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BRADYKININ-EVOKED RELEASE OF [^{3}H]NORADRENALINE FROM THE HUMAN NEUROBLASTOMA SH-SY5Y

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Abstract—Bradykinin (BK) evoked [^{3}H]noradrenaline ([^{3}H]NA) release from the human neuroblastoma SH-SY5Y and this was enhanced by pre-treatment with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) for 8 min. This effect of BK was inhibited by 500 μM [D-Phe^7]BK and 100 μM [Thi^{5,8},D-Phe^7]BK but not by 500 μM [Des-Arg^9,Leu^8]BK. The BK (B_1)-agonist [Des-Arg^9]BK did not evoke [^{3}H]NA release. This suggested that SH-SY5Y expressed BK (B_2)-receptors coupled to the release of [^{3}H]NA. BK acting at B_2-receptors, also elevated intracellular calcium and depolarized SH-SY5Y cells. Although pre-treatment of SH-SY5Y cells with TPA enhanced BK-evoked [^{3}H]NA release, the elevation of intracellular calcium [Ca^{2+}]; was decreased by about 50%. BK-evoked release of [^{3}H]NA in cells not pre-treated with phorbol ester was only 23% dependent on extracellular calcium. In comparison, following phorbol ester treatment approximately 40% of [^{3}H]NA release was dependent on extracellular calcium. Nifedipine (5 μM), $CoCl_2$ (1 mM) and $NiCl_2$ (1 mM) inhibited NA release in SH-SY5Y cells pre-treated with TPA by 16.0, 47 and 44%, respectively. The results of this study showed that BK, acting at B_2-receptors, activated [^{3}H]NA release in SH-SY5Y. Part of this effect appeared to be due to activation of L-type calcium channels but the majority of BK-evoked [^{3}H]NA release in SH-SY5Y cells appeared to depend on $[Ca^{2+}]_i$.

Key words: bradykinin; noradrenaline release; human neuroblastoma SH-SY5Y

Previous studies of this group [1, 2] showed that cultures of the human neuroblastoma SH-SY5Y, which had not been exposed to TPA§ for several days, expressed K^+- and nicotinic receptor-evoked release of [^{3}H]NA, which was dependent on extracellular calcium. In addition, this group has recently reported that depolarization-evoked release of [^{3}H]NA (stimulated by elevated K^+ [3] or activation of nicotinic receptors [2]) is coupled to L-type Ca^{2+} channels. Thus, cultures of SH-SY5Y can express an important property of mature sympathetic ganglionic neurones without exposure to TPA or other differentiation stimuli, such as retinoic acid or nerve growth factor. These observations are in contrast to previous work which found that SH-SY5Y cells expressed only properties of mature sympathetic ganglionic neurones following growth in the presence of TPA. Pahlman *et al.* [4] found that growth of SH-SY5Y cells for several days in the presence of 16 nM TPA induced neurosecretory granule formation, extension of neurites, elevation of NA and depolarization of the plasma membrane by increases in extracellular K^+ or by veratridine.

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§ Abbreviations: BK, bradykinin; NA, noradrenaline; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; $[Ca^{2+}]_i$, intracellular calcium ion concentration; [^{3}H]NA, [^{3}H]noradrenaline; IP_3, inositol 1,4,5-triphosphate; HBS, HEPES-buffered saline; ACE, angiotensin-converting enzyme; PLC, phospholipase C; PIP_2, phosphatidylinositol bisphosphate.

In addition to depolarization-evoked release of [^{3}H]NA, this group found that activation of muscarinic M_3 receptors evoked [^{3}H]NA release in SH-SY5Y [1]. In this case release was only partially dependent on extracellular calcium and was not coupled to activation of L-type Ca^{2+} channels [3]. Previous work has shown that muscarinic M_3-receptor subtypes in SH-SY5Y are coupled to phosholipase C and IP_3 induced changes in $[Ca^{2+}]_i$ [5, 6].

BK is a nonapeptide, formed by the cleavage of kininogens, which plays an important role in physiological processes associated with pain transmission, regulation of blood pressure and inflammation [7]. In addition, BK has been found to stimulate the transmission of nociceptive information from the periphery into the central nervous system [8]. BK has been reported to activate phospholipase C with associated formation of IP_3 and rises in $[Ca^{2+}]_i$ in neuronal cell lines [9–11]. More recently it has been found that activation of BK (B_2)-receptors evoked catecholamine release in chromaffin [12] and PC12[13] cells.

This paper reports that activation of BK (B_2)-receptors in SH-SY5Y cells resulted in: (1) an elevation of $[Ca^{2+}]_i$; (2) depolarization of the plasma membrane; (3) stimulation of [^{3}H]NA release.

MATERIALS AND METHODS

Materials. The human adrenergic clone SH-SY5Y

was kindly provided by Dr June L. Biedler of the Sloan-Kettering Institute for Cancer Research (Rye, NY, U.S.A.). Eagle's minimal essential medium, non-essential amino acids, Ham's F12 nutrient mixture, fetal calf serum and PBS were supplied by GIBCO (Paisley, U.K.). Amphertercin B, BK, [Des-Arg^9,Leu^8]-BK, [D-Phe^7]-BK [Thi^{5,8},D-Phe^7]-BK fura2/AM, pargyline and TPA were obtained from Sigma (Poole, U.K.).

