Mechanisms of Bradykinin-Induced Insulin Secretion in Clonal beta Cell Line RINm5F1

CHI YANG, BUMSUP LEE, TER-HSIN CHEN and WALTER H. HSU

Department of Veterinary Physiology and Pharmacology (C.Y., B.L., T.C., W.H.H.), Iowa State University, Ames, Iowa, Department of Veterinary Medicine National Chung Hsing University, Taichung, Taiwan 40227 (C.Y.), and Department of Comparative Medicine (T.H., W.H.H.), Pig Research Institute of Taiwan, Chunan, Miaoli, Taiwan 35099, Republic of China

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ABSTRACT

We investigated the mechanisms underlying bradykinin (BK)-induced rise in intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) and insulin secretion using clonal beta cell line RINm5F. Incubation with a range of concentrations of BK increased in concentration-dependent manners both insulin secretion (BK of 10 nM to 10 μM) and [Ca$^{2+}$] (BK of 100 nM to 100 μM). In Ca$^{2+}$-containing medium, BK (1 μM) induced a biphasic [Ca$^{2+}$] rise, which was characterized by a Ca$^{2+}$ peak and a sustained Ca$^{2+}$ phase. In the Ca$^{2+}$-free medium, BK failed to increase insulin secretion and induced only a Ca$^{2+}$ peak without the sustained Ca$^{2+}$ phase. Thapsigargin (1 μM), an inhibitor of the Ca$^{2+}$ pump in the endoplasmic reticulum, abolished the Ca$^{2+}$ peak and the sustained phase. Nimodipine (1 μM), a voltage-dependent Ca$^{2+}$ channel blocker, abolished the BK-induced sustained Ca$^{2+}$ phase and inhibited BK-induced insulin release. The BK receptor agonist des-Arg$^9$-BK (1 μM) did not change either [Ca$^{2+}$] or insulin secretion. Both the BK-induced insulin secretion and rise in [Ca$^{2+}$] were inhibited by a selective BK$\alpha$ receptor antagonist, HOE 140 (3.3–100 nM), in concentration-dependent manners but were not by a BK$\alpha$ receptor antagonist-des-Arg$^9$,Leu$^8$-BK (1 μM). Pretreatment with pertussis toxin (0.1 μg/ml) did not block the BK-induced insulin secretion or increase in [Ca$^{2+}$]. U-73122 (4, 6 and 8 μM), a phospholipase C inhibitor, antagonized both the BK-induced insulin secretion and the increase in [Ca$^{2+}$], in a concentration-dependent and parallel manner. BK increased intracellular concentrations of inositol-1,4,5-trisphosphate (IP$_3$). Neither (p-amylcinnamoyl)anthranilic acid (100 μM), a phospholipase A$_2$ inhibitor, nor N$^\omega$-nitro-L-arginine methyl ester (100 μM), a nitric oxide synthase inhibitor, inhibited these effects of BK. Taken together, these findings suggested that in beta cells, BK activates BK$\alpha$ receptors, which, in turn, activate a pertussis toxin-insensitive G protein. The G protein couples to phospholipase C, which promotes the formation of IP$_3$ and diacylglycerol. IP$_3$ releases [Ca$^{2+}$] from the intracellular Ca$^{2+}$ store, probably the endoplasmic reticulum, which triggers Ca$^{2+}$ influx via voltage-dependent Ca$^{2+}$ channels and thus increases insulin secretion.

The autacoid BK is a potent vasoactive nonapeptide that influences a number of biological processes; it regulates blood pressure and local blood flow (Pan et al., 1993), produces pain (Steranka et al., 1988) and inflammation (Pigueria et al., 1990), increases vascular permeability and localized edema (Carter et al., 1974), contracts smooth muscle (Collier et al., 1962), increases cell proliferation (Marceau and Tremble, 1986), increases glucose uptake (Sharp and Debnam, 1992) and decreases blood glucose concentration (Wicklmayr and Dietz, 1977). The effects of BK are mediated by at least two groups of BK receptors, BK$\alpha$ and BK$\beta$. BK$\alpha$ receptors mediate the acute inflammatory response, whereas BK$\beta$ receptors are responsible for most of the biological activities of kinins (Regoli et al., 1993).

