Neurotransmitter release from bradykinin-stimulated PC12 cells

Stimulation of cytosolic calcium and neurotransmitter release

Kenneth C. APPELL* and Dana S. BAREFOOT

Department of Molecular Biology, A. H. Robins Research Laboratories, Richmond, VA 23220, U.S.A.

The effect of bradykinin on intracellular free Ca^{2+} and neurotransmitter secretion was investigated in the rat pheochromocytoma cell line PC12. Bradykinin was shown to induce a rapid, but transient, increase in intracellular free Ca^{2+} which could be separated into an intracellular Ca^{2+} release component and an extracellular Ca^{2+} influx component. The bradykinin-induced stimulation of intracellular free Ca^{2+} displayed a similar time course, concentration dependencies and extracellular Ca^{2+} dependence as that found for neurotransmitter release, indicating an association between intracellular free Ca^{2+} levels and neurotransmitter secretion. The selective BK_1 -receptor antagonist des-Arg⁹,[Leu⁸]BK (where BK is bradykinin) did not significantly affect the stimulation of intracellular free Ca^{2+} or neurotransmitter release. In contrast, these effects of bradykinin were effectively blocked by the selective BK_2 -receptor antagonist [Thi^{5,8},D-Phe⁷]BK, and mimicked by the BK_2 partial agonist [D-Phe⁷]BK in a concentration-dependent manner. The stimulation of intracellular free Ca^{2+} and neurotransmitter release induced by bradykinin was shown not to involve voltage-sensitive Ca^{2+} channels, since calcium antagonists had no effect on either response at concentrations which effectively inhibit depolarization-induced responses. These results indicate that bradykinin, acting through the interaction with the BK_2 receptor, stimulates an increase in intracellular free Ca^{2+} leading to neurotransmitter secretion. Furthermore, bradykinin-induced responses involve the release of intracellular Ca^{2+} and the influx of extracellular Ca^{2+} that is not associated with the activation of voltage-sensitive Ca^{2+} channels.

INTRODUCTION

Bradykinin is a nonapeptide which has been postulated to influence many biological processes including inflammation (Bell *et al.*, 1980), pain generation (Regoli & Barabe, 1980), and gastrointestinal (Manning *et al.*, 1982) and cardiovascular (Regoli *et al.*, 1977) regulation. Bradykinin has also been recognized as a possible neurotransmitter in the central nervous system and may play a regulatory role in neuronal function (Snyder, 1980; Perry & Snyder, 1984).

In an attempt to understand the mechanisms underlying neurosecretory processes, several investigators have utilized the rat pheochromocytoma cell line, PC12, as a model with which to study the properties of several stimuli in evoking neurotransmitter release (Greene & Tischler, 1982). Much of this work has focused on the ability of such stimuli as elevated extracellular potassium and nicotinic cholinergic agonist to induce neurotransmitter release. These agents were thought to cause an influx of Ca^{2+} through depolarization-induced activation of voltage-sensitive Ca^{2+} channels, thereby triggering exocytosis (Greene & Rein, 1977; Ritchie, 1979; DiVirgilio *et al.*, 1987). In addition to depolarizing stimuli, muscarinic receptor activation was shown to induce neurotransmitter secretion through a Ca^{2+} -dependent process. Increased intracellular free Ca^{2+} levels, $[\text{Ca}^{2+}]_i$, were found to occur through agonist-stimulated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to yield inositol 1,4,5-triphosphate (IP_3) (Vin-

centini *et al.*, 1985; Rabe *et al.*, 1987). Muscarinic receptor-evoked neurotransmitter release was believed to be dependent on this IP_3 -induced Ca^{2+} mobilization.

In a recent study, vanCalker *et al.* (1987) have shown that bradykinin receptors on PC12 cells can stimulate the rapid accumulation of IP_3 , presumably through the hydrolysis of PIP_2 . Bradykinin-induced release of IP_3 might be expected to mobilize intracellular Ca^{2+} which then might evoke secretion from these cells. We therefore investigated the effects of bradykinin on $[\text{Ca}^{2+}]_i$ and neurotransmitter release in PC12 cells. In the work presented here, we report that bradykinin, acting through the BK_2 receptor subtype, rapidly stimulates the mobilization of calcium which then leads to neurotransmitter release. Furthermore, bradykinin-induced responses involve the influx of extracellular Ca^{2+} , but not through the activation of voltage-dependent Ca^{2+} channels.