Tissue culture. SH-SY5Y cells were cultured in a 1:1 mixture of Ham's F12 and Eagle's minimal essential medium containing non-essential amino acids, supplemented with 10% fetal calf serum in a 98% air–2% CO_2 humidified incubator at 37°. The cell monolayers were harvested, when confluent, by incubation at 37° for 10 min with PBS (without Ca^{2+} or Mg^{2+}) in the absence of trypsin. The procedure used was modified from previous reports [1, 2] to exclude trypsin in order to select against the epitheloid SH-EP cells which can occur in cultures of SH-SY5Y [14, 15]. The cell suspension was triturated by three passes through a 0.8 mm gauge needle and the cell number estimated using a Coulter Counter. Release of [^{3}H]NA was measured from cells subcultured in 24-well plates at a seeding density of 10^5 cells/mL. Measurements of $[Ca^{2+}]_i$ and patch-clamp studies were carried out on cells plated onto glass coverslips in 35 mm Petri dishes at the same seeding density.

Measurement of [^{3}H]*NA release.* The release of [^{3}H]NA was measured after 4–5 days in culture using the method described previously [1]. Briefly, culture medium was removed from SH-SY5Y cells in 24-well plates, the cell layer was rinsed twice with PBS containing Ca^{2+} and replaced with 0.4 mL of HBS (135 mM NaCl, 5 mM KCl, 0.6 mM $MgCl_2$, 2.5 mM $CaCl_2$, 10 mM HEPES, 6 mM glucose, 0.2 mM pargyline and 0.2 mM ascorbic acid, pH 7.4) containing 40 nM [^{3}H]NA. After incubation at 37° for 1 hr the unaccumulated [^{3}H]NA was removed by washing the monolayer consecutively with four aliquots (0.4 mL) of HBS each for 10 min. Where indicated in the relevant figures, cell layers were exposed to TPA (100 nM) for 8 min before initiating release by exposing the cell layers to K^+, carbachol or BK for 4 min. In solutions containing elevated K^+, the Na^+ concentration was decreased by a corresponding amount in order to maintain osmolarity. Unreleased [^{3}H]NA in the cell layer was extracted with 0.4 M perchloric acid. The amount of [^{3}H]NA released was expressed as a percentage of the total amount of radioactivity in the cell layer at the beginning of the incubation period.

Measurement of $[Ca^{2+}]_i$. Medium was removed from SH-SY5Y cells attached to glass coverslips and the cell layers were rinsed with 2 mL HBS. Cell layers were incubated with HBS containing 10 μM fura2/AM at room temperature for at least 1 hr. The cell layer was rinsed with 2 mL HBS and the glass coverslip placed in a thermostatted holder on the stage of an inverted Nikon microscope. Changes in $[Ca^{2+}]_i$ were measured using a Joyce Loebel PhoCal apparatus. The cell monolayer was perfused with HBS and $[Ca^{2+}]_i$ in a group of cells (5–6) was measured from the fluoresence emitted at 510 nm due to alternating excitation at 340 and 380 nm using a rotating filter wheel. The ratio, R, of the fluoresence at these wavelengths was converted to $[Ca^{2+}]_i$ using the equation $[Ca^{2+}]_i = K_d[(R - R_{min})/(R_{max} - R)]$ [16], where K_d of fura2/AM = 224 nM [17]. R_{max} (2.47) was the fluoresence ratio observed when the dye was saturated with Ca^{2+}; this was obtained by exposing cell layers to increasing concentrations of ionomycin (up to 5 μM) and R_{min} (0.35) represents the fluoresence ratio for free dye. Changes in $[Ca^{2+}]_i$ were recorded continuously following perfusion with carbachol or BK.

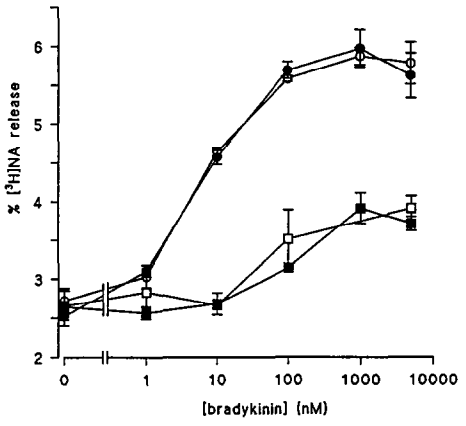

Fig. 1. Effect of TPA on BK-evoked release of [^{3}H]NA. Release was evoked by increasing concentrations of BK in the absence of TPA (■,□) or from cell layers pre-treated with 100 nM TPA for 8 min before the addition of BK (●,○). (□,○) represents release carried out in the presence of 10 μM MK 422 and 50 μM diprotin A. Results are expressed as percentage release and are means ± SEM (bars) of four experiments.

