et al., 1977; Rorsman et al., 2000), when these stimuli are applied in the absence of metabolic fuels. Therefore, we used a high external concentration of K^+ in the absence of fuel secretagogues to stimulate the first rapid phase secretion to deplete RRP in the insulinoma cell line, MIN6N8a. As shown in Fig. 2A, the insulin secretion was completely abolished upon re-admission of 55 mM KCl in the MIN6N8a cells. These results indicated that almost all of the RRP was exhausted in response to incubation with 55 mM KCl for 30 min. However, PMA, which stimulates sustained phase insulin secretion, stimulated insulin secretion in the RRP depleted MIN6N8a cells (Fig. 2B). This result indicates that PMA may stimulate sustained insulin secretion and induce vesicle mobilization (Zawalich et al., 1991; Yu et al., 2000). Next, we examined the effects of Anx I on insulin secretion in RRP depleted MIN6N8a cells. As shown in Fig. 2C, treatment with Anx I resulted in stimulated insulin secretion in the depleted cells identical to control.

Several explanations of the second phase insulin secretion have been suggested. However, it is not yet clear which of the many metabolic, cationic, or second messenger events underlie this biphasicity, although a number of studies (Ganesan et al., 1990; Ganesan et al., 1992; Zawalich et al., 1995; Vadakekalam et al., 1996; Zawalich et al., 2000; Nesher et al., 2002) have suggested that the PLC/PKC pathway is particularly important in this phenomenon. In addition, inhibition of PKC activity markedly decreased the second sustained phase insulin secretion response produced in response to a high glucose concentration in isolated and perifused rat islets (Zawalich et al., 1991). Taken together, these results suggest that Anx I is able to stimulate the second phase of insulin secretion in MIN6N8a cells.

