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Noradrenaline release from rat sympathetic neurones triggered by activation of B_2 bradykinin receptors

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1 The role of bradykinin receptors in the regulation of sympathetic transmitter release was investigated in primary cultures of neurones dissociated from superior cervical ganglia of neonatal rats. These cultures were loaded with [^{3}H]-noradrenaline and the outflow of radioactivity was determined under continuous superfusion.

2 Bradykinin (100 nmol l^{-1} applied for 10 min) caused a transient increase in tritium outflow that reached a peak within four minutes after the beginning of the application and then declined towards the baseline, despite the continuing presence of the peptide. ATP (100 μmol l^{-1}) and nicotine (10 μmol l^{-1}) caused elevations in ^{3}H outflow with similar kinetics, whereas outflow remained elevated during a 10 min period of electrical field stimulation (0.5 ms, 50 mA, 50 V cm^{-1}, 1.0 Hz).

3 When bradykinin was applied for periods of 2 min, the evoked ^{3}H overflow was half-maximal at 12 nmol l^{-1} and reached a maximum of 2.3% of cellular radioactivity. The preferential B_1 receptor agonist des-Arg^9-bradykinin failed to alter ^{3}H outflow. The B_2 receptor antagonists, [D-Phe^7]-bradykinin (1 μmol l^{-1}) and Hoe 140 (10 nmol l^{-1}), *per se* did not alter ^{3}H outflow, but shifted the concentration-response curve for bradykinin-evoked ^{3}H overflow to the right by a factor of 7.9 and 4.3, respectively.

4 Bradykinin-induced overflow was abolished in the absence of extracellular Ca^{2+} and in the presence of either 1 μmol l^{-1} tetrodotoxin or 300 μmol l^{-1} Cd^{2+}, as was electrically-induced overflow. Activation of α_2-adrenoceptors by 1 μmol l^{-1} UK 14,304 reduced both bradykinin- and electrically-triggered overflow. The Ca^{2+}-ATPase inhibitor thapsigargin (0.3 μmol l^{-1}) failed to alter either type of stimulated overflow. Caffeine (10 mmol l^{-1}) enhanced bradykinin-induced overflow, but reduced overflow triggered by electrical field stimulation.

5 Inclusion of Ba^{2+} (0.1 to 1 mmol l^{-1}) in the superfusion medium enhanced electrically induced overflow by approximately 100% and potentiated bradykinin-triggered overflow by almost 400%. Application of 1 mmol l^{-1} Ba^{2+} for periods of 2 min triggered ^{3}H overflow, and this overflow was abolished by 1μmol l^{-1} tetrodotoxin and enhanced by 10 mmol l^{-1} caffeine. In contrast, inclusion of tetraethylammonium (0.1 to 1 mmol l^{-1}) in the superfusion buffer caused similar increases of bradykinin- and electrically evoked ^{3}H overflow (by about 100%), and tetraethylammonium, when applied for 2 min, failed to alter ^{3}H outflow.

6 Treatment of cultures with 100 ng ml^{-1} pertussis toxin caused a significant increase in bradykinin-, but not in electrically-, evoked tritium overflow. Treatment with 100 ng ml^{-1} cholera toxin reduced both types of stimulated ^{3}H overflow.

7 These data reveal bradykinin as a potent stimulant of action potential-mediated and Ca^{2+}-dependent transmitter release from rat sympathetic neurones in primary cell culture. This neurosecretory effect of bradykinin involves activation of B_2-receptors, presumably linked to pertussis- and cholera toxin-insensitive G proteins, most likely members of the Gq family. Results obtained with inhibitors of muscarinic K^+ (K_M) channels, like caffeine and Ba^{2+}, indicate that the secretagogue action of bradykinin probably involves inhibition of these K^+ channels.

Keywords: B_2 bradykinin receptor; GTP binding proteins; sympathetic neurones; noradrenaline release; K_M channels

Introduction

Bradykinin, a nonapeptide formed from plasma protein precursors after tissue injury, is widely accepted as an important mediator of inflammation and pain (Farmer & Burch, 1992; Hall, 1992). Actions of bradykinin are mediated by at least two types of receptors, B_1 and B_2 bradykinin receptors (Farmer & Burch, 1992; Hall, 1992), and the existence of a third member of the family, a B_3 receptor, has also been suggested (e.g. Farmer & DeSiato, 1994). While B_1 and B_2 receptors can be characterized by the use of various agonists and antagonists, pharmacological tools to discriminate unequivocally between B_2 and B_3 receptors are lacking (see Farmer & Burch, 1992; Hall, 1992, for reviews). B_1 receptors are mainly restricted to the vasculature, whereas B_2 receptors have been detected in most types of tissues including the central and peripheral nervous system (e.g. Hall, 1992; Hall & Geppetti, 1995; Walker *et al*., 1995).

Peripheral sensory neurones are believed to represent the predominant mediators of bradykinin-dependent inflammatory pain (Dray & Perkins, 1993; Walker *et al*., 1995): the stimulation of nociceptive nerve terminals may cause the sensation of pain and bradykinin-induced release of neuropeptides from sensory nerve terminals may contribute to the inflammatory response (Geppetti, 1993). Nevertheless, there is compelling evidence to suggest that the sympathetic nervous system is also involved in some of the actions of bradykinin, including hyperalgesia (e.g. Levine *et al*., 1986) and plasma extravasation (Miao *et al*., 1996), phenomena which are abolished after sympathectomy.

The mechanisms by which sympathetic neurones might contribute to the aforementioned bradykinin-induced responses remained controversial. On the one hand, the nonapeptide has been found to increase depolarization-evoked transmitter release from sympathetic nerve terminals of rats *in vivo* and *in vitro* (e.g. Llona *et al*., 1991; Dominiak *et al*., 1992; Chulak *et al*., 1995), on the other hand, bradykinin has been shown to

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reduce electrically-induced noradrenaline release in rabbit pulmonary arteries and hearts (Starke *et al*., 1977). In a recent study, bradykinin was suggested to modulate transmitter release from sympathetic axon terminals in mouse vasa deferentia in a bimodal way, causing inhibition via B_1 and facilitation via B_2 receptors (Maas *et al*., 1995).

