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CALCIUM EFFLUX FROM CULTURED BOVINE ADRENAL CHROMAFFIN CELLS INDUCED BY BRADYKININ

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Abstract—The effect of bradykinin on Ca^{2+} efflux from cultured bovine adrenal chromaffin cells was examined. Bradykinin enhanced the efflux of $^{45}Ca^{2+}$ from the cells in a concentration dependent manner (10^{-9}–10^{-6} M). This effect was inhibited by a specific bradykinin B_2-receptor antagonist, but not by a B_1-receptor antagonist. Nifedipine, Co^{2+} and Cd^{2+} did not inhibit the bradykinin-stimulated $^{45}Ca^{2+}$ efflux from the cells. 12-*O*-Tetradecanoyl phorbol 13-acetate, an activator of protein kinase C, also had no effect on the efflux of $^{45}Ca^{2+}$ from the cells. The increase in bradykinin-stimulated $^{45}Ca^{2+}$ efflux was reduced by removal of extracellular Na^+. These results suggest that bradykinin stimulates Na^+/Ca^{2+} exchange in cultured bovine adrenal chromaffin cells.

The nonapeptide BK† influences several physiological processes including pain generation [1], blood pressure [2] and cardiovascular regulation [3]. It has also been suggested to be a central nervous system neurotransmitter and to play a role in the regulation of neuronal function [4, 5].

In neural cell lines, BK increases the level of cyclic GMP [6], stimulates production of inositol phosphates [7, 8] and increases the cytosolic free Ca^{2+} concentration [9]. The increase in the intracellular free Ca^{2+} level, $[Ca^{2+}]_i$, was found to occur through agonist-stimulated influx of extracellular Ca^{2+} and hydrolysis of PIP_2 to yield IP_3 [10, 11].

Previously we reported that BK increases the intracellular levels of inositol phosphates, free Ca^{2+} and possibly diacylglycerol in pheochromocytoma PC-12 cells [12]. These effects increased the activities of protein kinases (calcium/calmodulin-dependent protein kinase and protein kinase C), resulting in stimulation of the pathway of catecholamine formation [12, 13]. Also in adrenal chromaffin cells, BK has been shown to stimulate the formation of inositol phosphates and to increase $[Ca^{2+}]_i$. However, little is known of the mechanism of the fall in $[Ca^{2+}]_i$ elevation in adrenal chromaffin cells on BK stimulation. Hence the effect of BK on calcium efflux from cultured bovine adrenal chromaffin cells was studied. BK was found to enhance the efflux of $^{45}Ca^{2+}$ from these cells in culture and suggested that its effect may be mediated in part by acceleration of Na^+/Ca^{2+} exchange.

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† Abbreviations: TPA, 12-*O*-tetradecanoyl phorbol 13-acetate; PIP_2, phosphatidyl inositol 4,5-bisphosphate; IP_3, inositol 1,4,5-triphosphate; BSS, balanced salt solution; BK, bradykinin.

MATERIALS AND METHODS

Cell preparation and culture. Bovine adrenal chromaffin cells were dispersed enzymatically as described previously [14]. Briefly, the medulla was sliced with a hand slicer, and the slices were digested in medium containing 0.1% collagenase, 0.01% soybean trypsin inhibitor, and 0.5% bovine serum albumin in BSS (135 mM NaCl, 5.6 mM KCl, 1.2 mM $MgSO_4$, 2.2 mM $CaCl_2$, 10 mM glucose and 20 mM HEPES/NaOH, pH 7.4). Cells were plated in 35-mm culture dishes at a density of 2×10^6 cells/dish for measuring $^{45}Ca^{2+}$ efflux or on 22 × 22 mm cover glasses in 35-mm culture dishes at a density of 1×10^6 cells/dish for measuring intracellular calcium, and maintained for 3 days as monolayer cultures in Eagle's basal medium supplemented with 5% heat-inactivated fetal calf serum, 2 mM glutamine, penicillin (100 units/mL), streptomycin (100 μg/mL), gentamycin (40 μg/mL), fungizone (2.5 μg/mL) and 10 μM cytosine arabinoside.