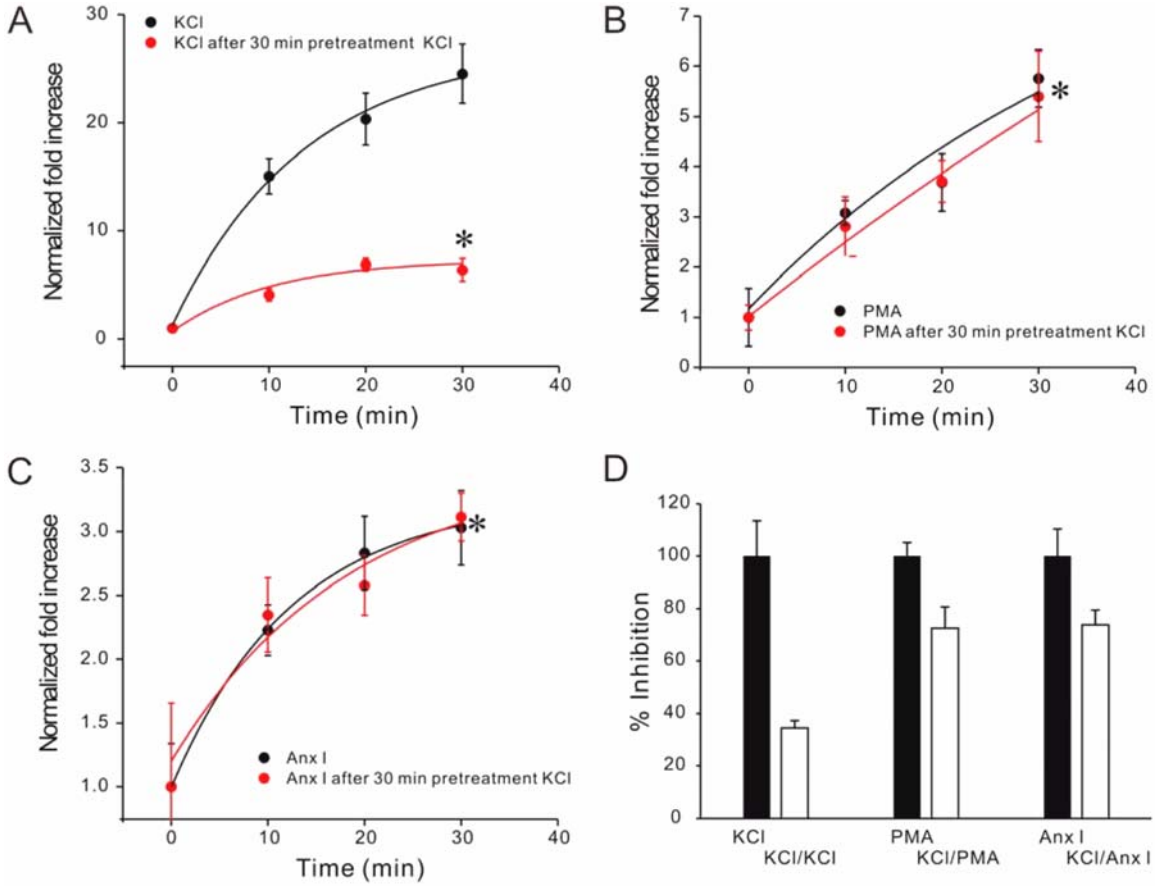

Fig. 2. Comparison with second sustained phase insulin secretion in RRP depleted cells. First phase insulin secretions were stimulated by treatment with 55 mM KCl. After 30 min, the cells were washed with mKRBH and then incubated in the presence of 55 mM KCl (A), 100 nM PMA (B) or 1 μM Anx I (C) for the indicated times. The amount of insulin secretion in the fraction was then measured by ELISA. Data are expressed as the mean value±SEM. *P<0.05 compared to 30 min KCl pretreatment as determined by a paired t-test. (D) The data are designed as a bar graph at the point of 30 min of (A), (B) and (C).

Involvement of PKC and cytoskeleton on the Anx I-stimulated insulin secretion

Generally, second phase insulin secretion requires the movement of insulin vesicles from the RP to the RRP, which is regulated by cytoskeleton reorganization and various signaling molecules (Li et al., 1994; Easom, 2000; Thurmond et al., 2003). Therefore, we examined the role that the cytoskeleton played on Anx I-stimulated insulin secretion using the microfilament or microtubules inhibitor. As shown in fig. 3A, Anx I-stimulated insulin secretion was inhibited (50% at 10 μM) by treatment with cytochalasin B, which is an actin filament destabilizing agent that is often used to inhibit vesicle translocation from the RP into the RRP, to inhibit vesicle mobilization form the RRP during exocytosis (Forscher and Smith, 1988; Smith, 1988). In contrast, treatment with microtubule inhibitor, colchicines did not alter the amount of exocytosis (Fig. 3B). We tested cell viability to examine cytotoxic effect of inhibitors by using MTT assay. Because we could not exclude the possibility that the small change of viability affected Anx I-stimulated insulin secretion. The cell viability of MIN6N8a cells was not affected by microtubule inhibitor. In the case of microfilament inhibitor, cell viability was not affected by cytochalasin B up to 1 μM. But, it was slightly reduced (15%±SEM) by 10 μM cytochalasin B (data not shown). However, most cells of them respond to same condition. It means that the inhibitory effect of cytochalasin B still has significant meaning and microfilament is involved in Anx I-stimulated insulin secretion rather than microtubule. The cytoskeleton network plays a key role in vesicle movement (Aunis and Bader, 1988; Sun et al., 1999; Gasman et al., 2004). In addition, it has been shown that actin remodeling is necessary for glucose-stimulated insulin secretion (Li et al., 1994; Wilson et al., 2001) and that the translocation of secretory granules is regulated by cortical actin network dynamics (Vitale et al., 1995). In addition, several studies have shown that a transient reduction of actin filaments activates granule movement, which, in turn, promotes refilling of the RRP (Hao et al., 2005). Furthermore, the results of a number of previously conducted studies support the idea that actin cytoskeleton reorganization is coupled with calcium-regulated exocytosis (Aunis and Bader, 1988;

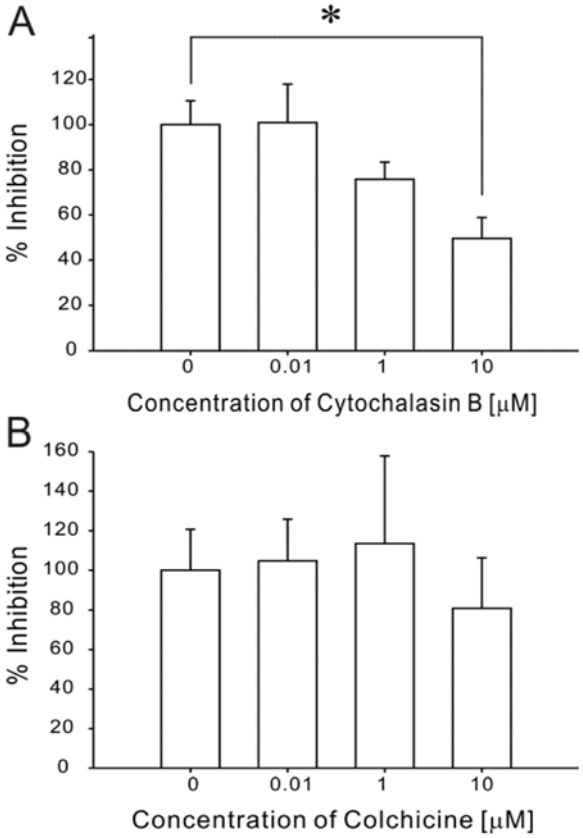

Fig. 3. Effects of cytoskeleton inhibitors on Anx I-stimulated insulin secretion. MIN6N8a cells were treated with various concentration of cytochalasin B (A) and colchicines (B) at 37°C for 30 min, after which they were washed with mKRBH and then incubated with 1 μM Anx I. The amount of insulin secretion in the fraction was then measured by ELISA. Data are expressed as the % values to the control±SEM. *P<0.05 when compared to cells that were not treated with cytochalasin B, as determined by a paired t-test.