Neurones dissociated from rat superior cervical ganglia represent a frequently used model to investigate how neurotransmitter receptors control the function of postganglionic sympathetic neurones (see Boehm & Huck, 1997, for a review). The present experiments were initiated to investigate the modulatory action of bradykinin on stimulation-evoked [^{3}H]-noradrenaline release in primary cultures of such neurones. However, our results showed that bradykinin *per se* triggers Ca^{2+}-dependent transmitter release from sympathetic neurones *in vitro*, an effect presumably related to its excitatory action in whole ganglia demonstrated previously (Lewis & Reit, 1965; Trendelenburg, 1966).

Methods

Cell culture

Primary cultures of neurones dissociated from superior cervical ganglia of neonatal rats were prepared as previously described in more detail (Boehm, 1994). Briefly, the ganglia were dissected from 2 to 6 day old Sprague Dawley rat pups and placed in ice-cold Ca^{2+}-free Tyrode solution containing (mmol l^{-1}): NaCl 150, KCl 4, $MgCl_2$ 2, glucose 10 and HEPES 10; adjusted to pH 7.4 with NaOH. After removal of adhering connective tissue and blood vessels, the ganglia were cut into 3 to 4 pieces and incubated in collagenase (1.5 mg ml^{-1}, Sigma, # 9891) and dispase (3.0 mg ml^{-1}, Boehringer Mannheim, # 165859) in Tyrode solution for 20 min at 36°C. Subsequently, the ganglia were treated with trypsin (0.25% trypsin in Tyrode solution; Worthington, # 3703) for 15 min at 36°C, dissociated by trituration, and plated onto 5 mm discs (about 40,000 cells per disc) coated with rat tail collagen (Biomedical Technologies). The cells were kept in a humidified 5% CO_2 atmosphere at 36°C for 7 days before the superfusion experiments, and one half of the medium was exchanged after 3 to 4 days.

Measurement of [^{3}H]-noradrenaline release

[^{3}H]-noradrenaline uptake and superfusion were performed as described by Boehm (1994). Cultures were labelled with 0.05 μmol l^{-1} [^{3}H]-noradrenaline (specific activity 71.7 Ci $mmol^{-1}$) in culture medium supplemented with 1 mmol l^{-1} ascorbic acid at 36°C for 1 h. After labelling, culture discs were transferred to small chambers and superfused with a buffer containing (mmol l^{-1}); NaCl 120, KCl 6.0, $CaCl_2$ 2.0, $MgCl_2$ 2.0, glucose 20, HEPES 10, fumaric acid 0.5, Na-pyruvate 5.0 and ascorbic acid 0.57, adjusted to pH 7.4 with NaOH. Superfusion was performed at 25°C at a rate of about 1.0 ml min^{-1}. The collection of either 2 min (experiments shown in Figure 1) or 4 min superfusate fractions (all remaining experiments) was started after a 60 min washout period. To compare the effects of bradykinin on tritium outflow with those of other secretory stimuli, 100 nmol l^{-1} bradykinin, 10 μmol l^{-1} nicotine, or 100 μmol l^{-1} adenosine 5′-triphosphate (ATP) were present in the superfusion medium, or monophasic rectangular pulses (0.5 ms, 1.0 Hz, 50 mA, 50 V cm^{-1}) were applied, from minute 70 to 80 of superfusion (see Figure 1). In most of the other experiments, ^{3}H overflow was induced by addition of bradykinin and/or related agonists or antagonists to the medium from minutes 72 to 74 of superfusion. Subsequently, electrical field stimulation as detailed above was applied from minute 92 to 94. In a subset of experiments (Figure 4c), the cultures were exposed to 1 mmol l^{-1} $BaCl_2$ and to 1 mmol l^{-1} tetraethylammonium chloride (TEA) instead of bradykinin or electrical field stimulation. Radioactivity released in response to electrical field stimulation from rat sympathetic neurones after labelling with tritiated noradrenaline, and under conditions similar to those of the present study, has previously been shown to consist predominantly of the authentic transmitter and to contain only small amounts ($\leqslant$15%) of metabolites (Schwartz & Malik, 1993). Hence, the outflow of tritium measured in this study was assumed to reflect primarily the release of noradrenaline and not of metabolites.

Modulatory drugs (tetrodotoxin, $CdCl_2$, UK 14,304, thapsigargin, caffeine, TEA, $BaCl_2$) were added to, or extracellular Ca^{2+} was removed from, the medium after 50 min of superfusion (i.e. 10 min before the start of sample collection). The buffer then remained unchanged until the end of experiments. Finally, the radioactivity remaining in the cells was extracted by immersion of the discs in 1.2 ml 2% (v/v) perchloric acid followed by sonication. Radioactivity in extracts and collected fractions was determined by liquid scintillation counting (Packard Tri-Carb 2100 TR).

Calculations

The fractional rate of ^{3}H outflow was obtained by dividing the radioactivity of a (2 or) 4 min sample by (2 or) 4 times the total radioactivity of cultures at the beginning of the corresponding (2 or) 4 min collection period. Stimulation-evoked overflow was calculated as the difference between the total ^{3}H outflow during and after stimulation and the estimated basal outflow which was assumed to decline linearly from the sample preceding stimulation to the sample 8–12 min after the beginning of the stimulus. This difference was expressed as a percentage of the total radioactivity in the cultures at the beginning of the respective stimulation (S%). Effects of modulatory agents upon the overflow evoked by bradykinin and electrical fields, respectively, are expressed as percentage of the corresponding control values. Effects on basal ^{3}H outflow were determined by comparing the fractional rate of outflow between min 68 and 72 of superfusion in the absence with that in the presence of modulatory compounds.

All data are given as arithmetic means$\pm$s.e.mean; n = number of cell culture discs. Unless stated otherwise, differences between single data points were evaluated by unpaired Student's t test. Concentration-response curves were fitted to experimentally obtained data by the ALLFIT programme (DeLean *et al*., 1978), which determines differences between single concentration-response curves by simultaneous fitting with shared parameters and subsequent calculation of the F-statistic on the resulting 'extra sum of squares'.

