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Ca^{2+}-dependent and -independent mechanism of cyclic-AMP reduction: mediation by bradykinin B_2 receptors

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1 Bradykinin caused a transient reduction of about 25% in the cyclic AMP level in forskolin prestimulated DDT_1 MF-2 smooth muscle cells (IC_{50}: 36.4±4.9 nM) and a pronounced, sustained inhibition (40%) of the isoprenaline-stimulated cyclic AMP level (IC_{50}: 37.5±1.1 nM).

2 The Ca^{2+} ionophore, ionomycin, mimicked both the bradykinin-induced transient reduction in the forskolin-stimulated cyclic AMP level and the sustained reduction in the isoprenaline-stimulated cyclic AMP level.

3 The Ca^{2+}-dependent effect on cyclic AMP induced by bradykinin was mediated solely by Ca^{2+} release from internal stores, since inhibition of Ca^{2+} entry with $LaCl_3$ did not reduce the response to bradykinin.

4 The involvement of calmodulin-dependent enzyme activities, protein kinase C or an inhibitory GTP binding protein in the bradykinin-induced responses was excluded since a calmodulin inhibitor, calmidazolium, a PKC inhibitor, staurosporine and pertussis toxin, respectively did not affect the decline in the cyclic AMP level.

5 Bradykinin enhanced the rate of cyclic AMP breakdown in intact cells, which effect was not mimicked by ionomycin. This suggested a Ca^{2+}-independent activation of phosphodiesterase activity by bradykinin in DDT_1 MF-2 cells.

6 The bradykinin B_1 receptor agonist, desArg^9-bradykinin, did not affect cyclic AMP formation in isoprenaline prestimulated cells, while the bradykinin B_2 receptor antagonists, Hoe 140 (D-Arg[Hyp^3, Thi^5, D-Tic^7, Oic^8]-BK) and D-Arg[Hyp^3, Thi^{5,8}, D-Phe^7]-BK completely abolished the bradykinin response in both forskolin and isoprenaline prestimulated cells.

7 Bradykinin caused an increase in intracellular Ca^{2+}, which was antagonized by the bradykinin B_2 receptor antagonists, Hoe 140 and D-Arg[Hyp^3, Thi^{5,8}, D-Phe^7]-BK. The bradykinin B_2 receptor agonist, desArg^9-bradykinin, did not evoke a rise in cytoplasmic Ca^{2+}.

8 It is concluded, that stimulation of bradykinin B_2 receptors causes a reduction in cellular cyclic AMP in DDT_1 MF-2 cells. This decline in cyclic AMP is partly mediated by a Ca^{2+}/calmodulin independent activation of phosphodiesterase activity. The increase in $[Ca^{2+}]_i$ mediated by bradykinin B_2 receptors inhibited forskolin- and isoprenaline-activated adenylyl cyclase differently, most likely by interfering with different components of the adenylyl cyclase signalling pathway.

Keywords: Cyclic AMP; Ca^{2+}; phosphodiesterase; bradykinin B_2 receptor; DDT_1 MF-2 cell

Introduction

Bradykinin is an important mediator of various biological processes, including regulation of blood pressure, neurotransmission and bronchoconstriction. The existence of bradykinin B_1 and B_2 receptors has been firmly established (Hall, 1992). Recently, a novel bradykinin B_3 receptor was proposed (Pyne & Pyne, 1993; 1994).

It was reported that bradykinin induces phospholipase C activation resulting in the formation of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) and the accumulation of cytoplasmic Ca^{2+} in DDT_1 MF-2 smooth muscle cells (Gerwins & Fredholm, 1992; Dickenson & Hill, 1992). Besides activation of phospholipase C, bradykinin has been reported to affect the cellular adenosine 3': 5'-cyclic monophosphate (cyclic AMP) level. In cultured tracheal smooth muscle cells, bradykinin elicited accumulation of cyclic AMP (Stevens *et al.*, 1994). In contrast, bradykinin evoked inhibition of cyclic AMP accumulation was observed in D384-human astrocytoma cells (Balmforth *et al.*, 1992; Altiok & Fredholm, 1993) and in NCB-20 hybrid neuronal cells (Garritsen *et al.*, 1992b). These inhibitory actions were caused by a simultaneous increase in cytoplasmic Ca^{2+}. Recently, inhibition of adenylyl cyclase activity caused by an inhibitory GTP binding protein was reported on stimulation of P_{2U} purinoceptors in DDT_1 MF-2 cells (Sipma *et al.*, 1994).