In a previous report, we demonstrated that BK stimulated insulin secretion via BK$\alpha$ receptors in a concentration-dependent manner from the perfused rat pancreas (Yang and Hsu, 1995). However, the mechanisms underlying BK-induced insulin secretion remain unknown. In general, kinin receptors are coupled to G proteins that may be PTX sensitive or insensitive (Bhoola et al., 1992). At least four possible signal transduction pathways may have been associated with the effects of BK in other cellular systems: (1) PLC, which hydrolyzes PIP$_2$ to form IP$_3$ and DAG; IP$_3$ mobilizes [Ca$^{2+}$] from the intracellular Ca$^{2+}$ store of the ER (Takeuchi et al., 1988); (2) PLA$_2$, which increases the formation of arachidonic acid (Birch and Axelrod, 1987). Arachidonic acid, in turn, mobilizes Ca$^{2+}$ from the ER and opens VDCCs (Fernandez

ABBREVIATIONS: ACA, (p-amylcinnamoyl)anthranilic acid; AM, acetoxymethyl ester; BK, bradykinin; DAG, diacylglycerol; ER, endoplasmic reticulum; HOE 140, IP$_3$, inositol-1,4,5-trisphosphate; KRB, Krebs-Ringer bicarbonate solution; L-NAME, N$^\omega$-nitro-L-arginine methyl ester; PI$\beta_2$, phosphatidylinositol-4,5-bisphosphate; PTX, pertussis toxin; PLA$_2$, phospholipase A$_2$; PLC, phospholipase C; TG, thapsigargin; VDCC, voltage-dependent Ca$^{2+}$ channel.
and Balsinde, 1991); (3) adenyl cyclase, which increases cAMP formation (Suidan et al., 1991); and (4) NO synthase, which increases the formation of NO (Mombouli and Vanhoutte, 1995). Therefore, in this study, we intended to determine which signal transduction pathway would mediate the effects of BK on insulin secretion from clonal beta cell line RINm5F. Specifically, we determined whether (1) BK induced insulin secretion through an increase in $\left[Ca^{2+}\right]_i$, (2) the effects of BK were mediated by BK$_\alpha$ or BK$_\beta$ receptors, (3) a PTX-sensitive G protein and PLC mediated the effects of BK, (4) VDCCs mediated BK-induced $Ca^{2+}$ influx, and (5) PLA$_2$ and NO played a role in BK-induced insulin secretion.

### Methods

#### Cell culture.
A clonal beta cell line RINm5F (donated by Dr. S. B. Pek of the University of Michigan, Ann Arbor, MI) was maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and aerated with 5% CO$_2$/95% air, as previously described (Chen and Hsu, 1994). The cells were cultured for 5 days, and passages from day 42 to 55 were used in these experiments.

#### Insulin secretion.
RINm5F cells were plated onto 24-well plates (Corning Glass Works, Corning, NY) at $2 \times 10^6$ cells/130-mm well and grown for 5 days. During the experiments, the culture medium was removed and replaced with KRB solution containing (in mM) 136 NaCl, 4.8 KCl, 1.2 CaCl$_2$, 1.2 KH$_2$PO$_4$, 1.2 MgSO$_4$, 5 NaHCO$_3$, 10 HEPES, 4 d-glucose and 0.1% bovine serum albumin, pH 7.4. The cells were preincubated for 15 min at 37°C and then incubated in KRB with the test agent. When needed, cells were pretreated with HOE 140, des-Arg$_9$,Leu$_8$-BK, U-73122 (1-(6-[[17beta-3-methoxyestra-1,3,5(10)-tri-en-17-yl]amino]hexyl)-2,5-pyrrolidine-dione), ACA, L-NAME or nimodipine for 100 sec before BK administration. The supernatant fluids were collected, kept at 4°C and subsequently assayed within 12 hr for insulin by using radioimmunoassay as previously described (Hsu et al., 1991a).