$^{45}Ca^{2+}$ efflux assay. Adrenal chromaffin cells were cultured in 35-mm dishes for 3 days. Then they were washed and incubated as described above in BSS containing $^{45}CaCl_2$ (3 μCi/mL) for 1 hr at 37°. After incubation, the cells in each well were washed 15 times with 1 mL volumes of BSS at intervals for 30 sec to remove unincorporated $^{45}Ca^{2+}$. The cells were then incubated 15 times with 1 mL volumes of BSS for 30 sec periods to determine basal efflux levels. Then, they were incubated 15 times with 1 mL volumes of reaction mixture with or without test agents for 30 sec periods to determine agonist-stimulated efflux levels. After agonist stimulation, the cells were solubilized in 1 mL 1% Triton X-100 to determine their residual $^{45}Ca^{2+}$. Samples were counted in 10 mL of liquid scintillation fluid for 2 min periods. The total radioactivity of $^{45}Ca^{2+}$ in each well was determined as the sum of the radioactivity in each fraction and the residual radioactivity, and this value was used to calculate the fractional release of Ca^{2+} in each period.

Measurement of intracellular calcium by fura-2. Intracellular Ca^{2+} level in single chromaffin cell was measured using the fluorescent Ca^{2+} indicator fura-2. The cells, which were cultured on cover glass, were incubated at 37° for 30 min with 1 mL BSS containing 2 μM fura-2/acetoxy methyl ester. Then the cells on the cover glass were transferred to a small incubation bath (approx. 0.5 mL) on the platform of a microscope. The temperature was maintained at 37° and the incubation bath was perfused with BSS at a rate of 0.8 mL/min during the experimental period. Fluorescence was measured in single chromaffin cell on the cover glass using a fluorescence spectromicroscope (excitation, 340/380 nm, emission, 510 nm). The intracellular Ca^{2+} level was determined using the equation described previously [15].

Chemicals. $^{45}CaCl_2$ was obtained from Amersham Corp. (Tokyo, Japan). BK, Des-Arg^9-[Leu^8]-BK and D-Arg-[Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin were obtained from the Peptide Institute (Osaka, Japan). Nifedipine was obtained from Wako Pure Chemical Co. (Osaka, Japan). 12-*O*-Tetradecanoyl phorbol 13-acetate (TPA) and amiloride were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other chemicals used were commercial products of reagent grade.

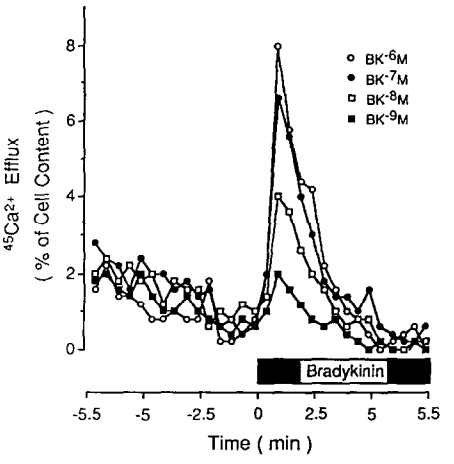

Fig. 1. Effects of different concentrations of BK on $^{45}Ca^{2+}$ efflux from cultured bovine adrenal chromaffin cells. Cells were preloaded with $^{45}Ca^{2+}$ as described in Materials and Methods, and then incubated with the indicated concentrations of bradykinin (10^{-9}–10^{-6} M). Data are shown as percentages of cell contents, and are means for four to six separate experiments. Maximal SE was ±10.6%.

RESULTS

Effects of bradykinin and BK antagonists on $^{45}Ca^{2+}$ efflux

Figure 1 shows the effluxes of $^{45}Ca^{2+}$ from adrenal chromaffin cells in culture induced by various concentrations of BK. The stimulatory effect of BK on $^{45}Ca^{2+}$ efflux was dose-dependent at concentrations of 10^{-9}–10^{-6} M BK. The efflux of $^{45}Ca^{2+}$ increased to a peak value within about 1 min after BK addition. The peak value with 10^{-6} M BK was 8.2 ± 0.7% (N = 6) of the total $^{45}Ca^{2+}$ in the cells. After the peak, efflux decreased rapidly in the next 5 min. Figure 2 shows the effects of the BK-receptor antagonists Des-Arg^9-[Leu^8] BK (B_1-receptor antagonist [16]) and D-Arg-[Hyp^3,Thi^{5,8}, D-Phe^7]-BK (B_2-receptor antagonist [17]) on the submaximal $^{45}Ca^{2+}$ efflux from the cells induced by 10^{-7} M BK. This efflux was inhibited 81% by 10^{-6} M D-Arg-[Hyp^3,Thi^{5,8}, D-Phe^7]-BK, but was not inhibited by Des-Arg^9-[Leu^8]-BK. This result suggests that the $^{45}Ca^{2+}$ efflux induced by bradykinin was mediated through the BK B_2-receptor.