In this study we describe a bradykinin-induced decrease in the cyclic AMP level in DDT_1 MF-2 cells. Depending on the agonist used to activate adenylyl cyclase, this decline in cyclic AMP was either transient or sustained in nature. Furthermore we classified the bradykinin receptor subtype mediating these responses and studied the mechanisms underlying the effect of bradykinin on the cyclic AMP level.

Methods

Cell culture

DDT_1 MF-2 cells, derived from a Syrian hamster vas deferens (Norris *et al.*, 1974) were cultured in Dulbecco's modified essential medium supplemented with 7 mM $NaHCO_3$, 10 mM HEPES at pH 7.2 (DMEM) and 10% foetal calf serum at 37°C in 5% CO_2 (Molleman *et al.*, 1989).

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Experimental design and sample preparation

DDT_1 MF-2 cells were growm in monolayers in 9.6 cm^2 plastic wells as described earlier (Hoiting et al., 1990). The medium was replaced by 2 ml DMEM at 20°C, 30 min before starting the experiment by adding agonists. Before adding bradykinin some cell preparations were pre-exposed to forskolin (1 μM) for 10 min or to isoprenaline (10 μM, 10 min). Antagonists were added 2 min before exposure to bradykinin.

Cyclic AMP phosphodiesterase activity in intact cells was measured as previously described by Garritsen et al. (1992a). Isoprenaline pretreated (1 μM, 10 min) cells were quickly washed 3 times by aspiration and addition of new solution supplemented with the β_2-adrenoceptor antagonist, propranolol (100 nM). Under these conditions *de novo* synthesis of cyclic AMP was abolished.

Reactions were stopped by removal of the medium and addition of 400 μl 5% trichloroacetic acid (TCA). Samples were placed on ice for at least 45 min and subsequently washed 3 times with 800 μl water-saturated diethylether and neutralised with KOH (25 μl, 0.2 mM).

Measurements of cyclic AMP

A radioligand binding assay was used to determine cyclic AMP concentrations, using a standard curve of cyclic AMP in ether-extracted trichloroacetic acid solution (Brown et al., 1971). To reach equilibrium, 50 μl sample was incubated with a buffer composed of 50 mM Tris-HCl (pH 7.4), 4 mM EDTA, 20 μg cyclic AMP binding protein, 200 μg bovine serum albumin and 10 μl [^{3}H]-cyclic AMP (190 nM, 40 Ci $mmol^{-1}$) to a total volume of 160 μl at 4°C for at least 2 h. The reaction was terminated by adding 500 μl of a charcoal suspension (Norit A special, 3.5 g l^{-1}) followed by centrifugation to remove the excess [^{3}H]-cyclic AMP. Radioactivity in the supernatant was measured by scintillation counting.

Measurements of intracellular calcium

Cells (10^6 cells ml^{-1}) were washed with and resuspended in a buffer solution containing (mM): NaCl 145, KCl 5, $MgSO_4$ 0.5, $CaCl_2$ 1, glucose 10, bovine serum albumin 2% and HEPES 10, (pH 7.4) (Hesketh et al., 1983) and were loaded with Fura-2 AM (3 μM) for 45 min at 37°C. The cells were collected by centrifugation (20 s, 1000 g) and washed two times before the fluorescence measurement with buffer without bovine serum albumin. Calcium-free solution contained Mg^{2+} (6.2 mM) to prevent membrane leakage and EGTA (0.4 mM) to remove extracellular Ca^{2+} (Den Hertog, 1981). Fura-2 fluorescence of the cells (excitation: 340 nM and 380 nM; emission 510 nm) was measured at 22°C. The cell suspension was continuously magnetically stirred. The internal calcium concentration was calculated (Hesketh et al., 1983) using 0.015% of Triton X-100 as permeabilizing agent.