Recordings of membrane potential. Fragments of coverslip with attached cells were transferred to a recording chamber (80 μL) and continuously perfused (flow rate approximately 1 mL/min) with a solution of composition: NaCl 135 mM, KCl 5 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 2.5 mM, HEPES 10 mM, glucose 10 mM (pH 7.4, 21–24°). Membrane potential was recorded in individual cells using either the whole-cell or perforated-patch configuration of the patch-clamp technique under current-clamp [18]. Patch electrodes contained: NaCl 10 mM, KCl 117 mM, $MgSO_4$ 4.09 mM, $CaCl_2$ 0.356 mM, EGTA 10 mM, HEPES 10 mM, ATP 2 mM (pH 7.2). For perforated patch recordings ATP was omitted from the solution and amphotercin B was included (final concentration 240 μg/mL, from stock solution in DMSO of 60 mg/mL) to perforate the area of membrane under the patch electrode and gain electrical access to the cell interior without dialysis [19]. Records were filtered at 2 kHz and stored on computer on-line for subsequent analysis using VCAN software (kindly provided by Dr J. Dempster, Strathclyde University, U.K.).

RESULTS

Effect of BK on [^{3}H]*NA release*

BK evoked [^{3}H]NA release from SH-SY5Y cells

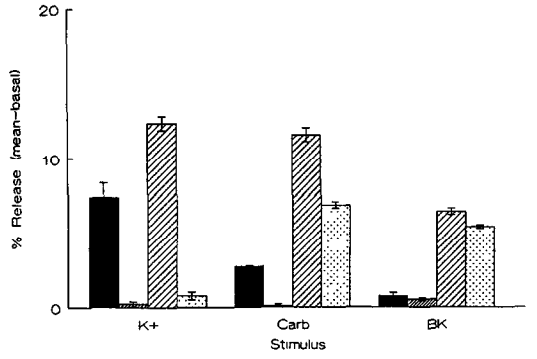

Fig. 2. Effect of Ca^{2+} on [^{3}H]NA release. Release was evoked by 100 mM (K^+), 3 μM BK or 1 mM carbachol (Carb) from cell layers either pre-treated with TPA (100 nM for 8 min; ▨, ▩) or in the absence of TPA (■, ▩). Cell layers were incubated in HBS (■, ▨) or in HBS in which 2.5 mM $CaCl_2$ was replaced with 100 μM EGTA (▩, ▩) for 4 min before evoking release. Results are expressed as percentage release from which release in HBS (basal) has been subtracted and are means ± SEM (bars) of four experiments.

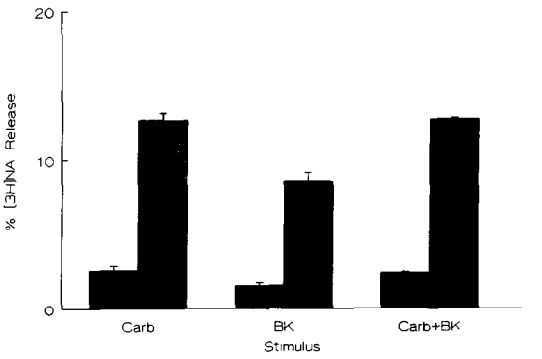

Fig. 3. Effect of co-addition of BK and carbachol on [^{3}H]NA release. Release was evoked by 1 mM carbachol (Carb), 3 μM BK or both (Carb + BK) from cell layers either pre-treated with TPA (100 nM for 8 min; ■) or in the absence of TPA (■). Results are expressed as percentage release and are means ± SEM (bars) of four experiments.

over the concentration range of 1–5000 nM with maximal stimulation of 1–2% above basal by 1000 nM (Fig. 1; EC_{50} approximately 100 nM). Pre-treatment of cell layers with 100 nM TPA for 8 min before the addition of BK enhanced [^{3}H]NA release at all concentrations to a maximum of between 4–6% above basal release, with a decrease in the EC_{50} value to approximately 10 nM (Fig. 1). Inclusion of the angiotensin converting enzyme inhibitor, MK 422 (10 μM) and the inhibitor of dipeptidyl peptidase IV (Diprotin A; 50 μM) did not effect the dose–response curve for BK either in the presence or absence of TPA (Fig. 1). This suggested that peptidase activity was not sufficiently high to alter the concentration of BK during the assay. Thus peptidase inhibitors were not routinely included in the assays. Furthermore, no increase in [^{3}H]NA release was observed using the BK B_1-specific agonist [Des-Arg^9]BK (data not shown). Exposure of SH-SY5Y cell layers (which had not been pre-treated with TPA) to Ca^{2+}-free HBS for 2 min before evoking release completely inhibited K^+ and carbachol-evoked release of [^{3}H]-NA. Under these conditions however, BK-evoked [^{3}H]NA release was only reduced by 23% (Fig. 2). No K^+-evoked release was observed under these conditions (Fig. 2). When the cell layers were pre-treated with 100 nM TPA for 8 min, approximately 80% of BK- and 60% of carbachol-evoked [^{3}H]NA release was still observed following exposure of the cell layers to calcium-free HBS for 2 min (Fig. 2). If cell layers, pre-treated with TPA, were exposed to Ca^{2+}-free HBS for 4 min then 60% of BK- and 40% of carbachol-evoked release remained (data not shown). Furthermore, exposure of cell layers pre-treated with TPA to Ca^{2+}-free HBS for up to 8 min resulted in no further inhibition of BK- evoked release of [^{3}H]NA. Coaddition of 1 mM carbachol and 3 μM BK did not increase [^{3}H]NA release above that observed with 1 mM carbachol alone (Fig. 3).