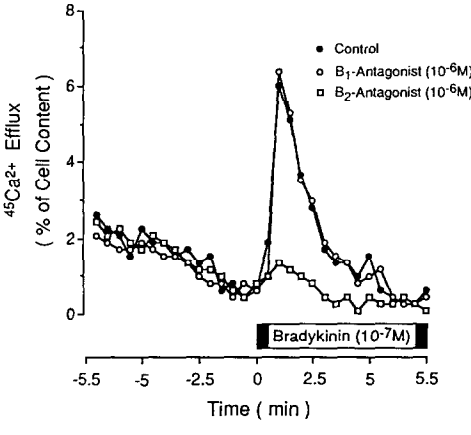

Fig. 2. Effects of BK antagonists on BK-induced $^{45}Ca^{2+}$ efflux from cultured bovine adrenal chromaffin cells. Cells were preloaded with $^{45}Ca^{2+}$ as described in Materials and Methods. The BK antagonist Des-Arg^9-[Leu^8]-BK (B_1-receptor antagonist; 10^{-6} M) or D-Arg-[Hyp^3,Thi^{5,8},D-Phe^7]-BK (B_2-receptor antagonist; 10^{-6} M), was added 150 sec before BK (10^{-7} M). Data are shown as percentages of cell contents, and are means for three to four separate experiments. Maximal SE was ±9.6%.

Effects of various agents on $^{45}Ca^{2+}$ efflux

To determine whether BK-stimulated $^{45}Ca^{2+}$ efflux is mediated by activation of Ca^{2+} channels, we examined whether it was inhibited by Ca^{2+} channel blockers. Nifedipine, an organic voltage-dependent Ca^{2+} channel blocker, had no effect on $^{45}Ca^{2+}$ efflux from the cells induced by BK (Fig. 3). The inorganic Ca^{2+} channel blockers, which inhibit voltage-dependent and receptor-operated Ca^{2+} channels, Co^{2+} and Cd^{2+} also did not inhibit BK stimulated $^{45}Ca^{2+}$ efflux (Fig. 3). Thus stimulation of $^{45}Ca^{2+}$ efflux by bradykinin is probably not due to increased Ca^{2+} flux through Ca^{2+} channels.

The stimulations of BK, histamine and muscarinic acetylcholine receptors are reported to induce breakdown of PIP_2 in cultured bovine adrenal chromaffin cells [18, 19]. The breakdown products of PIP_2 (IP_3 and diacylglycerol) increase the intracellular $[Ca^{2+}]_i$ level and stimulate protein kinase C [20]. The effects of bradykinin, histamine, acetylcholine and TPA (an activator of protein kinase C) on $^{45}Ca^{2+}$ efflux from the cells were

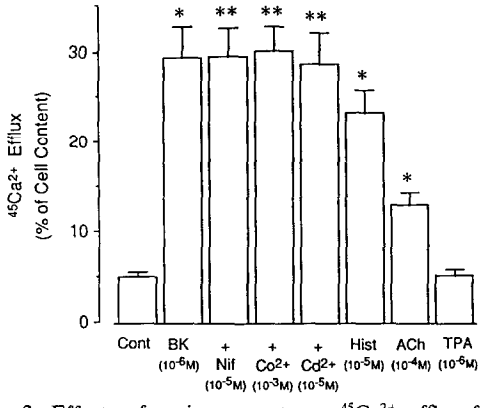

Fig. 3. Effects of various agents on $^{45}Ca^{2+}$ efflux from cultured bovine adrenal chromaffin cells. Cells were preloaded with $^{45}Ca^{2+}$ as described in Materials and Methods and then incubated in the presence or absence of BK (10^{-6} M), histamine (Hist; 10^{-5} M), acetylcholine (ACh; 10^{-4} M) or TPA (10^{-6} M). Nifedipine (Nif; 10^{-5} M), Co^{2+} (10^{-3} M) or Cd^{+2} (10^{-5} M) was added 150 sec before BK (10^{-6} M). $^{45}Ca^{2+}$efflux in 5 min after addition of agents was calculated. Data are means ± SE for three to four separate experiments. * Significant difference from control ($P < 0.01$). ** No significant difference from BK stimulated $^{45}Ca^{2+}$ efflux.