Data analysis

Data are presented as mean ± s.e.mean and were considered significantly different from control values at $P<0.05$ using Student's unpaired t test. A Sigma plot logistic curve fitting programme (Jandel Scientific, U.S.A.) was used to determine EC_{50} and IC_{50} values and to analyse binding parameters obtained from the respective radioligand binding assays.

Chemicals

Adenosine 3':5'-cyclic monophosphate (cyclic AMP) was from Boehringer (Germany). Forskolin, isoprenaline, bradykinin, desArg^9-bradykinin, pertussis toxin, 3-isobutyl-1-methyl-xanthine and staurosporine were purchased from Sigma, (U.S.A.). D-Arg[Hyp^3, Thi^{5,8}, D-Phe^7]-BK was from Hoechst AG (Germany). D-Arg[Hyp^3, Thi^5, D-Tic^7, Oic^8]-BK (Hoe 140) was from Peninsula Laboratories Inc. (U.S.A.). Calmidazolium chloride was obtained from Biomol (U.S.A.). [2,8-^{3}H]-adenosine 3':5'-cyclic monophosphate was obtained from Du Pont-New England Nuclear (U.S.A.). $LaCl_3$ and all other chemicals were from Merck (Germany).

Results

Forskolin and isoprenaline induced cyclic AMP accumulation

Stimulation of DDT_1 MF-2 cells with forskolin (1 μM) resulted in an increase in basal cellular cyclic AMP from 2.1±0.2 pmol/10^6 cells to 8.6±0.3 pmol/10^6 cells. In the presence of the β-adrenoceptor agonist, isoprenaline (1 μM), intracellular cyclic AMP reached a maximum of 65.6±3.5 pmol/10^6 cells. Both forskolin and isoprenaline induced cyclic AMP formation were maximal after 10 min of exposure to the agonists and remained stable for at least 10 min afterwards as described in detail elsewhere (Sipma et al., 1995).

Bradykinin and cyclic AMP

Stimulation of DDT_1 MF-2 cells with bradykinin (1 μM, 2 min) did not affect the basal cyclic AMP level (not shown). Bradykinin evoked a transient decline in cellular cyclic AMP in forskolin (1 μM, 10 min) pretreated cells (Figure 1a). The cellular cyclic AMP level showed a minimum between 0.5 and 2 min and returned to its original value after 5 min of exposure to bradykinin. This action of bradykinin on forskolin pretreated cells was concentration-dependent (Figure 2a, IC_{50}: 36.4±4.9 nM). Bradykinin induced a pronounced, sustained decrease in the cyclic AMP level in isoprenaline (1 μM, 10 min) pretreated cells (Figure 1b). Bradykinin also elicited a pronounced response when cells were pretreated with a lower concentration of isoprenaline (10 nM), resulting in a similar rise in cyclic AMP concentration as seen with forskolin (Figure 1c). The reduction of isoprenaline (1 μM)-induced cyclic AMP exhibited a similar concentration-dependency on bradykinin (Figure 2b, IC_{50}: 37.5±1.1 nM) as observed for forskolin pretreated cells. Exposure of DDT_1 MF-2 cells to bradykinin has previously been shown to elicit Ca^{2+} release from Ins(1,4,5)P_3-sensitive internal stores and to provoke Ca^{2+}-entry (Gerwins & Fredholm, 1992; Dickenson & Hill, 1992). Exposure of cells to forskolin or isoprenaline did not change the basal cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) or the bradykinin-induced increase in $[Ca^{2+}]_i$ (Table 1).