Materials

(-)-[Ring-2,5,6-^{3}H]-noradrenaline was obtained from NEN (Dreieich, Germany); bradykinin, des-Arg^9-bradykinin, [D-Phe^7]-bradykinin, tetrodotoxin (TTX), pertussis toxin, cholera toxin, thapsigargin, caffeine, tetraethylammonium chloride (TEA) from Sigma (Diesenhofen, Germany); D-Arg-[Hyp^3, Thi^5, D-Tic^7, Oic^8]-bradykinin (Hoe 140) and 5-bromo-N-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine (UK 14,304) from Research Biochemicals Inc. (Natick, MA, U.S.A.).

Results

Time-course of bradykinin-evoked ^{3}H overflow and comparison with other secretory stimuli

After being loaded with [^{3}H]-noradrenaline and subsequent to a 60 min washout period, rat superior cervical ganglion neurones steadily released tritium into the superfusion buffer. The fractional outflow of radioactivity amounted to 0.0027$\pm$0.0001 min^{-1} during min 68 through 72 of superfusion which corresponded to 0.0508$\pm$0.0016 nCi min^{-1} (n = 181). Inclusion of 100 nmol l^{-1} bradykinin in the superfusion buffer for 10 min caused a marked increase in ^{3}H outflow within 2 minutes after the onset of application. Bradykinin-induced over-

flow peaked after 4 min and then declined towards the levels of basal outflow, despite the continuing presence of the peptide (Figure 1a). Previously, agonists at other neurotransmitter receptors, for instance at P2X-purinoceptors (Boehm, 1994) and at nicotinic acetylcholine receptors (e.g. Boehm & Huck, 1995), have been found to trigger noradrenaline release from rat sympathetic neurones. Figure 1 shows that the time course of bradykinin-evoked tritium overflow is similar to that induced by either 100 μmol l^{-1} ATP (Figure 1b) or 10 μmol l^{-1} nicotine (Figure 1d). In all these cases, agonist-evoked overflow declined during the presence of the secretory stimuli. In contrast, overflow triggered by 1.0 Hz electrical field stimulation (0.5 ms pulses, 50 mA, 50 V cm^{-1}) was fairly stable during a 10 min stimulation period (Figure 1c).

Pharmacological characterization of the receptor mediating the secretory action of bradykinin

When applied for periods of 2 min, secretagogue effects of bradykinin were half-maximal at 12.0 ± 4.5 nmol l^{-1} and reached a maximum of $2.34 + 0.16$% of cellular radioactivity (Figure 2b and c). Des-Arg^9-bradykinin (10 and 100 nmol l^{-1}), an agonist at bradykinin B_1 receptors (see Regoli *et al.*, 1990; Hall, 1992), failed to enhance ^{3}H outflow (Figure 2a). Likewise, [D-Phe^7]-bradykinin (1 μmol l^{-1}) and Hoe 140 (10 nmol l^{-1}), antagonists at B_2 bradykinin receptors (Regoli *et al.*, 1990; Rhaleb *et al.*, 1991; Hall, 1992), did not induce any alteration in ^{3}H outflow. Nevertheless, these two peptides caused rightward shifts of the concentration-response curve for bradykinin-evoked overflow (Figure 2b and c). In both cases, the apparent half maximal concentration of bradykinin was significantly increased ($P < 0.01$) and amounted to 95.1 ± 33.4 nmol l^{-1} in the presence of 1 μmol l^{-1} [D-Phe^7]-bradykinin and to 52.0 ± 19.3 nmol l^{-1} in the presence of 10 nmol l^{-1} Hoe 140, respectively.

Ca^{2+}-dependence, tetrodotoxin- and Cd^{2+}-sensitivity of bradykinin-evoked ^{3}H overflow

In the subsequent experiments, neuronal cell cultures were first stimulated by 100 nmol l^{-1} bradykinin from min 72 to 74 of superfusion and then by electrical field stimulation at 1.0 Hz from min 92 to 94. This experimental procedure was chosen to compare directly bradykinin-evoked with electrically-induced ^{3}H overflow (Figure 3a). When Ca^{2+} was omitted from the superfusion medium, neither bradykinin nor electrical field stimulation caused any alteration in ^{3}H outflow (Figure 3a).

Transmitter release from sympathetic neurones in cell culture elicited by electrical field stimulation involves the propagation of Na^+-dependent action potentials and subsequent

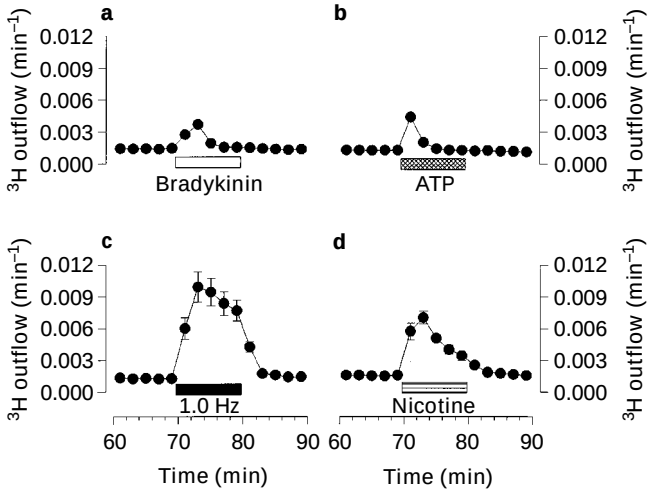

Figure 1 Tritium overflow induced by bradykinin, ATP, electrical field stimulation and nicotine in primary cultures of rat sympathetic neurones previously loaded with [^{3}H]-noradrenaline. After labelling, cultures were superfused and subjected to a 60 min washout period. Thereafter, 2 min fractions of superfusate were collected. From minutes 70 to 80, the superfusion buffer contained 100 nmol l^{-1} bradykinin (a), 100 μmol l^{-1} ATP (b), or 10 μmol l^{-1} nicotine (d). Alternatively, 0.5 ms electrical pulses (50 mA, 50 V cm^{-1}) were applied at a frequency of 1.0 Hz for the same period of time (c). The radioactivity retrieved in the fractions collected is depicted as fractional outflow (min^{-1}), $n = 5$ to 6.