MATERIALS AND METHODS

Materials

Bradykinin was obtained from United States Biochemical Corporation; [Thi^{5,8},D-Phe⁷]BK was from Cambridge Research Biochemicals; [D-Phe⁷]BK and des-Arg⁹,[Leu⁸]BK was from Sigma. Calcium antagonists were generously provided by Tanabe Seijaku Co. Ltd., Osaka, Japan (1-*cis*-diltiazem); Janssen Pharmaceutical, Beerse, Belgium (flunarizine); Miles Laboratories, New Haven, CT, U.S.A. (nimodipine); and Knoll Pharma-

Abbreviations used: $[\text{Ca}^{2+}]_i$, intracellular free calcium concentration; DA, dopamine; BSS, balanced salt solution; IP_3 , inositol 1,4,5-trisphosphate; PIP_2 , phosphatidylinositol 4,5-bisphosphate; IP_4 , inositol 1,3,4,5-tetrakisphosphate; BK, bradykinin (in names of synthetic analogues).

* To whom all correspondence and reprint requests should be sent, at present address: Life Sciences Research Laboratories, Building 320, Eastman Kodak Company, Rochester, NY 14650, U.S.A.

ceutical, Whippany, NJ, U.S.A. (verapamil). AHR-5360 {1-[4-(3-{4-[bis(4-fluorophenyl)methyl]-1-piperidinyl}propoxy)-3-methoxy phenyl]ethanone mandelate} was synthesized at A. H. Robins Co., Richmond, VA, U.S.A.

Cell culture

The commercially obtained rat pheochromocytoma cell line PC12 (CRL-1721; American Type Culture Collection, Rockville, MD, U.S.A.) was cultured as monolayers in polystyrene flasks as described by Greene & Tischler (1976). Cells were maintained in RPMI 1640 medium supplemented with 10% horse serum (Hazleton), 5% newborn calf serum (Whittaker), 2 mM-glutamine and containing penicillin (100 units/ml) and streptomycin (100 µg/ml). PC12 cells were harvested by gently streaming the culture medium on the surface of the monolayers and then dissociated to yield single cells by passage through a syringe with a 20 gauge needle. Cells were prepared for experimentation by seeding the PC12 cells on poly-D-lysine-coated ACLAR (Allied Chemical Corp.) plastic strips {14 mm × 64 mm for $[Ca^{2+}]_i$ measurements or 22 mm × 60 mm for $[^3H]$ -dopamine ($[^3H]$ DA) release assays} at a density of 190 000–280 000 cells/cm² as described by Reynolds & Dubyak (1986). Cells attached to the ACLAR plastic strips were maintained in culture medium and used 1–2 days later.

Measurements of $[Ca^{2+}]_i$

Intracellular free Ca^{2+} concentrations were determined using the fluorescence Ca^{2+} indicator Fura-2. In these experiments, culture medium was replaced with a balanced salt solution (BSS) containing (millimolar): NaCl, 115; KCl, 5.6; KH_2PO_4 , 1.2; $MgCl_2$, 0.6; $CaCl_2$, 1.8; glucose, 5.6; Hepes/NaOH, 25, pH 7.4, and supplemented with 0.1% bovine serum albumin. Cells attached to the ACLAR plastic were washed with 4 ml of buffer and were then incubated in buffer containing 5 µM-Fura-2AM (Molecular Probes Inc.) for 40 min at 37 °C. The free Fura-2AM was removed from the cells by two 4 ml washes of buffer and incubated for an additional 20 min at 37 °C. At the end of the incubations, the strips were cut into 14 mm × 16 mm coverslips, placed in 4 ml of BSS and stored at room temperature. The coverslips were positioned diagonally into a 1 cm² quartz cuvette containing a stir bar and 2 ml of BSS. Intracellular Fura-2 fluorescence changes were measured at 25 °C in a Perkin-Elmer LS-5 spectrofluorometer (excitation 495 ± 5 nm; emission, 505 ± 10 nm). Bradykinin or KCl was added from 100× concentrated stock solutions. Intracellular free calcium concentrations were determined using the equation (Grynkiewicz *et al.*, 1985):

$$[Ca^{2+}]_i \text{ (nM)} = (135 \text{ nM}) (F - F_{\min.}) / (F_{\max.} - F)$$

where F was the measured relative fluorescence intensity, $F_{\max.}$ was the relative fluorescence intensity after the addition of ionomycin (10 µM final concentration) and $F_{\min.}$ was the relative fluorescence intensity obtained after the subsequent addition of EGTA to a concentration of 10 mM plus sufficient Tris base to raise the pH to greater than 8.3.