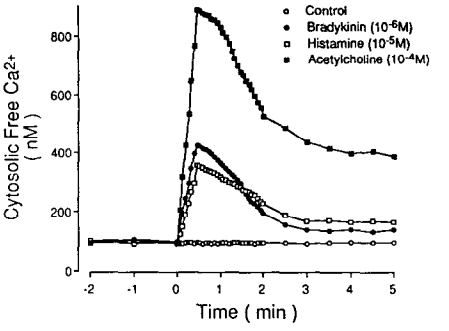

Fig. 4. Effects of BK, histamine and acetylcholine on intracellular free Ca^{2+} concentration in cultured bovine adrenal chromaffin cells. Cells were preloaded with 2 μM fura-2/acetoxy methyl ester as described in Materials and Methods and then incubated in the presence or absence of BK (10^{-6} M), histamine (10^{-5} M) or acetylcholine (10^{-4} M). Data are means for four to five separate experiments. Maximal SE was ±9.7%.

examined (Fig. 3). Bradykinin (10^{-6} M) and histamine (10^{-5} M) increased the $^{45}Ca^{2+}$ efflux to about 460 and 350% the control level. Acetylcholine (10^{-4} M) increased $^{45}Ca^{2+}$ efflux from the cells slightly, but significantly, whereas TPA (10^{-6} M) had no effect on $^{45}Ca^{2+}$ efflux. These results suggest that bradykinin-stimulated $^{45}Ca^{2+}$ efflux from the cells is related to the formation of IP_3, but not to activation of protein kinase C.

Effects of BK, histamine and acetylcholine on intracellular free Ca^{2+} concentration

To determine whether the increased $^{45}Ca^{2+}$ efflux induced by BK is dependent on the elevation of intracellular $[Ca^{2+}]_i$ level, effects of BK, histamine and acetylcholine on intracellular free Ca^{2+} concentration were examined. As shown in Fig. 4, BK (10^{-6} M) and histamine (10^{-5} M) increased the intracellular $[Ca^{2+}]_i$ to approximately 430 and 360 nM. Acetylcholine (10^{-4} M) increased it to about 890 nM. However, acetylcholine-stimulated $^{45}Ca^{2+}$ efflux from the cells was less than BK-or histamine-stimulated $^{45}Ca^{2+}$ efflux (Fig. 3). Therefore, BK-stimulated $^{45}Ca^{2+}$ efflux from the cells may not be dependent on the elevation of intracellular $[Ca^{2+}]_i$ level in the cells.

Effects of extracellular sodium deprivation and amiloride on BK stimulated $^{45}Ca^{2+}$ efflux

To determine whether the increased $^{45}Ca^{2+}$ efflux induced by BK is Na^+-dependent, we carried out a series of experiments in the absence of extracellular Na^+. As shown in Fig. 5, complete replacement of Na^+ by sucrose significantly blocked the enhanced $^{45}Ca^{2+}$ efflux from the cells induced by bradykinin.

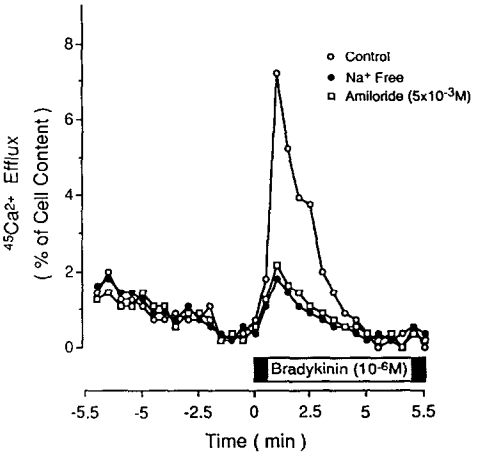

Fig. 5. Effects of Na^+-free medium and amiloride on BK induced $^{45}Ca^{2+}$ efflux from cultured bovine adrenal chromaffin cells. Cells were preloaded with $^{45}Ca^{2+}$ as described in Materials and Methods. The medium was changed to Na^+-free medium (with sucrose instead of all Na^+) 150 sec before adding BK (10^{-6} M). In the case of the amiloride experiment, amiloride (5×10^{-3} M) was added 150 sec before BK (10^{-6} M) in normal BSS medium. Data are shown as percentages of cell contents, and are means for three to four separate experiments. Maximal SE was ±11.8%.

Amiloride, an inhibitor of the Na^+/Ca^{2+} exchanger [21], also significantly inhibited BK-stimulated $^{45}Ca^{2+}$ efflux from the cells. Therefore, the effect of BK in stimulating Ca^{2+} efflux across the plasma membrane may be mediated in part by a Na^+/Ca^{2+}exchange mechanism.