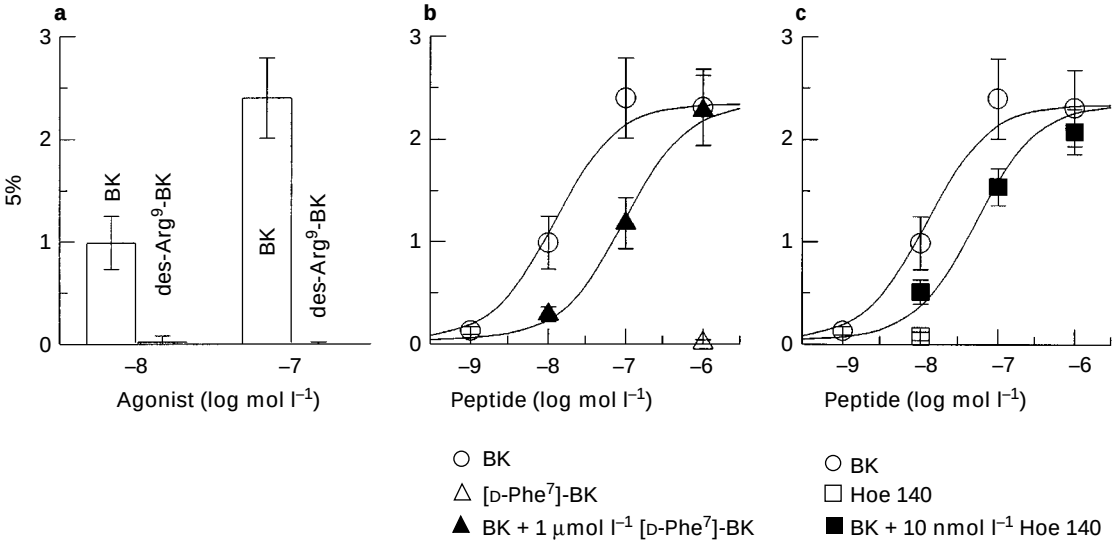

Figure 2 Characterization of the receptor mediating bradykinin-triggered tritium overflow from rat sympathetic neurones previously loaded with [^{3}H]-noradrenaline. After labelling, cultures were superfused and subjected to a 60 min washout period. Thereafter, 4 min fractions of superfusate were collected. From minutes 72 to 74, the superfusion buffer contained the indicated concentrations of bradykinin and/or bradykinin receptor agonists and antagonists, respectively. Stimulation-evoked overflow is presented as percentage of the radioactivity in the cultures at the beginning of stimulation (S%). (a) Comparison of the amounts of overflow induced by 10 or 100 nmol l^{-1} bradykinin (BK) and des-Arg^9-bradykinin, respectively, $n = 6$ to 9. (b) Shows bradykinin-induced overflow in the absence and presence of 1 μmol l^{-1} [D-Phe^7]-bradykinin and the effect of 1 μmol l^{-1} [D-Phe^7]-bradykinin alone; $n = 8$ to 9. (c) Shows bradykinin-induced overflow in the absence and presence of 10 nmol l^{-1} Hoe 140 and the effect of 10 nmol l^{-1} Hoe 140 alone; $n = 8$ to 9.

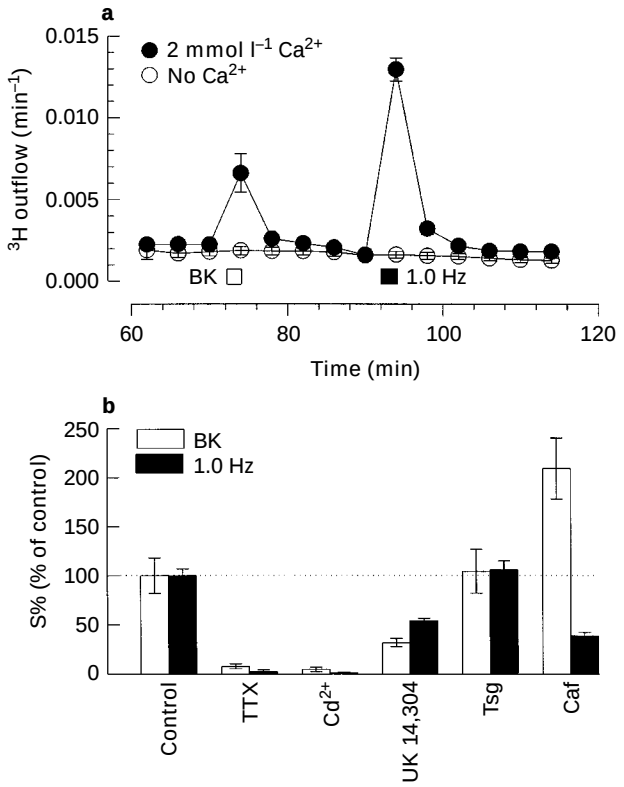

Figure 3 Characterization of bradykinin-evoked tritium overflow from rat sympathetic neurones previously loaded with [^{3}H]-noradrenaline by comparison with electrically-induced overflow. After labelling, cultures were superfused and subjected to a 60 min washout period. Thereafter, 4 min fractions of superfusate were collected. From minutes 72 to 74, the superfusion buffer contained 100 nmol l^{-1} bradykinin (BK), and from minutes 92 to 94, 120 monophasic rectangular pulses (0.5 ms; 50 mA; 50 V cm^{-1}) were applied at a frequency of 1.0 Hz. (a) The time course of fractional ^{3}H outflow (min^{-1}) in such experiments in the presence and absence of 2 mmol l^{-1} Ca^{2+}, respectively; $n=6$. (b) The amount (S%) of bradykinin- and electrically evoked overflow in the presence of 1 μmol l^{-1} tetrodotoxin (TTX), 300 μmol l^{-1} $CdCl_2$ (Cd^{2+}), 1 μmol l^{-1} UK 14,304, 0.3 μmol l^{-1} thapsigargin (Tsg) and 10 mmol l^{-1} caffeine (Caf), $n=6$ to 8. Results are shown as percentage of the respective stimulation-dependent overflow in the absence of these agents (% of control).