Measurement of $[^3H]$ DA release

PC12 cells, attached to the ACLAR plastic strips, were washed and incubated at 37 °C for 60 min in 4 ml of BSS containing 1.2 mM-sodium ascorbate and supplemented

with 0.5 µCi of $[^3H]$ DA/ml (47 Ci/mmol; Amersham). Following the incubation, the cells were washed and incubated at 37 °C for an additional 60 min in 4 ml of buffer without $[^3H]$ DA. The ACLAR strips were then cut into 15 mm × 22 mm coverslips and placed in 2.2 ml of buffer. The assay was performed in an 8-well multiplate (Miles Scientific) placed in a shaking water bath at 25 °C. Immediately before use, the coverslips were washed three times in 1.5 ml of buffer and then incubated for the indicated times at 25 °C in 1.5 ml of buffer, then in 1.5 ml of buffer containing the appropriate concentrations of bradykinin or KCl, and finally in 1.5 ml of buffer alone. In the experiments in which the KCl concentration was raised by 50 mM, the NaCl concentration was decreased accordingly to maintain iso-osmolality. At the end of these incubations, the coverslip was transferred to 10 ml of scintillation fluid to solubilize the cells and 1 ml of each incubation well was sampled and radioactivity was determined by liquid scintillation counting. The addition of the monoamine oxidase inhibitor pargyline (0.1 µM) had no effect on the total accumulation of $[^3H]$ DA or bradykinin- and KCl-stimulated release of $[^3H]$ DA, and therefore pargyline was not used routinely in the experiments reported.

The $[^3H]$ DA contained in the cells at the beginning of each experiment (200 000–300 000 d.p.m.) was calculated for each coverslip by summing the radioactivity in the incubation buffers and the solubilized cells. The amount of $[^3H]$ DA released from the cells within the time of exposure to bradykinin or KCl is expressed as a percentage of the total $[^3H]$ DA contained in the cells at the beginning of the release experiment. The amount of spontaneously released $[^3H]$ DA, obtained in the absence of stimuli, was routinely determined both just before and after the addition of stimulus. Net $[^3H]$ DA release produced by stimulus was calculated by taking the mean of these two spontaneous release values and subtracting it from the amount released in response to stimulus.

RESULTS

Bradykinin-induced stimulation of $[Ca^{2+}]_i$

In PC12 cells, bradykinin has been shown to cause a rapid but transient release of IP_3 through the degradation of PIP_2 (vanCalker *et al.*, 1987). Since the release of IP_3 is thought to act as an intracellular second messenger for the mobilization of Ca^{2+} , we have investigated the effect of bradykinin on $[Ca^{2+}]_i$ using Fura-2 loaded PC12 cells. In BSS containing 1.8 mM- Ca^{2+} , the resting $[Ca^{2+}]_i$ was found to be 35 ± 2 nM (mean $\pm$ S.E.M. for 15 separate cell preparations). Addition of bradykinin (1 µM) to these cells resulted in a rapid increase in $[Ca^{2+}]_i$ that reached maximal levels (210 ± 10 nM, $n = 15$) within 20–30 s and then slowly declined to resting $[Ca^{2+}]_i$ within 4–6 min after the addition of bradykinin (Fig. 1a). Readdition of bradykinin (1 µM) 6 min after the initial stimulation caused no further effect on $[Ca^{2+}]_i$, indicating that the transient properties of $[Ca^{2+}]_i$ were not due to the loss of bradykinin activity (results not shown). The role of extracellular Ca^{2+} was also investigated in cells incubated in BSS without added Ca^{2+} for 5 min prior to the addition of bradykinin (Fig. 1b). Under these conditions, resting $[Ca^{2+}]_i$ (25 ± 2 nM, $n = 4$) was slightly reduced and addition of bradykinin (1 µM) caused a rapid, but attenuated, transient stimulation of $[Ca^{2+}]_i$ (maximal stimulation, 87 ± 11 nM). When the incubation buffer was made

Fig. 1. Extracellular Ca^{2+} -dependence of bradykinin-induced stimulation of $[Ca^{2+}]_i$.

Cells grown on ACLAR coverslips were loaded with Fura-2, and $[Ca^{2+}]_i$ was determined as described in the Materials and methods section. Prior to stimulation, the cells were washed four times with 2 ml of BSS with (a) or without (b) added Ca^{2+} (1.8 mM). After a 5 min preincubation, bradykinin (BK) was added to a final concentration of 1 μ M. In (b), 1.8 mM- Ca^{2+} (final) was added 4 min after bradykinin addition.

1.8 mM- Ca^{2+} , a second transient increase of $[Ca^{2+}]_i$ was observed. This increase in $[Ca^{2+}]_i$ reached a maximum $[Ca^{2+}]_i$ of 151 ± 15 nM ($n = 4$) and then slowly declined to a new steady state level near the resting $[Ca^{2+}]_i$ observed in Fig. 1(a). The magnitude of this second transient response was found to be sensitive to extracellular Ca^{2+} concentrations added. Thus, reducing the extracellular Ca^{2+} from 1.8 mM- Ca^{2+} (Fig. 1b) to 1.0 mM- and 0.5 mM- Ca^{2+} (results not shown) produced a progressive decrease in the maximal bradykinin response. A decrease in the magnitude of the overall response was also observed when cells, incubated in the continuous presence of reduced extracellular Ca^{2+} , were stimulated with bradykinin as outlined in Fig. 1(a) (results not shown). These results suggest that at least part of the bradykinin-induced increase in $[Ca^{2+}]_i$ was derived from extracellular Ca^{2+} .