Involvement of an inhibitory G-protein

The involvement of inhibitory GTP binding proteins (G_i) in the effects of bradykinin on cyclic AMP was studied by use of pertussis toxin (PTX). Pretreatment of DDT_1 MF-2 cells with PTX (100 ng ml^{-1}, 24 h) neither affected the forskolin induced formation of cyclic AMP nor reduced the bradykinin-induced decline in the forskolin-stimulated cyclic AMP level (Figure 3a). Remarkably, the isoprenaline-induced formation of cyclic AMP was reduced after pre-exposure of cells to PTX. Under these experimental conditions, bradykinin still decreased the isoprenaline-stimulated cyclic AMP level (Figure 3b).

Ionomycin and cyclic AMP

In order to investigate the contribution of cytoplasmic Ca^{2+} to the effects observed, ionomycin (5 μM) was used to permeabilize plasma membranes and to deplete internal Ca^{2+} stores. In the presence of extracellular Ca^{2+} this treatment resulted in a transient decline in the forskolin-induced cyclic AMP level and a maintained decrease in the isoprenaline-induced cyclic AMP level (Figure 4). The characteristics of the cyclic AMP response in the presence of ionomycin were similar to those obtained with bradykinin (Figure 1).

Contribution of Ca^{2+}-entry

In order to estimate the relative contribution of Ca^{2+} entry and Ca^{2+} mobilization from internal stores to the bradykinin induced decline in cyclic AMP levels, Ca^{2+} entry was blocked with lanthanum ions ($LaCl_3$). The bradykinin-induced maximal increase in $[Ca^{2+}]_i$ was reduced to about 65% of the control response (Figure 5, Table 1) in the presence of $LaCl_3$ (50 μM). Moreover, the slowly declining phase of the Ca^{2+} response, representing Ca^{2+}-entry, was completely abolished by $LaCl_3$ (Figure 5). Inhibition of Ca^{2+} entry with $LaCl_3$ (50 μM) did not change the forskolin or isoprenaline-stimulated cyclic AMP level and did not affect the bradykinin induced decline in the forskolin- or isoprenaline-induced cyclic AMP level (Table 2).