Ca^{2+} entry through voltage-activated Ca^{2+} channels (see Boehm & Huck, 1997, for a review). Accordingly, blockade of Na^+ channels by 1 μmol l^{-1} tetrodotoxin, or of Ca^{2+} channels by 300 μmol l^{-1} Cd^{2+}, abolished tritium overflow due to electrical field situation. These manipulations also prevented overflow triggered by 100 nmol l^{-1} bradykinin (Figure 3b), but failed to affect spontaneous ^{3}H outflow significantly (Table 1).

Modulation of bradykinin-evoked ^{3}H overflow via presynaptic α_2-autoreceptors

A hallmark of sympathetic transmitter release is its modulation via presynaptic α_2-autoreceptors (Starke, 1987) which can also be demonstrated in primary cultures of rat sympathetic neurones (Hill *et al*., 1993; Boehm & Huck, 1995). The selective α_2-adrenoceptor agonist UK14,304 (1 μmol l^{-1}) reduced both, bradykinin- and electrically evoked overflow (Figure 3b). Basal tritium outflow was not altered by 1 μmol l^{-1} UK 14,304 (Table 1).

Effects of thapsigargin and caffeine on bradykinin-evoked ^{3}H overflow

B_2 bradykinin receptors are known to mobilize intracellular Ca^{2+}, an effect that can be prevented by thapsigargin (Thastrup, 1990; Reiser *et al*., 1992). In addition, thapsigargin slightly increases intracellular Ca^{2+} in sympathetic neurones (Foucart *et al*., 1995). Neither bradykinin- nor electrically-evoked overflow was altered in the continuous presence of 0.3 μmol l^{-1} thapsigargin (Figure 3b).

Caffeine transiently increases intracellular Ca^{2+} and thereafter depletes Ca^{2+} stores of sympathetic neurones (Thayer *et al*., 1988). Inclusion of 10 mmol l^{-1} caffeine in the superfusion buffer increased bradykinin-evoked overflow by more than 100%, but reduced electrically-induced overflow by about 60% (Figure 3b). Spontaneous tritium outflow was not significantly altered by either thapsigargin or caffeine (Table 1).

Table 1 Modulation of spontaneous tritium outflow from rat sympathetic neurones previously labelled with [^{3}H]-noradrenaline by tetrodotoxin, $CdCl_2$ (Cd^{2+}), UK 14,304, thapsigargin, and caffeine, and by the removal of extracellular Ca^{2+}

Modulator	*Concentration* (mmol l^{-1})	*^{3}H outflow* (min^{-1})	n
Control	–	0.0024±0.0001	15
Ca^{2+}	0	0.0018±0.0002	6
Tetrodotoxin	0.001	0.0020±0.0003	6
Cd^{2+}	0.3	0.0018±0.0001	6
UK 14,304	0.001	0.0028±0.0004	6
Thapsigargin	0.0003	0.0031±0.0004	8
Caffeine	10	0.0021±0.0004	6

After labelling, cultures were superfused and subjected to a 60 min washout period. Thereafter, 4 min fractions of superfusate were collected. The agents indicated above were added to, or Ca^{2+} was ommited from, the superfusion buffer after 50 min of superfusion (i.e. 10 min before the start of sample collection). Thereafter, the buffer was not changed until the end of experiments, and spontaneous outflow was determined between min 68 and 72 of superfusion. Bonferroni's multiple comparison procedure did not reveal any significant difference between data obtained under control and any other of the above conditions, respectively.

Modulation of bradykinin-evoked ^{3}H overflow by K^+ channel blockers

Apart from raising intracellular Ca^{2+}, caffeine exerts modulatory effects on various ion channels of sympathetic neurones, including an inhibition of muscarinic K^+ (K_M) channels (Akaike & Sadoshima, 1989). Bradykinin also inhibits K_M channels (e.g. Jones *et al*., 1995). To reveal whether a blockade of K_M channels may indeed preferentially facilitate bradykinin-triggered ^{3}H overflow, the selective K_M channel blocking agent Ba^{2+} (0.1 to 1 mmol l^{-1}) was included in the superfusion buffer. Ba^{2+} enhanced electrically-induced overflow in a concentration-dependent manner, by up to 100%, and potentiated bradykinin-triggered overflow by up to 400% (Figure 4a). To reveal whether a blockade of K^+ channels other than K_M might also differentiate between bradykinin- and electrically-evoked tritium overflow, TEA (0.1 to 1 mmol l^{-1}), which blocks Ca^{2+}-activated K^+ channels (Marsh & Brown, 1991) but not K_M channels (e.g. Robbins *et al*., 1992), was used instead of Ba^{2+}. TEA increased both types of overflow to the same extent, by up to about 100% (Figure 4b). Neither the inclusion of Ba^{2+}, nor of TEA, in the superfusion buffer altered spontaneous ^{3}H outflow (not shown).