Fig. 2. Concentration dependence of bradykinin-induced stimulation of $[Ca^{2+}]_i$.

Changes in $[Ca^{2+}]_i$ were determined from the maximal bradykinin-induced increase in $[Ca^{2+}]_i$ obtained 20–30 s after the addition of the appropriate concentrations of bradykinin. The stimulated increase in $[Ca^{2+}]_i$, obtained in BSS containing 1.8 mM- Ca^{2+} , was corrected for the initial basal value. The solid line represents the non-linear best fit of the data to the Hill equation. Results are from a representative experiment of three separate cell preparations.

Fig. 2 demonstrates that bradykinin concentration dependencies for the maximal increase in $[Ca^{2+}]_i$ obtained in BSS containing 1.8 mM- Ca^{2+} . A plot of the log of the bradykinin concentration versus the maximal increase in $[Ca^{2+}]_i$, measured 20–30 s after the addition of bradykinin, shows the half-maximal stimulation at 3.7 ± 1.5 nM ($n = 3$), with the maximal response occurring at approx. 1 μ M.

Bradykinin-induced stimulation of $[^3H]DA$ release

In the previous subsection, it was shown that the addition of bradykinin to PC12 cells induces a rapid but transient increase in $[Ca^{2+}]_i$, reaching a maximum within 20–30 s (Fig. 1a). Maximal $[Ca^{2+}]_i$ then slowly declined to prestimulated levels. Because the stimulation of neurotransmitter release is believed to be dependent on $[Ca^{2+}]_i$ (Katz & Miledi, 1970; Reichardt & Kelly, 1983), we investigated the effect of bradykinin on $[^3H]DA$ release in an attempt to determine whether changes in $[Ca^{2+}]_i$ accompanied the stimulation of secretion by bradykinin. Cells, grown on plastic coverslips were loaded with $[^3H]DA$ and release was measured in the presence of bradykinin. As illustrated in Fig. 3(a), bradykinin produced a substantial increase in the rate of $[^3H]DA$ release, reaching maximal rates within 30 s of the addition of 1 μ M-bradykinin. The stimulated rate of release then declined during the continuous presence of bradykinin. The net cumulative response further illustrates the initial rapid rate of release followed by a slower rate of response, reaching a plateau (no net release) within 2 min of the addition of bradykinin. Thus, bradykinin produces a rapid but transient increase in the rate of neurotransmitter release. The concentration dependence for bradykinin-induced stimulation of $[^3H]DA$ release is

Fig. 3. Time course and concentration-dependence of bradykinin-induced stimulation of $[^3\text{H}]\text{DA}$ release

Cells grown on ACLAR strips were loaded with $[^3\text{H}]\text{DA}$, and bradykinin-induced release was determined as described in the Materials and methods section. In (a), $[^3\text{H}]\text{DA}$ release (bars) was measured during successive 0.5 min incubations in the absence (B) or presence of $1\ \mu\text{M}$ -bradykinin, and is expressed as the amount released as a percentage of the total $[^3\text{H}]\text{DA}$ contained in the cell at the beginning of the release experiment. Values represent the means $\pm$ S.E.M. obtained from three separate cell preparations. The net cumulative $[^3\text{H}]\text{DA}$ release ($\bullet$) was calculated by subtracting the mean spontaneous release (B) obtained in the absence of bradykinin from the amount released in response to $1\ \mu\text{M}$ -bradykinin. The percentage $[^3\text{H}]\text{DA}$ release (b) was measured during a 4 min stimulation with the indicated concentrations of bradykinin and corrected for basal release. The solid line represents the non-linear best fit of the data to the Hill equation. Results are from a representative experiment of three separate cell preparations.

shown in Fig. 3(b). The stimulation was half-maximal at $1.8 \pm 0.2\ \text{nM}$ ($n = 3$) and reached a plateau at approx. $1\ \mu\text{M}$.