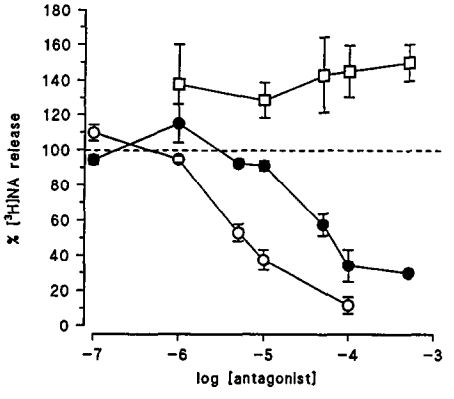

Fig. 4. Effect of antagonists on BK-evoked release. Release was effected by 30 nM BK in the presence of 100 nM TPA and increasing concentrations of [Thi^{5,8}, D-Phe^7]BK (○), [D-Phe^7]BK (●) or [Des-Arg^9,Leu^8]BK (□). Results are expressed as a percentage of release evoked by 30 nM BK (which represents 100%) and are means ± SEM (bars) of three to five experiments.

Figure 4 shows that BK-evoked release of [^{3}H]NA was 90% inhibited by 100 μM [Thi^{5,8}, D-Phe^7]BK (IC_{50} 6 μM) and 80% inhibited by 500 μM [D-Phe^7]BK (IC_{50} 66 μM). Using the equation $K_i = I_{50}/(1 + s/K_a)$ [20], K_i values of 2.4 and 18.9 μM, respectively, were calculated with s = 30 nM for [Thi^{5,8},D-Phe^7]BK and 50 nM for [D-Phe^7]BK, and K_a (the concentration of BK giving half-maximal release of [^{3}H]NA) = 10 nM. In contrast, no inhibition of [^{3}H]NA release was observed with 500 μM [Des-Arg^9,Leu^8]BK (Fig. 4).