#### Measurement of $[Ca^{2+}]_i$.
Cultured RINm5F cells of $30 \times 10^6$ cells were loaded with 2 $\mu$M fura-2 AM in KRB for 30 min at 37°C. The loaded cells were centrifuged (2000 x g), resuspended in KRB at a concentration of 10$^6$/ml and kept at 24°C for $[Ca^{2+}]_i$ measurement. Aliquots of 1.5 $\times 10^6$ cells were used for $[Ca^{2+}]_i$ measurement at 24°C. In the absence of extracellular Ca$^{2+}$, cells were centrifuged (2000 x g) and resuspended in Ca$^{2+}$-free/EGTA (10 mM) medium. The 400 nm/380 nm fluorescence ratios were monitored in an SLM-8000 fluorescence spectrophotometer (SLM, Urbana, IL). $[Ca^{2+}]_i$ was calculated after cell lysis as previously described (Hsu et al., 1991a). When needed, cells were pretreated with HOE 140, des-Arg$_9$,Leu$_8$-BK, U-73122, ACA, L-NAME or nimodipine for 5 min before BK administration. The supernatant fluids were collected, kept at 4°C and subsequently assayed within 12 hr for insulin by using radioimmunoassay as previously described (Hsu et al., 1991a).

#### Measurement of IP$_3$.
Intracellular IP$_3$ concentration of RINm5F cells was measured using a radioreceptor binding assay kit purchased from DuPont (Boston, MA). Then, 1.5 $\times 10^6$ cells in 1 ml KRB were placed in a polypropylene tube, and the treatment with BK was terminated in 10 sec by the addition of 20% (v/v) ice-cold trichloroacetic acid. The concentration of IP$_3$ was determined according to instructions from the manufacturer.

#### Data analyses.
Data are expressed as mean ± S.E. Analysis of variance was used to determine the treatment or dose effect. The least significant difference test was used to determine the differences between means of end points for which the analysis of variance indicated a significant (P < .05) F ratio.

#### Materials.
All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), except HOE 140, which was donated by Hoechst-Roussel Pharmaceuticals (Somerville, NJ); U-73122, U-73343 and ACA were purchased from BIOMOL Research Laboratory (Plymouth Meeting, PA), nimodipine was purchased from Research Biochemicals Inc. (Natick, MA) and fura-2 AM was purchased from Molecular Probes (Eugene, OR).

### Results

**Effects of BK on insulin secretion and $[Ca^{2+}]_i$ increase.** BK (10 nM to 10 $\mu$M) increased insulin secretion in a concentration-dependent manner (fig. 1A). BK (1 $\mu$M) increased insulin concentration in the wells to 3.7 times the basal level. BK (1 $\mu$M) failed to induce insulin secretion in Ca$^{2+}$-free medium (control = 0.34 ± 0.02 ng/well/min; BK = 0.35 ± 0.01 ng/well/min, n = 3, P > .05). BK (100 nM to 10 $\mu$M) increased $[Ca^{2+}]_i$ in a concentration-dependent manner (fig. 1B). BK (1 $\mu$M) increased $[Ca^{2+}]_i$ to ~2 times (at the peak) over the basal level. In Ca$^{2+}$-containing medium, BK (1 $\mu$M) induced a transient $Ca^{2+}$ increase, which reached the peak (238 ± 6 nM, n = 8) at ~20 sec. This peak was followed by a sustained $Ca^{2+}$ plateau phase that gradually declined to the basal level over 4 min (fig. 2). In Ca$^{2+}$-free medium, BK (1 $\mu$M) induced only a transient increase in $[Ca^{2+}]_i$ (168 ± 5 nM, n = 8) without the sustained $Ca^{2+}$ phase (fig. 2). The basal $[Ca^{2+}]_i$ in Ca$^{2+}$-free/EGTA medium was 88 ± 4 nM (n = 8) (fig. 2).

**Effects of BK receptor agonists and antagonists on insulin secretion and $[Ca^{2+}]_i$ increase.** BK (1 $\mu$M) increased insulin secretion (fig. 3) and $[Ca^{2+}]_i$ (at the peak) (fig. 4), but des-Arg$_9$,BK (1 $\mu$M), a BK$_\alpha$ receptor agonist, did not increase insulin secretion (fig. 3) or $[Ca^{2+}]_i$ (fig. 4). HOE 140 (1 $\mu$M), a BK$_\beta$ receptor antagonist, inhibited both the BK-induced insulin secretion (fig. 3) and increase in $[Ca^{2+}]_i$ (fig. 4). In contrast, des-Arg$_9$,Leu$_8$-BK (1 $\mu$M), a BK$_\alpha$ receptor antagonist, failed to alter this effect of BK (figs. 3 and 4). HOE 140 (3 to 100 nM) inhibited both the BK-induced insulin secretion (fig. 5A) and $[Ca^{2+}]_i$ increase in a concentration-dependent manner (fig. 5B). HOE 140 at 100 nM abolished