The bradykinin receptor can be separated into two subtypes, BK_1 and BK_2 , which differ in sensitivity to synthetic bradykinin analogues (Regoli & Barabe, 1980). The bradykinin receptor subtype responsible for mobilizing calcium and neurotransmitter release was investigated using synthetic bradykinin analogues (Table 1). Addition of the BK_1 antagonist des-Arg⁹, [Leu⁸]BK to PC12 cells

4 min before stimulation with $10\ \text{nM}$ -bradykinin had no effect on either $[^3\text{H}]\text{DA}$ release or $[\text{Ca}^{2+}]_i$ at concentrations up to $50\ \mu\text{M}$. In contrast, the BK_2 antagonist [Thi^{5,8}, D-Phe⁷]BK caused a concentration-dependent inhibition of bradykinin-induced responses. These results suggest that the bradykinin receptor subtype in PC12 cells is exclusively BK_2 . Furthermore, when [D-Phe⁷]BK, a BK_2 partial agonist (Whalley *et al.*, 1987), was added directly to the cells, agonist activity for both $[^3\text{H}]\text{DA}$ release and $[\text{Ca}^{2+}]_i$ was observed, further supporting the presence of a BK_2 receptor subtype in PC12 cells.

Depolarization-induced stimulation of $[\text{Ca}^{2+}]_i$ and $[^3\text{H}]\text{DA}$ release

In an attempt to further understand the mechanism of bradykinin-induced Ca^{2+} mobilization and neurotransmitter release, we have compared the properties of bradykinin-stimulated responses with those of depolarization with KCl. We first examined the extracellular Ca^{2+} dependence on depolarization-induced changes in $[\text{Ca}^{2+}]_i$ from Fura-2 loaded cells. As illustrated in Fig. 4, depolarization induces a rapid increase in $[\text{Ca}^{2+}]_i$ that reaches the maximal levels ($582 \pm 55\ \text{nM}$; 10 separate cell preparations) within 15–30 s of the addition of $50\ \text{mM}$ -KCl. Maximal levels of $[\text{Ca}^{2+}]_i$ then decline, reaching a new steady state well above the initial resting $[\text{Ca}^{2+}]_i$. In the absence of added Ca^{2+} , resting $[\text{Ca}^{2+}]_i$ was slightly decreased, and upon depolarization $> 95\%$ of the $[\text{Ca}^{2+}]_i$ response was lost. Thus, while the bradykinin-induced changes in $[\text{Ca}^{2+}]_i$ were only partially dependent on the presence of extracellular Ca^{2+} , essentially all of the calcium responsible for the depolarization-induced increase in $[\text{Ca}^{2+}]_i$ was derived from extracellular Ca^{2+} .

Depolarization of cells preloaded with $[^3\text{H}]\text{DA}$ caused a rapid rate of release, being maximal within 1 min of the addition of $50\ \text{mM}$ -KCl (Fig. 5). Furthermore, when the rate of $[^3\text{H}]\text{DA}$ release was measured at earlier time points, a maximal rate of release was observed within 30 s of the addition of KCl (results not shown). As illustrated by the net cumulative rate of release (corrected for basal) in the continuous presence of KCl, the rate of $[^3\text{H}]\text{DA}$ release declined from maximum levels, reaching a new steady state rate of release significantly above basal levels that was maintained for at least 5 min after depolarization.

Removal of extracellular calcium was shown in Fig. 1(b) to produce a small but significant bradykinin-induced stimulation of $[\text{Ca}^{2+}]_i$. Release of $[^3\text{H}]\text{DA}$ was also investigated under these conditions (Fig. 6). In the absence of added extracellular Ca^{2+} , bradykinin ($1\ \mu\text{M}$) induced the release of $[^3\text{H}]\text{DA}$, but this release was approx. 40% of the response observed in incubation buffer containing $1.8\ \text{mM}$ - Ca^{2+} . In contrast, removal of extracellular Ca^{2+} prior to depolarization resulted in the complete loss of KCl-induced release of $[^3\text{H}]\text{DA}$. Thus, the bradykinin-induced stimulation of $[^3\text{H}]\text{DA}$ release is only partially dependent on extracellular Ca^{2+} , while the depolarization-induced response depends entirely on the presence of extracellular Ca^{2+} .

Effect of selected calcium antagonists on stimulated $[^3\text{H}]\text{DA}$ release and $[\text{Ca}^{2+}]_i$

To determine whether activation of voltage-dependent Ca^{2+} channels plays a role in bradykinin-induced responses, selected calcium antagonists were used in an attempt to block the effects of bradykinin on Ca^{2+}

Table 1. Characteristics of the effects of bradykinin on the stimulation of [³H]DA release and [Ca²⁺]_i

[Ca²⁺]_i and [³H]DA release from PC12 cells were determined as described in the Materials and methods section. The bradykinin antagonists des-Arg⁹,[Leu⁸]BK and [Thi^{5,8},D-Phe⁷]BK were added 4 min prior to stimulation with 10 nM-bradykinin. [D-Phe⁷]BK was used as an agonist in place of bradykinin. Net [³H]DA release was determined over a 4 min stimulation period. Net stimulation of [³H]DA release in the presence of 10 nM-bradykinin alone was 3.51 ± 0.23% (*n* = 9). Data are expressed as percentages of control values obtained during 4 min stimulation with 10 nM-bradykinin. Results are the means ± S.E.M. for three separate experiments.