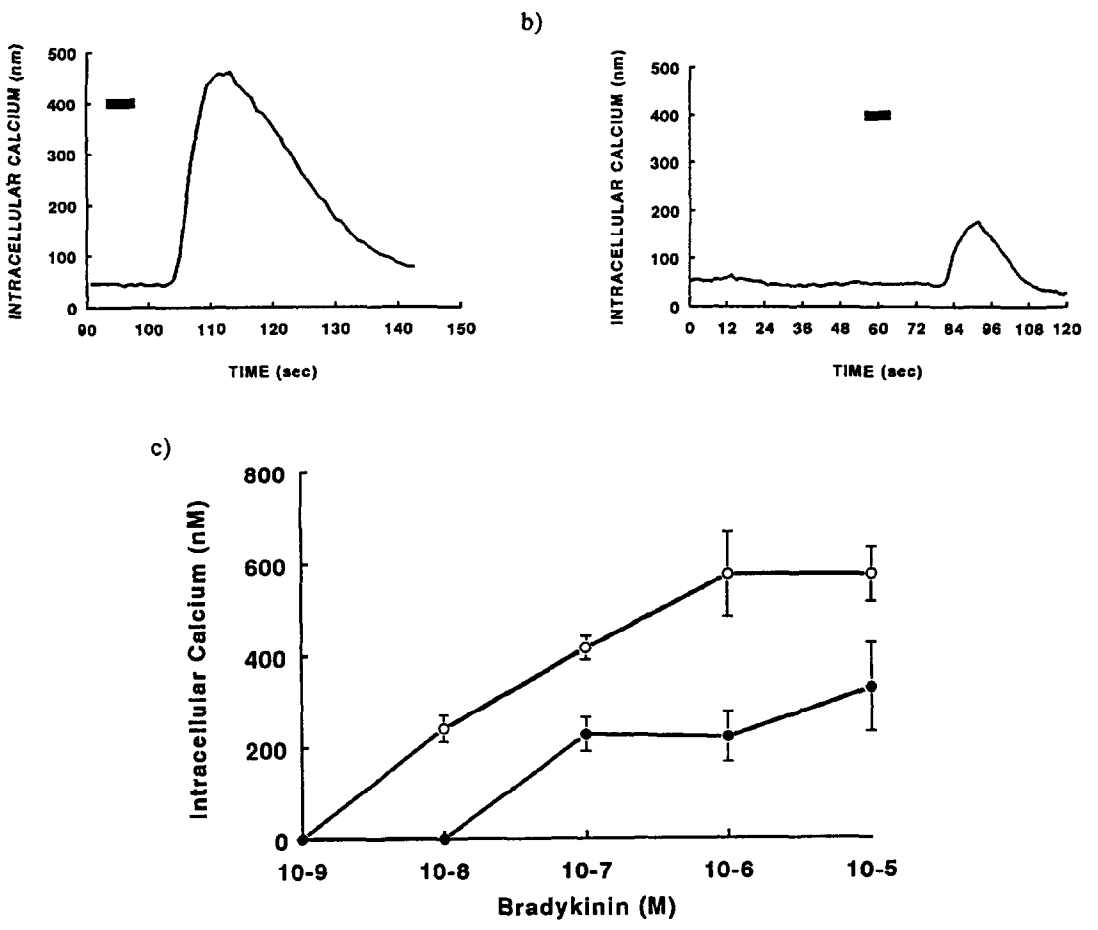

Fig. 5. BK-induced changes in $[Ca^{2+}]_i$. SH-SY5Y cells were grown on glass coverslips and loaded with fura2/AM. Changes in $[Ca^{2+}]_i$ were induced by perfusing the cell layer with BK (1 μM, horizontal line) for cells in the absence of TPA (a) or following pre-treatment with 100 nM TPA for 8 min (b). The effect of increasing concentrations of BK on $[Ca^{2+}]_i$ (maximal nanomolar concentrations corrected for basal Ca^{2+} levels, mean ± SEM, N = 3–5) is shown in (c) either for cell layers pre-treated with TPA (●) or untreated cells (○).

Instead, a slight (approximately 50%) enhancement of release was observed (Fig. 4).

Effect of BK on $[Ca^{2+}]_i$

BK (1 μM) increased $[Ca^{2+}]_i$ from basal values of 39.1 ± 4.2 to 411 ± 62.8 nM (mean ± SEM, N = 8; Fig. 5a). Pre-treatment of the cell layers with 100 nM TPA for 10 min decreased the elevation of $[Ca^{2+}]_i$ by 1 μM BK by 30% to 293.8 ± 68.8 nM (Fig. 5b). BK increased $[Ca^{2+}]_i$ in a concentration-dependent manner with maximal stimulation between 1 and 10 μM (Fig. 5c). In contrast, application of 10 μM [Des-Arg^9]BK to the cell layer did not result in elevation of $[Ca^{2+}]_i$. Pre-treatment of SH-SY5Y cell layers with 100 nM TPA decreased the change in $[Ca^{2+}]_i$ by approximately 50% and shifted the dose–response curve to higher concentrations. The elevation in $[Ca^{2+}]_i$ observed with 500 nM BK was progressively inhibited by increasing concentrations of [D-Phe^7]BK to 80% with 500 μM [D-Phe^7]BK. In contrast, no inhibition of BK induced elevation of

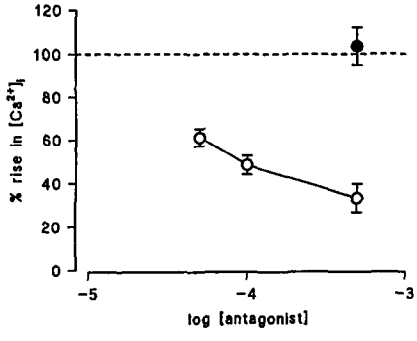

Fig. 6. Effect of antagonists on BK-induced changes in $[Ca^{2+}]_i$. SH-SY5Y cells were grown on glass coverslips in the absence of TPA. Changes in $[Ca^{2+}]_i$ were measured by perfusing the cell layer with BK (500 nM) in the presence of either [Des-Arg^9,Leu^8]BK (●) or [D-Phe^7]BK (○). Results are expressed as a percentage of the increase in $[Ca^{2+}]_i$ caused by BK (500 nM) in the absence of antagonist (100%) and are means ± SEM (bars), N = 4.