DISCUSSION

In the present study we examined the mechanisms

involved in stimulation of Ca^{2+} efflux from cultured bovine adrenal chromaffin cells by BK. BK is known to stimulate the formation of inositol phosphates in cultured bovine adrenal chromaffin cells [18, 19] and to increase $[Ca^{2+}]_i$ in a number of cell types [7, 8, 12, 22]. However, this increased $[Ca^{2+}]_i$ should be restored to a physiological level in response to further stimulus.

BK has been found to stimulate Ca^{2+} efflux from Swiss 3T3 fibroblasts [23], guinea pig tracheal cells [24] and human IMR-90 lung fibroblasts [25]. As shown in Fig. 1, it also increased Ca^{2+} efflux from cultured bovine adrenal chromaffin cells in a concentration-dependent manner. The concentration range of BK for this effect is similar to that for its stimulation of PIP_2 breakdown in the cells. We have reported that increases in inositol phosphates in pheochromocytoma PC-12 cells by BK is a B_2-mediated response [12]. Here we report the pharmacological characterization of BK receptors in cultured bovine adrenal chromaffin cells determined by $^{45}Ca^{2+}$ efflux studies. The selective B_2-receptor antagonist D-Arg-[Hyp^3,Thi^{5,8},D-Phe^7]-BK [17] inhibited the $^{45}Ca^{2+}$ efflux from the cells induced by bradykinin, whereas the B_1-receptor antagonist Des-Arg^9-[Leu^8]-bradykinin [16] did not (Fig. 2). Thus this effect of BK may be mediated through the B_2-receptor.

The increased Ca^{2+} efflux induced by BK was not inhibited by nifedipine, Co^{2+} or Cd^{2+} (Fig. 3) suggesting that this efflux does not involve calcium channels in the cell membrane. In previous studies with pheochromocytoma PC-12 cells we found that BK had no effect on voltage-dependent calcium channels for Ca^{2+} uptake [12]. BK, therefore, does not seem to influence Ca^{2+} fluxes through calcium channels in adrenal chromaffin cells.

The increases in breakdown of PIP_2 in adrenal chromaffin cells induced by BK or histamine have been examined [18]. Diacylglycerol is produced concurrently with IP_3 on breakdown of PIP_2 by phospholipase C. Diacylglycerol is thought to activate protein kinase C by increasing the affinity of the enzyme for calcium [26–28]. Acetylcholine also induces production of IP_3 through the cholinergic muscarinic-receptor in adrenal chromaffin cells, though its efflux is less than these of BK and histamine [27]. In our study, BK and histamine treatments increased Ca^{2+} efflux from the cells approximately 5.6-and 4.5-fold, and acetylcholine increased it approximately 2.5-fold (Fig. 3). TPA, an activator of protein kinase C, had no influence on Ca^{2+} efflux from the cells (Fig. 3). Therefore, the production of IP_3 induced by BK may regulate Ca^{2+} efflux from adrenal chromaffin cells.

The level of increase in $[Ca^{2+}]_i$ might influence Ca^{2+} efflux from the cells. However, treatments with 10^{-6} M BK, 10^{-5} M histamine and 10^{-4} M acetylcholine increased $[Ca^{2+}]_i$ in cultured bovine adrenal chromaffin cells to 430, 360 and 890 nM, respectively, as measured with fura-2 (Fig. 4). Thus the $[Ca^{2+}]_i$ after acetylcholine stimulation was higher than those after BK and histamine stimulations, whereas the increase in Ca^{2+} efflux from the cells after acetylcholine treatment was less than those after BK and histamine treatments (Figs 3 and 4). Therefore, elevation of $[Ca^{2+}]_i$ did not apparently increase Ca^{2+} efflux from adrenal chromaffin cells.

Enhanced Ca^{2+} efflux from cultured bovine adrenal chromaffin cells following cell stimulation with a cholinergic agonist was found to depend on extracellular Na^+ [29]. We observed enhanced $^{45}Ca^{2+}$ efflux from cultured bovine adrenal chromaffin cells during stimulation with BK and found here that this bradykinin-stimulated $^{45}Ca^{2+}$ efflux was inhibited in Na^+-free medium and by amiloride treatment (Fig. 5). Thus BK potentiates Na^+/Ca^{2+} exchange mechanisms in cultured bovine adrenal chromaffin cells. It is not clear if it is involved in some other BK-stimulated $^{45}Ca^{2+}$ efflux mechanisms because extracellular Na^+ deprivation and amiloride could not completely abolish this efflux.

We conclude from this study that BK increases Ca^{2+} efflux from cultured bovine adrenal chromaffin cells, and that this efflux may be explained by stimulation of Na^+/Ca^{2+} exchange mechanisms.

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