BK analogue	Selectivity	[BK analogue] (μM)	Stimulation (% of control)	
			[³ H]DA release	[Ca ²⁺] _i
Des-Arg ⁹ ,[Leu ⁸]BK	BK ₁ antagonist	50	98 ± 4	98 ± 4
		10	101 ± 5	—
		1	101 ± 5	—
[Thi ^{5,8} ,D-Phe ⁷]BK	BK ₂ antagonist	10	5.3 ± 1.6	10 ± 5
		1	51 ± 4	34 ± 7
		0.1	92 ± 1	73 ± 6
[D-Phe ⁷]BK	BK ₂ partial agonist	10	60 ± 3	39 ± 1
		1	23 ± 2	10 ± 1
		0.1	1.5 ± 0.6	1.7 ± 0.9

Fig. 4. Extracellular Ca²⁺-dependence of depolarization-induced stimulation of [Ca²⁺]_i

Cells grown on ACLAR coverslips were loaded with Fura-2, and [Ca²⁺]_i was determined as described in the Materials and methods section. Prior to stimulation, the cells were washed four times in BSS with (upper trace) or without (lower trace) added Ca²⁺ (1.8 mM). After 5 min pre-incubation, KCl was added to a final concentration of 50 mM.

mobilization and neurotransmitter release. PC12 cells have previously been reported to possess dihydropyridine-sensitive voltage-dependent calcium channels which when activated by cellular depolarization allow the stimulation of Ca²⁺ influx, leading to neurotransmitter release (Rabe & McGee, 1983; Meldolesi *et al.*, 1984; DiVirgilio *et al.*, 1987). These depolarization-induced responses were shown to be effectively blocked by several calcium antagonists. Furthermore, voltage-dependent calcium channels have been identified by [³H]dihydropyridine receptor binding (Toll, 1982; Albus *et al.*, 1984; Messing *et al.*, 1985). In agreement with these binding studies, we have shown that [³H]nimodipine binds to a saturable,

Fig. 5. Time course of depolarization-induced stimulation of [³H]DA release

Cells grown on ACLAR strips were loaded with [³H]DA and depolarization-induced release was determined as described in the Materials and methods section. [³H]DA release (bars) was measured during successive 1 min incubations in the absence (B) or presence of 50 mM-KCl. The data are expressed as the amount released from the cells within the time of exposure to KCl as a percentage of the total [³H]DA contained in the cells at the beginning of the release experiment. Values represent the means ± S.E.M. obtained in three separate cell preparations. The net cumulative [³H]DA release (●) was calculated by subtracting the mean spontaneous release (B) obtained in the absence of KCl from the amount released in response to 50 mM-KCl.

single class of binding sites with *K_d* of 0.47 ± 0.05 nM (*n* = 3) and *B_{max}* of 0.049 ± 0.006 pmol/mg when binding was performed on isolated PC12 cell membranes (results not shown). The presence of functional dihydropyridine-sensitive voltage-dependent Ca²⁺ channels in PC12 cells was shown by determining the inhibition of depolarization-induced stimulation of [Ca²⁺]_i with several classes

Fig. 6. Extracellular Ca^{2+} -dependence of bradykinin- and depolarization-induced stimulation of [^3H]DA release

[^3H]DA release was determined as described in the Materials and methods section. Cells were washed four times with BSS buffer in the presence (open bars) or absence (hatched bars) of added Ca^{2+} (1.8 mM). After a 5 min preincubation, bradykinin (1 μM) or KCl (50 mM) was added. Results are the means $\pm$ S.E.M. from three separate cell preparations.

Fig. 7. Effect of calcium antagonists on depolarization-induced stimulation of $[\text{Ca}^{2+}]_i$

Cells grown on ACLAR coverslips were loaded with Fura-2 and $[\text{Ca}^{2+}]_i$ was determined as described in the Materials and methods section. Prior to stimulation, the cells were washed three times with buffer containing the indicated concentrations of nimodipine (▲), AHR-5360 (◆), flunarizine (●), verapamil (▼) or diltiazem (■). After a 1 h preincubation, KCl was added to a final concentration of 50 mM. Data are expressed as a percentage of $[\text{Ca}^{2+}]_i$ obtained in the absence of drug with basal levels subtracted. Each data point represents the mean of two to four separate cell preparations. The solid lines represent the non-linear least squares best fit of the data to the Hill equation. The slope factor (Hill coefficient, n) and IC_{50} (half-saturating response) were computed using the following equation: $\text{response (\%)} = \{1 - ([X]^n / \text{IC}_{50}^n + [X]^n)\} \times 100$, where $[X]$ is the effector drug concentration.