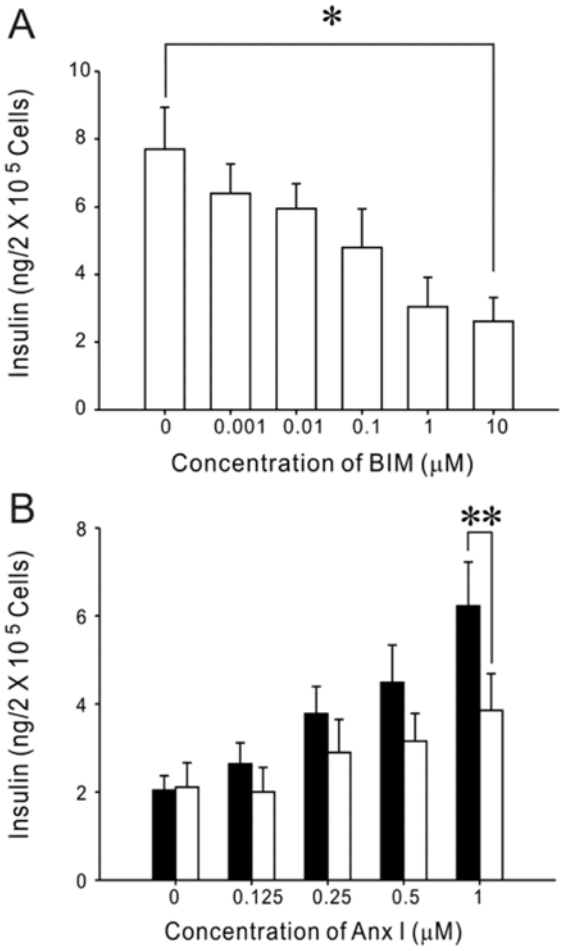

Fig. 4. Effects of PKC down-regulation on Anx I-stimulated insulin secretion. (A) MIN6N8a cells were treated with various concentration of BIM at 37°C for 30 min, washed with mKRBH, and then incubated with 1 μM Anx I. (B) MIN6N8a cells were incubated overnight with (white bar) or without (black bar) 100 nM PMA at 37°C, washed with mKRBH, and then incubated with 1 μM Anx I. The amount of insulin secretion in the fraction was then measured by ELISA. Data are expressed as the mean value±SEM. *P<0.05 compared to cells that were not treated with BIM. **P<0.05 compared to cells that were not treated with PMA, as determined by a paired t-test.

Eitzen, 2003; Bader et al., 2004). Therefore, these findings imply that cytoskeleton is involved in Anx I-stimulated insulin secretion.

Several studies have shown that PKC is involved in control of cortical actin network dynamics in chromaffin cells (Trifaro et al., 2002; Park et al., 2006; Trifaro et al., 2008). In addition, inhibition of PKC activity is known to affect the second sustained insulin secretion in isolated and perifused rat islets (Li et al., 1994). These findings indicated that regulation of PKC activity by Anx I may effect cytoskeleton reorganization in insulin secreting cells. Therefore, we evaluated whether PKC was involved in Anx I-stimulated insulin secretion using the PKC inhibitor, BIM, or by down-regulation of PKC activity. Anx I-stimulated insulin secretion was inhibited by BIM (Fig. 4A) and overnight treatment with PMA to deplete PKC activity (Fig. 4B). Several studies have suggested that cytoskeleton activity and PKC activity are correlated. For example, in chromaffin cells, disassembly of cortical F-actin allows movement of the vesicles by controlling Ca^{2+} entry and PKC activation (Trifaro et al., 2002). In addition, phosphorylation by PKC is critically involved in vesicle release through F-actin disassembly and vesicle translocation (Park et al., 2006). Therefore, these results are consistent with the cytoskeleton being regulated by PKC.

In the present study, we evaluated the role that Anx I plays during glucose-stimulated insulin secretion. Our results suggest that Anx I signaling pathway was involved with the second phase insulin secretion by glucose, and that this event may be involved with cytoskeleton reorganization and vesicle trafficking via PKC activation. In sum, these findings suggest that Anx I reinforces insulin secretion through vesicle mobilization. However, further study is required to determine the mechanism by which PKC affects remodeling of the cytoskeleton.

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