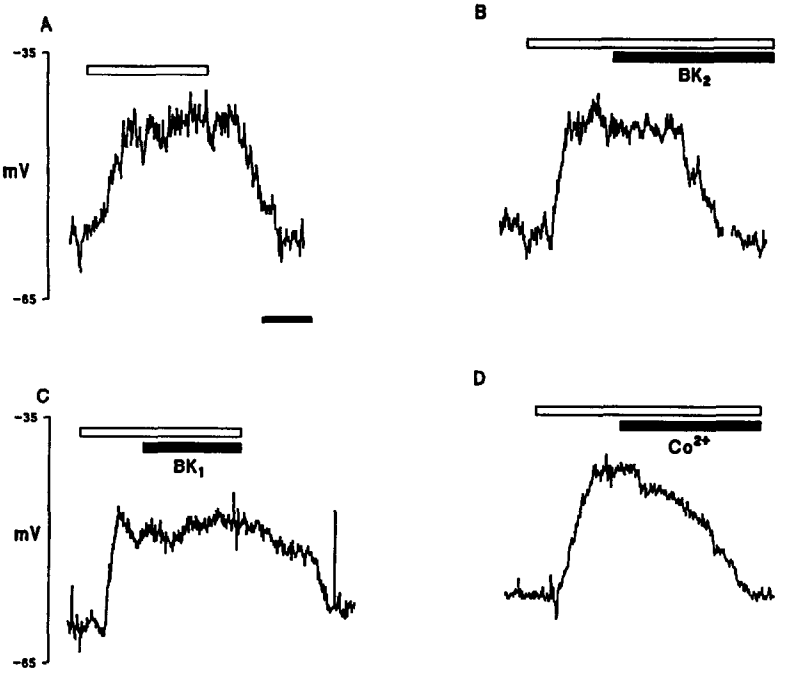

Fig. 7. Representative examples of whole-cell patch recordings of membrane potential in single SH-SY5Y cells. In all traces the period of BK exposure is indicated by the open bar. (A) Effects of bath application of 1 μM BK for 40 sec. Depolarization was sustained for 10–15 sec after removal of BK before decaying to resting values. (B) Bath application of 1 mM [D-Phe^7]-BK (BK_2) 40 sec after application of BK reversed membrane depolarization. (C) Bath application of 1 mM [Des-Arg^9,Leu^8]-BK (BK_1) for 60 sec did not affect BK-induced depolarization. (D) Bath application of 1 mM $CoCl_2$ reversed the membrane potential. Time bar [below trace (A)] represents 60 sec for (A) and (C), and 30 sec for (B) and (D).

$[Ca^{2+}]_i$ was observed with 500 μM [Des-Arg^9,Leu^8]BK (Fig. 6).

Effect of nickel, cobalt and nifedipine on NA release

In this groups previous patch-clamp studies it was shown that 5 μM nifedipine inhibited calcium currents maximally by about 35% [21]. It was also shown that 5 μM nifedipine completely inhibited dimethylphenylpiperazinium iodide (a nicotinic receptor agonist)-evoked [^{3}H]NA release in SH-SY5Y [2]. In contrast 5 μM nifedipine inhibited only BK-evoked release by 16.0 ± 1.5% (N = 4) in cells pre-treated with TPA. Similarly, 1 mM $NiCl_2$ and 1 mM $CoCl_2$ inhibited BK-evoked release of [^{3}H]-NA by 44.2 ± 6.6 (N = 9) and 46.9 ± 5.2% (N = 11), respectively, in SH-SY5Y cells pre-treated for 8 min with TPA. The stimulation of [^{3}H]NA release by BK in the absence of TPA-pre-treatment was too small to accurately measure the effects of calcium-channel antagonists on release.

Effects of BK on membrane depolarization

Bath application of BK (1 μM) always evoked a depolarization which recovered when BK was removed by washing (Fig. 7A). BK-induced depolarizations were sustained during applications of between 30 and 180 sec. In a minority of cells (15%) depolarization was preceded by a small, transient hyperpolarization (e.g. Fig. 7D). Pre-treatment with 100 nM TPA for 10 min at 37° did not change the degree of depolarization caused by BK (Table 1). Similar degrees of depolarization were also observed when the membrane potential was recorded using the perforated-patch technique (see Materials and Methods), indicating that dialysis of cells with the patch pipette solution (which contained 10 mM EGTA) did not alter cell responses to BK (Table 1) in either TPA-treated or untreated cells.

Bath application of the B_2-receptor antagonist [D-Phe^7]BK (1 mM) reversed the depolarizing effects of 1 μM BK (Fig. 7B, representative of eight cells). In contrast, the BK_1-receptor antagonist [Des-Arg^9,Leu^8]BK (1 mM) was without effect on depolarization evoked by 1 μM BK (Fig. 7C, representative of six cells). Application of the B_1-agonist [Des-Arg^9,Leu^8]BK (1 μM) failed to alter the membrane potential in the five cells examined (data not shown).

Depolarization caused by BK was inhibited by 1 mM $CoCl_2$ (Fig. 7D, representative of eight cells) and also by 1 mM $NiCl_2$ (N = 9; data not shown). However, nifedipine (5 μM) was without effect on membrane depolarization caused by 1 μM BK in any of the seven cells tested (data not shown).