To corroborate that inhibition of K_M channels, but not of Ca^{2+}-dependent K^+ channels, may indeed be sufficient to elicit action potential-dependent tritium overflow, sympathetic neurones were exposed for periods of 2 min to 1 mmol l^{-1} Ba^{2+} and 1 mmol l^{-1} TEA instead of bradykinin and electrical filed stimulation. Ba^{2+}, but not TEA, caused small, but significant ($P<0.01$), elevations in ^{3}H outflow, and this overflow was abolished in the presence of 1 μmol l^{-1} tetrodotoxin (Figure 4c). As the inclusion of Ba^{2+} in the superfusion med-

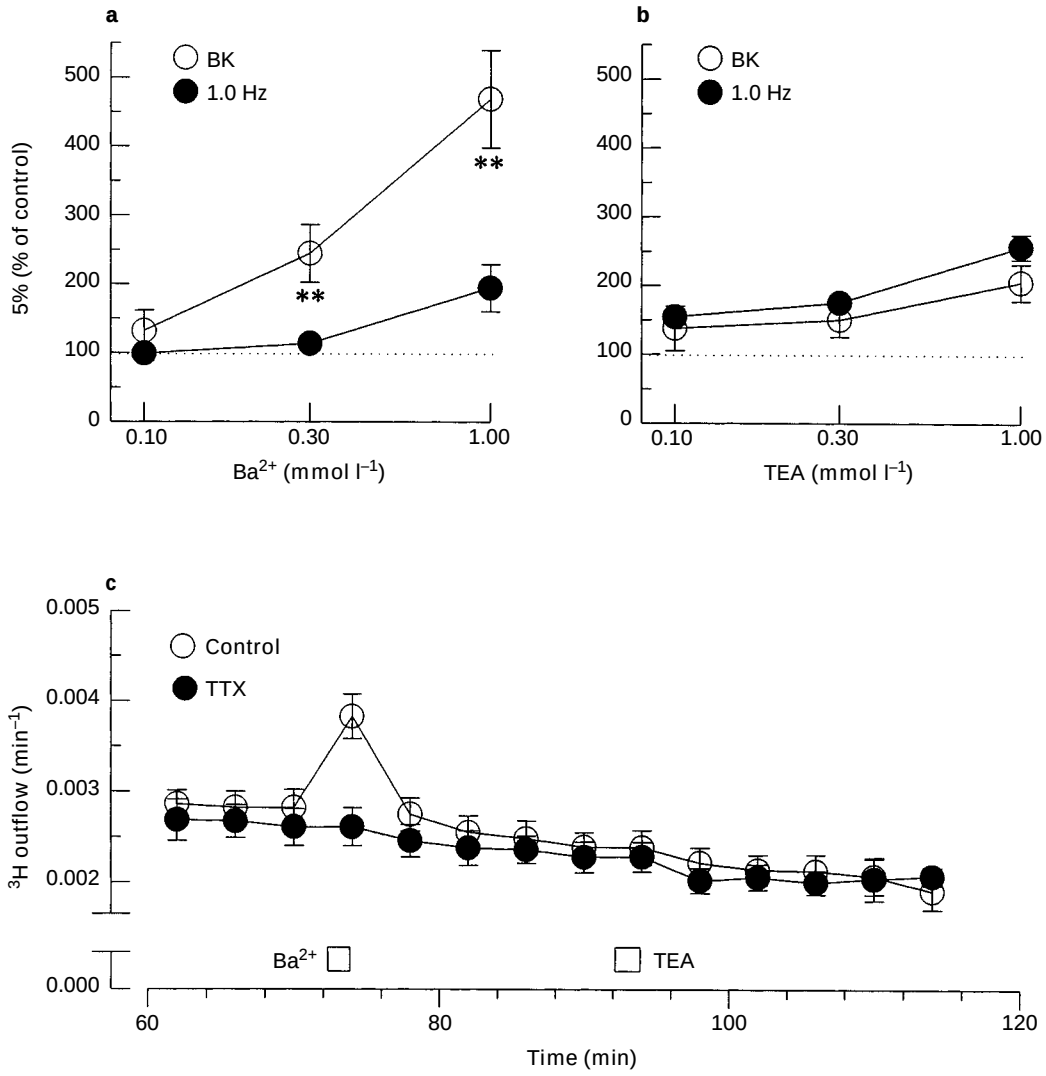

Figure 4 Modulation of bradykinin- and electrically-evoked tritium overflow, as well as spontaneous tritium outflow, from rat sympathetic neurones previously loaded with [^{3}H]-noradrenaline by K^+ channel inhibitors. After labelling, cultures were superfused and subjected to a 60 min washout period. Thereafter, 4 min fractions of superfusate were collected. From minutes 72 to 74, the superfusion buffer contained 100 nmol l^{-1} bradykinin, and from minutes 92 to 94, 120 monophasic rectangular pulses (0.5 ms; 50 mA; 50 V cm^{-1}) were applied at a frequency of 1.0 Hz. (a) The amount (S) of bradykinin- and electrically evoked (1.0 Hz) overflow in the presence of the indicated concentrations of $BaCl_2$ as percentage of the respective stimulation-dependent overflow in the absence of $BaCl_2$ (% of control); $n=8$ to 12. (b) The amount (5%) of bradykinin- and electrically evoked (1.0 Hz) overflow in the presence of the indicated concentrations of tetraethylammonium chloride (TEA) as percentage of the respective stimulation-dependent overflow in the absence of TEA (% of control); $n=8$ to 12. **Indicate significance of differences ($P<0.01$) between the effects of Ba^{2+} on bradykinin- and electrically-evoked overflow, respectively. (c) Shows the time course of fractional ^{3}H outflow (min^{-1}) in the absence and presence of 1 μmol l^{-1} tetrodotoxin (TTX). From minutes 72 to 74 the buffer contained 1 mmol l^{-1} $BaCl_2$ and from minutes 92 to 94 1 mmol l^{-1} tetraethylammonium chloride (TEA). $n=6$ to 9.

ium failed to alter basal ^{3}H outflow when determined after 20 min of its presence (see above), the secretagogue action of Ba^{2+} must be transient, as is the action of bradykinin (see Figure 1a). In the presence of 10 mmol l^{-1} caffeine, overflow triggered by 1 mmol l^{-1} Ba^{2+} increased from 0.53 ± 0.04% of cellular radioactivity (control, $n=6$) to 2.14 ± 0.27% ($n=6$).

Effects of pertussis or cholera toxin on bradykinin-evoked ^{3}H overflow

In neuroblastoma cells and sensory neurones, for example, bradykinin has been shown to act via Gi and Go type G proteins (Higashida *et al*., 1986; Ewald *et al*., 1989). For this reason, we incubated cell cultures in 100 ng ml^{-1} pertussis toxin for 24 h, a treatment that abolishes the inhibition of voltage-activated Ca^{2+} currents of rat sympathetic neurones by, for example, muscarinic agonists under our experimental conditions (Freissmuth *et al*., 1996, and unpublished observations), as an indication to complete inactivation of Gi/Go type G proteins. In pertussis toxin-treated cultures, bradykinin-induced, but not electrically-evoked, overflow was significantly enhanced (Figure 5).