Fig. 8. Effect of diltiazem on bradykinin- and depolarization-induced stimulation of $[\text{Ca}^{2+}]_i$ and [^3H]DA release

Intracellular free calcium (a) and [^3H]DA (b) release were determined as described in the Materials and methods section. Prior to stimulation, the cells were washed three times in buffer containing 25 μM -diltiazem. After a 15 min preincubation, cells were stimulated with either bradykinin (1 μM) or KCl (50 mM). Data are expressed as a percentage of control, obtained in the absence of diltiazem and corrected for basal levels where control values for bradykinin and KCl respectively were 160 ± 5 and 643 ± 66 nM for $[\text{Ca}^{2+}]_i$ and 2.77 ± 0.22 and $3.72 \pm 0.05\%$ for [^3H]DA release. Results are the means $\pm$ S.E.M. for three separate cell preparations.

of calcium antagonists. Thus, preincubation of Fura-2-loaded PC12 cells with selected calcium antagonists produced a concentration-dependent inhibition of depolarization-induced stimulation of $[\text{Ca}^{2+}]_i$ (Fig. 7). The order of potency for these calcium antagonists was found to be nimodipine > AHR-5360 > flunarizine > verapamil > diltiazem. The IC_{50} (inhibitor concentration causing 50% inhibition) values ($n = 2-4$) for each of the compounds were determined by least-squares best fit of the data to the Hill equation (nM): nimodipine (1.4; 3.5), AHR-5360 (45 ± 10 , $n = 4$), flunarizine (140 ± 37 , $n = 3$), verapamil (410; 480) and diltiazem (1200 ± 260 , $n = 4$), with slopes of 1.0, 0.9, 1.2, 0.9 and 1.0 respectively, indicating a single class of binding sites.

The effects of the calcium antagonist diltiazem on

bradykinin- and depolarization-induced stimulation of $[Ca^{2+}]_i$ were investigated (Fig. 8a). Preincubation of cells with 25 μ M-diltiazem had no significant effect on stimulation of $[Ca^{2+}]_i$ by bradykinin (1 μ M). However, diltiazem greatly reduced the depolarization-induced rise in $[Ca^{2+}]_i$, resulting in the 95 % loss in stimulatory activity. Similar studies were performed in cells preloaded with $[^3H]DA$ (Fig. 8b). Addition of diltiazem had little effect on bradykinin-induced release of $[^3H]DA$, whereas depolarization-induced responses were reduced to 90 % of that found in the absence of diltiazem. Similar results were obtained with 200 nM-nimodipine (results not shown), indicating that the selective inhibition of voltage-sensitive calcium channels has no effect on bradykinin-induced responses at calcium antagonist concentrations which greatly reduced the response induced by depolarization.

DISCUSSION

In the present study, we have investigated the effect of bradykinin on the stimulation of intracellular free Ca^{2+} and neurotransmitter secretion in the rat pheochromocytoma cell line, PC12. This cell line offers the opportunity to study the molecular mechanisms involved in the mobilization of intracellular Ca^{2+} which, in turn, leads to a cellular response, i.e. neurotransmitter release.

The results presented here demonstrate that in Fura-2-loaded PC12 cells, bradykinin stimulates a rapid increase in $[Ca^{2+}]_i$ whose magnitude was found to be dependent on extracellular Ca^{2+} concentrations. Thus, in cells incubated in the absence of added Ca^{2+} , bradykinin induced a rapid but attenuated response, and readdition of extracellular Ca^{2+} produced a second transient increase in $[Ca^{2+}]_i$ (Fig. 1b). The initial rise in $[Ca^{2+}]_i$ induced by bradykinin in the absence of extracellular Ca^{2+} is believed to arise from the release of Ca^{2+} from intracellular stores through the involvement of IP_3 (Berridge & Irvine, 1984). Indeed, recent studies have shown that in PC12 cells, bradykinin stimulates the rapid hydrolysis of PIP_2 to yield IP_3 , an important second messenger for the mobilization of intracellular Ca^{2+} (vanCalcker *et al.*, 1987), with a similar time course and transient nature as that observed in Fig. 1(b). The second transient increase in $[Ca^{2+}]_i$, produced by the addition of extracellular Ca^{2+} in the continuous presence of bradykinin, is believed to be derived from the influx of extracellular Ca^{2+} across the plasma membrane. Thus, the bradykinin-induced response observed in Fig. 1(a) consists of an intracellular Ca^{2+} release component as well as a Ca^{2+} influx component. The precise mechanism for the activation of the Ca^{2+} influx component of the bradykinin response is not known, but several possibilities can be excluded: (a) since the intracellularly released Ca^{2+} was allowed to return to basal levels prior to the readdition of extracellular Ca^{2+} , this indicates that released Ca^{2+} is not responsible for this activation process; and (b) since calcium antagonists were ineffective in blocking the bradykinin-induced increase in $[Ca^{2+}]_i$ at concentrations which effectively block depolarization-induced $[Ca^{2+}]_i$ (Fig. 8), this suggests that the Ca^{2+} channel responsible for extracellular Ca^{2+} influx is distinct from the dihydropyridine-sensitive, L-type, voltage-dependent Ca^{2+} channel. Others have reported that inositol 1,3,4,5-tetrakisphosphate (IP_4) may activate plasma membrane Ca^{2+}