![Fig. 1. Effects of BK on insulin secretion (A) and peak $[Ca^{2+}]_i$ increase (B) in RINm5F cells. In this and following figures, static incubation for 15 min was performed to measure insulin secretion. $P < .05$, compared with (A) the insulin concentration of basal control group that was 0.35 ± 0.02 ng/well/ml ($n = 4$ cultures with 4 replicates in each culture) or (B) the basal $[Ca^{2+}]_i$, which was 120 ± 5 nM ($n = 4$).](image-url)
Effects of PTX and U-73122 on BK-induced insulin secretion and \([Ca^{++}]_{i}\) increase. Pretreatment with PTX (0.1 μg/ml) for 16 and 2 hr, respectively, failed to inhibit either BK (1 μM)-induced insulin secretion (BK = 1.31 ± 0.04 ng/well/min; PTX + BK = 1.26 ± 0.05 ng/well/min, n = 3; P > .05) or an increase in \([Ca^{++}]_{i}\) (BK = 212 ± 5 nm; PTX + BK = 210 ± 4 nM, n = 6; P > .05). PTX (0.1 μg/ml) alone did not induce insulin secretion (control = 0.35 ± 0.03 ng/well/min; PTX = 0.36 ± 0.02 ng/well/min, n = 3; P > .05) or increase \([Ca^{++}]_{i}\) (control = 110 ± 5 nM; PTX = 112 ± 3 nM, n = 6; P > .05).

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11.4 ± 2 nM over the basal level after U-73122 at 6 and 8 μM, respectively (n = 8 for each concentration level). [Ca++]i returned to the basal level within 100 sec of U-73122 administration.

**Effect of BK on intracellular concentrations of IP3.** The basal level of IP3 was 36 ± 2 pmol/10^6 cells. BK significantly increased intracellular IP3 concentrations to 52 ± 2 pmol/10^6 cells within 10 sec of administration.

**Effects of TG and nimodipine on BK-induced insulin secretion and [Ca++]i increase.** Pretreatment of RINm5F cells with TG (1 μM), an inhibitor of the Ca++ pump in the ER, for 30 min abolished the BK-induced transient Ca++ release phase and sustained Ca++ influx phase (fig. 7A). TG (1 μM) alone significantly increased [Ca++]i, with an onset of 5 sec and reached the peak that was 118 ± 9 nM (n = 6) over the basal level. TG-induced increase in [Ca++]i lasted <10 min. Pretreatment of RINm5F cells with nimodipine (1 μM), a VDCC blocker, for 5 min and 100 sec also inhibited BK-induced insulin secretion (control) and abolished the Ca++ influx phase (fig. 7B), respectively. Nimodipine (1 μM) alone did not change basal insulin secretion (control = 0.35 ± 0.02 ng/well/min; nimodipine = 0.39 ± 0.04 ng/well/min, n = 3; P > .05) or [Ca++]i (control = 112 ± 4 nM; nimodipine = 114 ± 2 nM, n = 6; P > .05).

**Failure of ACA and L-NAME to alter BK-induced insulin secretion.** Neither the PLA2 inhibitor ACA (100 μM) nor the NOS inhibitor L-NAME (100 μM) affected BK (1 μM)-induced insulin secretion (ACA + BK = 1.24 ± 0.04 ng/well/min; L-NAME + BK = 1.20 ± 0.04 ng/well/min; BK = 1.27 ± 0.03 ng/well/min, n = 3; P > .05, compared with each other).

**Discussion**

The results of the present study suggested that BK increases [Ca++]i and insulin secretion in RINm5F cells by activating BKβ receptors because BK, the nonselective BK receptor agonist (Farmer, 1992), increased insulin secretion and [Ca++]i, but a BKβ receptor agonist, des-Arg^2-BK (Farmer, 1992), did not. In addition, a specific BKβ receptor antagonist, HOE 140 (Hock et al., 1991), inhibited the BK-induced insulin secretion and [Ca++]i increase, but a specific BKα receptor antagonist. des-Arg^2,Leu^4-BK (Farmer, 1992). did not. These findings are consistent with those of our previous studies in the perfused rat pancreas (Yang and Hsu, 1995) and a clonal beta cell line, HIT-T15 (Saito et al., 1996). Further studies are needed to quantify BKβ receptors of beta cells using the radioligand binding technique.