DISCUSSION

This study demonstrated that BK evoked release

Table 1. Effects of bradykinin on membrane potential in SH-SY5Y cells

Recording conditions	Change in membrane potential (mV)	No. of cells tested
Whole cell + TPA	22.3 ± 2.5	7
Perforated patch + TPA	23.2 ± 3.2	7
Whole cell − TPA	20.0 ± 1.4	12
Perforated patch − TPA	21.7 ± 3.0	6

Mean depolarizing changes in membrane potential (±SEM) recorded from SH-SY5Y cells in response to bath application of 1 μM BK. Recordings were made in either the whole-cell or perforated-patch configuration of the patch clamp technique (see Materials and Methods). Resting membrane potentials before application of BK were always in the range −58 to −72 mV.

of [^{3}H]NA from cultures of the human neuroblastoma SH-SY5Y. Only a small amount of release (1–2% above basal) was observed in the absence of TPA. However, pre-treatment of cells with 100 nM TPA for 8 min increased BK- evoked [^{3}H]NA release by approximately 2–3-fold compared with release in the absence of TPA. This suggested that BK- evoked release was enhanced by activation of protein kinase C, as has previously been reported in SH-SY5Y for potassium- and muscarinic receptor M_3-evoked [22], and nicotinic receptor-evoked, release of [^{3}H]NA [2]. It also appeared that pre-treatment with TPA decreased EC_{50} for BK from approximately 100 to 10 nM (Fig. 1). This latter value was at least an order of magnitude greater than EC_{50} for BK in chromaffin cells [12] but agreed very closely with the value of 30 nM reported for PC12 cells [13]. The inhibition of BK-evoked release by the B_2-selective antagonists [23] [D-Phe^7]BK and [Thi^{5,8},D-Phe^7]BK, but not by the B_1-selective antagonist [Des-Arg^9,Leu^8]BK suggested that the B_2-subtype rather than the B_1-subtype of the BK-receptor was coupled to [^{3}H]NA release in SH-SY5Y. This conclusion was strengthened by the finding that the B_1-selective agonist [Des-Arg^9]BK did not evoke [^{3}H]NA release even in the presence of TPA. These observations are in keeping with the recent reports that B_2-receptors regulate NA release from PC12 cells [13] and from chromaffin cells [12].

This group's previous studies [24] showed that SH-SY5Y cells contained little peptidase activity. Thus, endopeptidase-24.11 could not be detected, but low levels of ACE and dipeptidyl peptidase IV, both able to degrade BK, were present. The observation that inclusion of an ACE inhibitor (MK 422) and an inhibitor of dipeptidyl peptidase (diprotin A) did not alter the concentration–response curve for BK suggested that little degredation of BK occurred during these assays.

The observation that pre-treatment with TPA for 8 min enhanced BK- evoked release of [^{3}H]NA was in agreement with this groups previous reports on the effect of TPA on depolarization- and muscarinic M_3-evoked release in SH-SY5Y [22, 2]. Of particular interest was the observation that approximately 80% of BK-evoked release and 60% of carbachol-evoked release in SH-SY5Y cells pre-treated with TPA occurred in the absence of extracellular calcium. This was in contrast to K^+-evoked release of [^{3}H]-NA which was inhibited by over 95% under the same conditions. These findings are in agreement with a recent report that IP_3 induces release of acetylcholine from NG108-15 cells by increases in $[Ca^{2+}]_i$ [25]. BK- evoked release in the presence of TPA was inhibited by only 16% by the L-type calcium channel antagonist nifedipine and 44.2% by Ni^{2+} and 46.9% by Co^{2+}, providing further evidence that the BK- evoked release was only partially dependent on extracellular calcium under these conditions. The percentage inhibition found in this study for nifedipine on BK-evoked [^{3}H]NA release agreed with the value of 15.6% reported previously [3] for the inhibition of carbachol-evoked release of NA by nifedipine. In contrast the percentage inhibitions for $NiCl_2$ and $CoCl_2$ on BK-evoked [^{3}H]-NA release were greater than the values of 13 and 34%, respectively, reported previously [3] for the inhibition of carbachol-evoked release of NA by these Ca^{2+} channel antagonists. Of particular interest was the observation that 5 μM nifedipine, which was previously found to inhibit Ca^{2+} currents in SH-SY5Y maximally by 35% [21], only slightly inhibited BK- evoked release in cells pre-treated with TPA and did not inhibit BK-induced membrane depolarization. Thus, although it was possible that the small amount of Ca^{2+}-dependent release of [^{3}H]-NA observed in the absence of TPA was a consequence of the depolarization of SH-SY5Y cells by BK it was unlikely that L-type Ca^{2+} channels were of importance. Nevertheless, the presence of BK-regulated, Ca^{2+} permeable channels (as opposed to L-type voltage gated Ca^{2+} channels) remains a distinct possibility. In contrast, the component of BK-evoked release dependent on activation of PKC occurred by a mechanism other than activation of Ca^{2+} channels, as it appeared to be independent of external calcium.

The situation in SH-SY5Y appeared to differ from that in chromaffin [12] and PC12 [13] cells in which over 80% of BK-evoked release is dependent on extracellular Ca^{2+}. Furthermore, Ca^{2+}-dependent BK-evoked release in chromaffin cells and PC12 cells was not inhibited by dihydropyridines, which suggested that calcium entry evoked by BK in these cell cultures did not occur via L-type Ca^{2+} channels. However, these studies with chromaffin and PC12

cells were carried out on cells which had not been pre-treated with TPA, so the effect of activation of PKC on BK-evoked catecholamine release is not known in these cell lines. It is of interest that 1 mM $CoCl_2$ and $NiCl_2$ inhibited BK-evoked release of [^{3}H]NA from PC12 cells by 68 and 50%, respectively [13], whereas neither nifedipine (10 μM) nor w-conotoxin (10 nM) caused any inhibition. The authors conclude that BK-evoked release in PC12 cells is not coupled to L- or N-type Ca^{2+} channels but that these cells expressed a BK-activated Ca^{2+} channel which coupled to [^{3}H]NA release. Further support for this suggestion was provided by the observation that Sr^{2+} could replace Ca^{2+} in supporting NA release from PC12 cells [13].