channels resulting in the influx of extracellular Ca^{2+} (Irvine & Moor, 1986). However, the role of IP_4 is a general second messenger for regulating Ca^{2+} channels is still a matter of conjecture (Crossley *et al.*, 1988). Whether the Ca^{2+} influx component of the bradykinin response occurs through IP_4 -activated 'receptor-operated' Ca^{2+} channels in PC12 cells remains to be determined.

Several lines of evidence suggest that the bradykinin-induced increase in $[Ca^{2+}]_i$ is associated with neurotransmitter release. In support of this interpretation is the observation that bradykinin stimulated an elevation of $[Ca^{2+}]_i$ with a similar time course as that observed for bradykinin-induced neurotransmitter release (Figs. 1a and 3a). The bradykinin concentration dependence for the increase in $[Ca^{2+}]_i$ also paralleled the stimulation of neurotransmitter release, further supporting the association between these two processes (Figs. 2 and 3b). As mentioned above, removal of extracellular Ca^{2+} prior to the addition of bradykinin resulted in an attenuated bradykinin-stimulated increase in $[Ca^{2+}]_i$. Under these conditions, bradykinin-induced transmitter release was also reduced to approximately one-third of the response observed in the presence of 1.8 mM- Ca^{2+} (Fig. 6), indicating that the Ca^{2+} -dependence of neurotransmitter release arises from the release of intracellular Ca^{2+} as well as the influx of extracellular Ca^{2+} .

As illustrated in Table 1, the bradykinin-induced stimulation of $[Ca^{2+}]_i$ and neurotransmitter release could be ascribed to the selective activation of the BK_2 receptor. The selective BK_2 receptor antagonist $[Thi^{5,8}, D-Phe^7]BK$ produced a concentration-dependent inhibition of these bradykinin-induced responses. Furthermore, direct stimulation was observed when the BK_2 partial agonists $[D-Phe^7]BK$ was added to PC12 cells. In contrast, the BK_1 -selective antagonist $des-Arg^9, [Leu^8]BK$ was without effect on either process. Thus, in PC12 cells, bradykinin-induced increase in $[Ca^{2+}]_i$ leading to neurotransmitter release occurs through the selective activation of BK_2 receptors.

Similar to the effects of bradykinin, depolarization stimulated an increase in $[Ca^{2+}]_i$, which rapidly reached a maximum and then decayed to a new steady state level well above the initial prestimulated level of $[Ca^{2+}]_i$ (Fig. 4). Neurotransmitter release induced by depolarization followed a similar time course, reaching maximal levels and then declining to a new steady state rate of release well above prestimulated rates (Fig. 5). Thus, in agreement with previously reported data (Meldolesi *et al.*, 1984; DiVirgilio *et al.*, 1987), depolarization produced a biphasic rate of release, a rapid component occurring within 1 min the addition of KCl and a slower rate of release that was maintained for several minutes after depolarization. Depolarization-induced stimulation of $[Ca^{2+}]_i$ and neurotransmitter release was shown to be entirely dependent on the presence of extracellular Ca^{2+} (Fig. 6). Furthermore, the influx of extracellular Ca^{2+} leading to neurotransmitter release appears to be derived through the activation of dihydropyridine-sensitive voltage-dependent calcium channels. Several classes of calcium antagonists produced a concentration-dependent inhibition of Ca^{2+} influx at concentrations which effectively blocked neurotransmitter release (Figs. 7 and 8). Thus, both bradykinin and depolarization stimulate a rapid rise in the rate of neurotransmitter release which can be associated with the stimulation of $[Ca^{2+}]_i$ levels. However, the mechanism for the increase in $[Ca^{2+}]_i$

induced by these two stimuli appears to occur through distinct processes.

In summary, we present evidence to suggest that bradykinin, acting through the activation of BK_2 receptors, stimulates a rapid increase in $[Ca^{2+}]_i$ which is comprised of an intracellular Ca^{2+} release component and an extracellular Ca^{2+} influx component. Both sources of Ca^{2+} contribute to a net increase in $[Ca^{2+}]_i$, which in turn is responsible for the stimulation of neurotransmitter release.

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