To test for a possible role of Gs type G proteins, a 24 h treatment with 100 ng ml^{-1} cholera toxin was performed, which eliminates Gsα from sympathetic neurones (Boehm *et al*., 1996). Cholera toxin reduced both bradykinin- and electrically induced ^{3}H overflow (Figure 5). Neither pertussis nor cholera toxin caused significant alterations in basal tritium outflow (not shown).

Discussion

Several neurotransmitters, including ATP (Boehm, 1994), acetylcholine (Boehm & Huck, 1995) and uridine 5′-triphosphate (UTP) (Boehm *et al*., 1995) have previously been shown

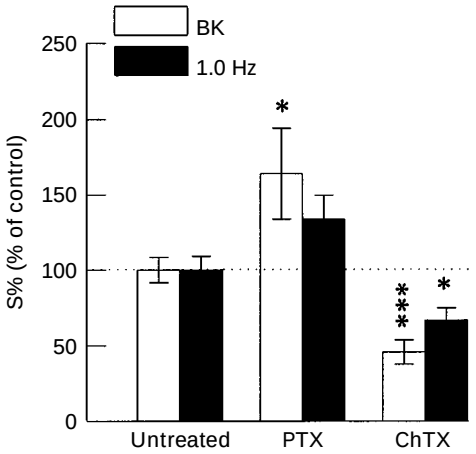

Figure 5 Modulation of bradykinin- and electrically-evoked tritium overflow from rat sympathetic neurones previously loaded with [^{3}H]-noradrenaline by a preceding 24 h treatment with 100 ng ml^{-1} pertussis toxin (Ptx) or cholera toxin (ChTx). After this treatment, cultures were labelled with [^{3}H]-noradrenaline, superfused, and subjected to a 60 min washout period. Thereafter, 4 min fractions of superfusate were collected. From minutes 72 to 74, the superfusion buffer contained 100 nmol l^{-1} bradykinin and from minutes 92 to 94, 120 monophasic rectangular pulses (0.5 ms, 50 mA; 50 V cm^{-1}) were applied at a frequency of 1.0 Hz. The amount (5%) of bradykinin- and electrically-evoked (1.0 Hz) overflow is shown as percentage (% of control) of the respective stimulation-dependent overflow in cultures not treated with any kind of toxin (=untreated). Significance of differences in comparison with control value, $*P<0.05$ and $***P<0.001$, respectively.

to trigger Ca^{2+}-dependent noradrenaline release from rat sympathetic neurones *in vitro*. In the present experiments, we demonstrated that bradykinin was also able to elicit transmitter release in this preparation. While ATP, acetylcholine and UTP have been found to be active at micromolar concentrations (Boehm & Huck, 1995; Boehm *et al*., 1995), bradykinin elicits noradrenaline release at nanomolar concentrations and thus represents the most potent secretagogue in primary cultures of rat sympathetic neurones.

Bradykinin may exert its actions via at least two different types of receptors, via B_1 and B_2 bradykinin receptors (Farmer & Burch, 1992; Hall, 1992). Rat superior cervical ganglia had been shown to express B_2 receptors which mediate bradykinin-induced depolarizations (Borkowski *et al*., 1995; Jones *et al*., 1995). In the present study, the following results indicated that B_2 receptors also mediated the secretory effects of the peptide. (i) The preferential B_1 receptor agonist des-Arg^9-bradykinin, at concentrations where bradykinin itself clearly triggered noradrenaline release, had no effect. (ii) [D-Phe^7]-bradykinin, a low affinity antagonist at B_2 receptors (Rhaleb *et al*., 1991), antagonized the secretory action of bradykinin: 1 μmol l^{-1} [D-Phe^7]-bradykinin shifted the concentration-response curve for bradykinin-triggered transmitter release to the right and caused an eight fold increase in the apparent half-maximal concentration of bradykinin. This is consistent with affinities of [D-Phe^7]-bradykinin for B_2 bradykinin receptors in the range of 1 μmol l^{-1} (Rhaleb *et al*., 1991; Dray & Perkins, 1993). [D-Phe^7]-bradykinin itself showed no agonistic activity in rat sympathetic neurones, a result that is in contrast to data previously obtained in rat vas deferens (Rhaleb *et al*., 1991). (iii) Hoe 140, a potent B_2 receptor antagonist (see Hall, 1992, for a review), also displayed purely antagonistic activity and, at 10 nmol l^{-1}, caused a rightward shift of the concentration-response curve for bradykinin-evoked release by a factor of 4. This is in line with previous findings which indicated affinities of Hoe 140 for B_2 receptors in the low nanomolar range (Hock *et al*., 1991; Griesbacher & Lembeck, 1992).

Molecolar cloning of the B_2 bradykinin receptor revealed a seven transmembrane structure and additional features typical for the superfamily of G protein coupled receptors (McEachern *et al*., 1991). Experimental evidence has been provided that B_2 bradykinin receptors elicit cellular responses via G proteins of either the Gq or the Gi/Go subfamily (e.g. Higashida *et al*., 1986; Gutowski *et al*., 1991; Hall, 1992). Therefore, it was not unexpected that the selective elimination of Gsα from sympathetic neurones by a 24 h treatment with cholera toxin (see Boehm *et al*., 1996) did not abolish the secretory effect of bradykinin. The reduction of electrically-evoked overflow by cholera toxin has previously been described in more detail and is related to longlasting changes in cyclic AMP-dependent signalling cascades (Boehm *et al*., 1996). When cultures had been treated with pertussis toxin in a manner that entirely inactivates Gi/Go type G proteins of sympathetic neurones (Nanoff *et al*., 1994; Freissmuth *et al*., 1996), the secretory effect of bradykinin was neither abolished nor reduced, but even enhanced. Hence, the initiation of transmitter release by B_2 bradykinin receptors did not involve G proteins of the Gi/Go subfamily.