Elevation of $[Ca^{2+}]_i$ by BK was inhibited by [D-Phe^7]BK but not by [Des-Arg^9, Leu^8]BK, suggesting that the B_2-receptor in SH-SY5Y was coupled to PLC and hence liberated IP_3. This is in agreement with the coupling of B_2-receptors to PLC in chromaffin [26, 12] and PC12 [13] cells and is in agreement with reports that hydrolysis of PIP_2 by PLC is one of the earliest effects of activation of BK receptors [27–29].

The observation that BK- and carbachol-evoked release of [^{3}H]NA was not additive (Fig. 3) suggested a common mechanism of receptor-evoked release for these two agonists in SH-SY5Y. Two possibilities are either that these receptors were coupled to a common pool of vesicles or to a common intracellular pool of calcium. Further studies are in progress to distinguish between these hypotheses.

The inhibition of BK-evoked membrane depolarization by [D-Phe^7]BK but not by [Des-Arg^9, Leu^8]BK suggested that activation of B_2-receptors also depolarized cell membranes. Similar degrees of depolarization were seen using perforated-patch recordings and conventional whole-cell recordings, in which cells were dialysed by an "intracellular" solution containing a high concentration of EGTA (10 mM; Table 1). This suggested that depolarization did not occur as a result of BK raising $[Ca^{2+}]_i$. Indeed, BK-induced depolarization was likely to be a contributory factor in elevating $[Ca^{2+}]_i$, as is the case in PC12 cells where BK activates an inward (depolarizing) current which is partly carried by Ca^{2+} ions [31]. In contrast, in NG108-15 cells BK caused a transient hyperpolarization followed by prolonged depolarization. The hyperpolarization resulted from elevation of $[Ca^{2+}]_i$ (released from internal stores) and depolarization due to inhibition of M-currents [30]. In this study BK only occasionally caused transient hyperpolarizations in SH-SY5Y cells, although this may reflect differences due to recording techniques, since hyperpolarizations were much more readily observed in cells impaled with citrate-rich microelectrodes [30].

Although the mechanism of depolarization caused by BK in SH-SY5Y cells has not been investigated, it appeared more likely that depolarization occurred via the activation of an inward current as in PC12 cells [31] rather than by inhibition of an M-current [30]. Thus, although M-currents are present in SH-SY5Y cells (Reeve and Peers, unpublished observations) these currents are inactive at −60 mV [32] and BK depolarizes SH-SY5Y cells from resting potentials around this value. In addition, $CoCl_2$ and $NiCl_2$ inhibited both [^{3}H]NA release and also depolarization. This suggested that BK activated a calcium-entry pathway which was important in release, as described in PC12 cells [31]. This would account for the observation that both $CoCl_2$ and $NiCl_2$ inhibited release in TPA-treated SH-SY5Y cells to approximately the same degree as removal of external calcium. Depolarization may also have been sufficient to cause partial activation of L-type Ca^{2+} channels since [^{3}H]NA release was reduced slightly by nifedipine.

Depolarization occurred in both untreated cells and cells which were exposed to TPA for 10 min. $CoCl_2$ blocked depolarization in both cases, and inhibited BK-evoked release of [^{3}H]NA to the same extent as removal of external calcium in TPA-treated cells. It is suggested, therefore, that BK-evoked depolarization of SH-SY5Y cells may arise due to activation of a conductance which is carried, at least in part, by Ca^{2+} ions. Furthermore, BK-evoked [^{3}H]NA release was inhibited by approximately 20% by nifedipine. This suggested that BK-evoked depolarization may have been sufficient to cause some activation of the voltage-gated L-type Ca^{2+} channels known to be present in these cells [1, 2, 21]. This activation of L-type Ca^{2+} channels would be sufficient to account for the small amount of BK-evoked NA release, observed in the absence of TPA, which was dependent on extracellular Ca^{2+}.

In conclusion, this study shows that activation of BK (B_2)-receptors in SH-SY5Y cells resulted in: (1) increases in $[Ca^{2+}]_i$; (2) membrane depolarization; (3) stimulation of [^{3}H]NA release. The results presented are consistent with BK-evoked release of [^{3}H]NA occurring by two distinct mechanisms. The first is dependent on extracellular Ca^{2+} with Ca^{2+} entry possibly occuring as a consequence of the opening of Ca^{2+}-permeable channels. This in turn caused cell depolarization which led to partial activation of L-type Ca^{2+} channels. The second mechanism operated following activation of protein kinase C with TPA and appeared to be independent of external Ca^{2+}.

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