The BKβ receptor protein has seven-transmembrane spanning domains, which are coupled to a G protein (Hess et al., 1994). There are PTX-sensitive G proteins, such as Gαs and Gαq (Helper and Gilman, 1992; Schmidt et al., 1991), and PTX-insensitive G proteins, such as the Gβα family (Helper and Gilman, 1992). In bovine aortic endothelial cells, BK activates both Gαs and Gαq (Liao and Homcy, 1993). We found that PTX at 0.1 μg/ml failed to inhibit the stimulatory effect of BK in the present study, but it abolished the inhibitory effect of alpha-2 adrenoceptor agonists on insulin secretion from RINm5F cells (Chen and Hsu, 1994a) and [Ca++]i in HIT cells (Hsu et al., 1991b). These results suggested that in beta cells, BKβ receptors are coupled to a PTX-insensitive G protein, probably Gαs, thereby increasing [Ca++]i, and insulin secretion.

The Gαs proteins are usually coupled to PLC (Helper and Gilman, 1992). The specific PLC inhibitor U-73122 inhibits PLC in a variety of cells, such as human neutrophils and platelets (Bleasdale et al., 1990), rat hepatocytes (Galan et al., 1991), pancreatic acinar cells (Yule and Williams, 1992) and beta cells (Chen and Hsu, 1994b). In the present study, BK-induced insulin secretion and [Ca++]i increase were inhibited by U-73122 in a concentration-dependent manner but were not altered by the analog U-73343, which does not inhibit PLC activity (Smith et al., 1990). By using neomycin (0.2 to 10 mM), a less specific PLC inhibitor that also inhibits VDCC (Redman and Silinsky, 1994), Saito et al. (1996) found that it inhibited BK-induced increase in [Ca++]i of HIT cells. Thus, these results suggested that BK activates PLC, which catalyzes the formation of IP3. IP3 in turn, increases Ca++ release from the ER (Berridge, 1993).

Intracellular Ca++ is a major signal in insulin secretion. In many cell types, the stimulus-response usually couples to the elevation of [Ca++]i through Ca++ release from the intracel-
lular store and Ca\(^{2+}\) influx. Secretagogues, including hor-
mones and neurotransmitters, increase [Ca\(^{2+}\)], which lead to
insulin secretion (Komatsu et al., 1989; Li et al., 1992). In
RINm5F cells, BK induced a transient Ca\(^{2+}\) peak that was
immediately followed by a sustained Ca\(^{2+}\) phase attributable
to Ca\(^{2+}\) influx. The transient Ca\(^{2+}\) peak is partly attrib-
uted to Ca\(^{2+}\) release from the intracellular store, prob-
ably the ER, because in Ca\(^{2+}\)-free medium, BK induced only a
Ca\(^{2+}\) peak without the sustained Ca\(^{2+}\) phase. TG inhibits
Ca\(^{2+}\) uptake by the Ca\(^{2+}\) pump into the ER, thereby deplet-
ing the intracellular Ca\(^{2+}\) store (Thastrup et al., 1990). Our
findings showed that TG abolished both the BK-induced transient
Ca\(^{2+}\) peak and sustained Ca\(^{2+}\) phase, suggesting that the
BK-induced Ca\(^{2+}\) influx depends on the Ca\(^{2+}\)-
releasing. The BK-induced [Ca\(^{2+}\)]\(i\) peak in Ca\(^{2+}\)-
containing medium was higher than that in Ca\(^{2+}\)-free medium.
Thus, the BK-induced Ca\(^{2+}\) peak in Ca\(^{2+}\)-containing me-
dium came partly from Ca\(^{2+}\) release and partly from Ca\(^{2+}\)
influx. The opening of voltage-independent Ca\(^{2+}\) channels and/or VDCCs may greatly contribute to the Ca\(^{2+}\) influx
(Fasolato et al., 1994). Our findings suggested that the BK-
induced Ca\(^{2+}\) influx is predominantly mediated through VD-
CCs because the sustained Ca\(^{2+}\) influx was abolished by a
VDCC blocker nimodipine.

BK stimulates the formation of NO in vascular endothelial
cells by activating a PTX-insensitive G protein (G\(_{12}\)) (Liao and
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Send reprint requests to: Dr. Walter H. Hsu, Department of Biomedical Sciences, Iowa State University, Ames, IA 50011.