The enhancement of bradykinin-triggered release by pertussis toxin might be explained by the loss of an α_2-adrenoceptor-mediated autoinhibitory feedback, as these receptors are linked to pertussis toxin-sensitive G proteins (Hill *et al*., 1993, and unpublished observations). However, the fact that electrically-evoked noradrenaline release was not significantly altered after pertussis toxin treatment argues against this interpretation. Alternatively, bradykinin itself might activate inhibitory, possibly presynaptic, receptors in addition to its release stimulating action; this could limit bradykinin-triggered transmitter release. Presynaptic inhibition of sympathetic transmitter release by bradykinin, either by a direct mechanism or via prostaglandins, has indeed been described in rabbit and mouse (Starke *et al*., 1977; Mass *et al*., 1995), but it is not known whether these effects are pertussis toxin-sensitive. However, bradykinin is known to reduce voltage-dependent Ca^{2+} currents via pertussis toxin-sensitive pathways in various neuronal preparations (Ewald *et al*., 1989; Boland *et al*., 1991), and the inhibition of Ca^{2+} channels is the major mechanism underlying presynaptic inhibition in sympathetic neurones (Boehm & Huck, 1995; 1996). Hence, the increase in bradykinin-evoked noradrenaline release after pertussis toxin treatment may be taken as an indication of presynaptic release-inhibiting bradykinin receptors in rat sympathetic neurones.

Since neither G proteins of the Gi/Go family, nor those of the Gs family, mediated the secretory action of bradykinin, proteins of the Gq class might be involved. Alternatively, the effect might occur independently of any kind of G protein. However, this appears unlikely in light of the structural features of B_2 bradykinin receptors (McEachern *et al*., 1991). Moreover, proteins of the Gq family have been shown to mediate the depolarizing action of B_2 bradykinin receptors in rat superior cervical ganglia (Borkowski *et al*., 1995; Jones *et al*., 1995). If, in fact, Gq type G proteins mediated the action of bradykinin, the question arises as to what the subsequent signalling mechanisms are. Most frequently, the activation of proteins of the Gq family leads to a rise in intracellular Ca^{2+} concentration (e.g. Hepler & Gilman, 1992). Liberation of Ca^{2+} from intracellular stores mediated the catecholamine releasing effect of bradykinin previously described in chromaffin cells (Owen *et al*., 1989) and neuronal cell lines (McDonald *et al*., 1994), as bradykinin-induced secretion occurred, at least in part, independently of extracellular Ca^{2+}. However, in the sympathetic neurones investigated in the present study, bradykinin-triggered release was completely tetrodotoxin-sensitive, entirely dependent on the presence of extracellular Ca^{2+} and abolished by Cd^{2+}, as was electrically-evoked release. These results indicated that bradykinin, similarly to electrical stimulation (see Boehm & Huck, 1997, for an overview), depolarized the neurones to an extent sufficient to trigger Na^+-dependent action potentials which then depolarized axon terminals, the only sites of transmitter release (Przywara *et al*., 1993), where finally transmembrane Ca^{2+}

entry occurred via voltage-activated Ca^{2+} channels. Thus, the secretory action of bradykinin appeared independent of the liberation of Ca^{2+} from intracellular stores. This assumption is also supported by the finding that thapsigargin, an inhibitor of Ca^{2+} ATPase (Thastrup, 1990) which prevents bradykinin-induced rises in intracellular Ca^{2+} (Reiser *et al*., 1992), did not alter bradykinin effects. Bradykinin potently blocks K_M channels in several neuronal cell lines (Brown & Higashida, 1988; Villaroel, 1996) and in neurones from rat superior cervical ganglia (Jones *et al*., 1995). Like activation of muscarinic receptors (Brown, 1983), this bradykinin action may cause neuronal depolarization (Jones *et al*., 1995). In the present study, results obtained with caffeine and Ba^{2+} indicated a central role of K_M channels in the secretagogue action of bradykinin. (1) Caffeine reduced electrically-evoked, but enhanced bradykinin-induced transmitter release. The inhibitory effect most likely reflected the Ca^{2+} channel blocking properties of this methylxanthine (Thayer *et al*., 1988). Since bradykinin-evoked release also depended on the function of voltage-activated Ca^{2+} channels (as shown by the inhibition by Cd^{2+}), one might expect an inhibitory effect of caffeine also on this type of stimulation-evoked release. However, caffeine additionally activates Ca^{2+} channels and blocks K_M channels of sympathetic neurones (Akaike & Sadoshima, 1989). The first effect would reduce rather than enhance stimulation-evoked noradrenaline release. Therefore, it was presumably the latter action, the inhibition of K_M channels, that caused the increase in bradykinin-evoked release. (2) Ba^{2+} at submillimolar concentrations selectively blocks K_M channels of sympathetic neurones (Stansfeld *et al*., 1993). Electrical stimulation-dependent transmitter release was increased by Ba^{2+} (0.1 to 1 mmol l^{-1}) by up to 100%, whereas bradykinin-triggered release was potentiated by up to 400%. In comparison, TEA at the same concentrations which block Ca^{2+}-activated K^+ channels (Marsh & Brown, 1991), but not K_M channels (Robbins *et al*., 1992), enhanced both bradykinin- and electrically-induced noradrenaline release to the same extent (<150%). Hence, blockade of K_M channels preferentially facilitated bradykinin-evoked transmitter release from sympathetic neurones. (3) Ba^{2+}, when applied for periods of 2 min, evoked tetrodotoxin-sensitive noradrenaline release which was markedly enhanced in the presence of caffeine. This indicated that, in rat sympathetic neurones a blockade of K_M channels may indeed be sufficient to trigger action potential-dependent transmitter release and that release evoked by an inhibition of K_M channels can be facilitated by caffeine.

In conclusion, the present experiments show that bradykinin by activation of B_2 receptors depolarizes rat sympathetic neurones to trigger action potentials and finally exocytotic transmitter release. This action presumably relies on the previously described (Jones *et al*., 1995) inhibition of K_M channels via Gq type G proteins.

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