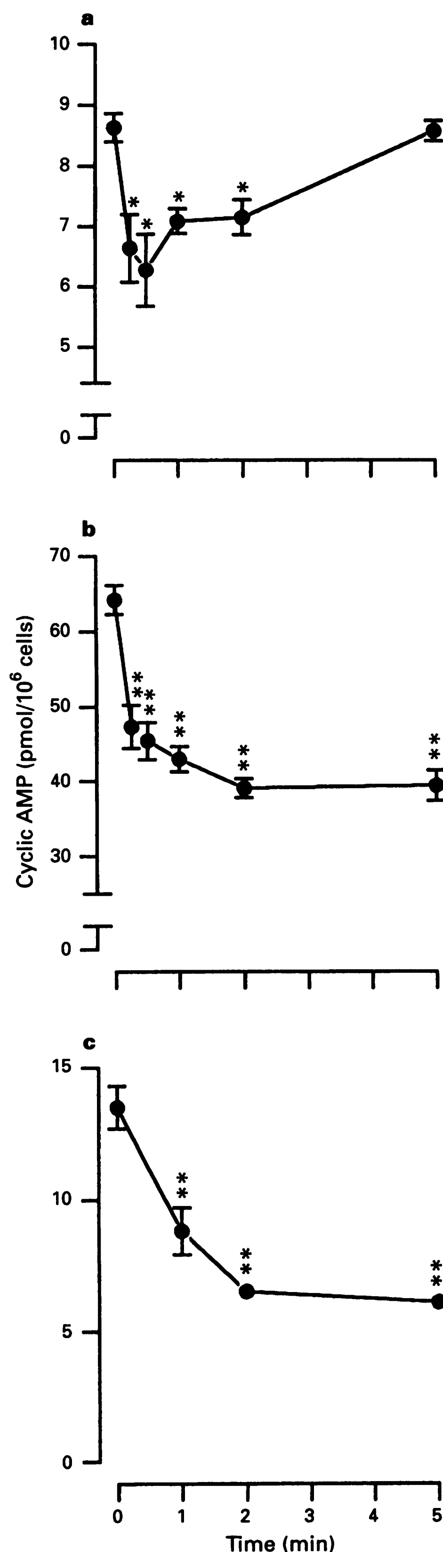

Figure 1 The time-dependent effect of bradykinin on the cyclic AMP level. (a) Bradykinin (1 μM) transiently reduced the cyclic AMP level in forskolin (1 μM, 10 min) prestimulated cells. (b) The bradykinin (1 μM)-induced reduction of cyclic AMP accumulation in isoprenaline (1 μM, 10 min) prestimulated cells is sustained. (c) The effect of bradykinin on cellular cyclic AMP after pretreatment of the cells with a low concentration of isoprenaline (10 nM, 10 min). *Different from values obtained in the absence of bradykinin, $P<0.05$; **$P<0.01$. Each point represents the mean ± s.e.mean of at least 5 experiments.

Involvement of protein kinase C and calmodulin dependent enzymes

Pretreatment of cells with the protein kinase C inhibitor, staurosporine (0.5 μM, 5 min), did not change the basal level of forskolin- or isoprenaline-induced cyclic AMP and did not affect the bradykinin-induced decline in the forskolin- and isoprenaline-stimulated cyclic AMP level (Table 3).

A calmodulin inhibitor was used to determine whether the inhibitory effect of Ca^{2+} on cyclic AMP levels was mediated by Ca^{2+}/calmodulin-dependent enzymes. Calmidazolium is known to inhibit calmodulin-dependent enzymes with high potency (Gietzen, 1983). Pre-exposure of cells to calmidazo-

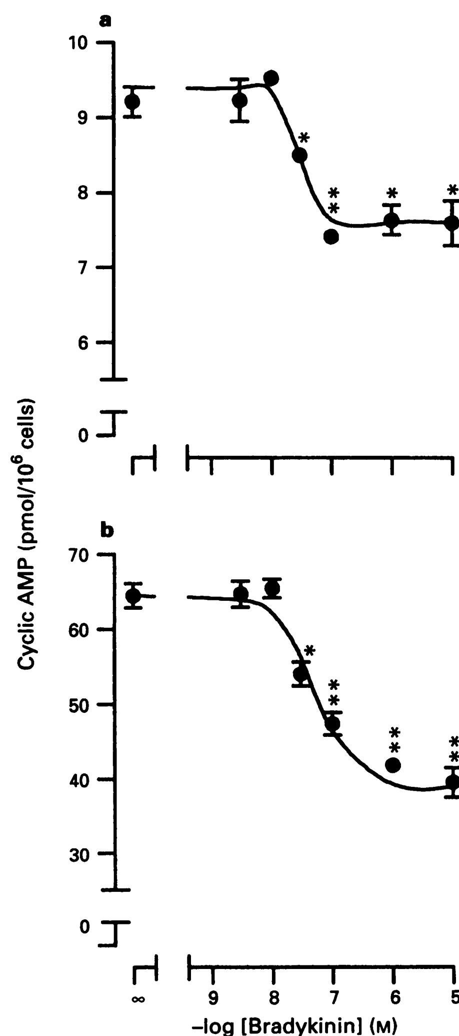

Figure 2 The concentration-dependent effect of bradykinin on the cyclic AMP level. (a) The bradykinin-induced reduction of cyclic AMP accumulation after 2 min in forskolin (1 μM, 10 min) pretreated cells. (b) The reduction in the cyclic AMP level in isoprenaline (1 μM, 10 min) pretreated cells. *Different from values obtained in the absence of bradykinin, $P<0.05$; **$P<0.01$. Each point represents the mean ± s.e.mean of at least 5 experiments.

Table 1 Bradykinin-induced increases in cytoplasmic Ca^{2+} in DTT_1 MF-2 cells

Treatment	*Basal* $[Ca^{2+}]_i$ *(nM)*	*Bradykinin induced increase in* $[Ca^{2+}]_i$ *(nM)*
None	153±9	155±22
Forskolin	160±12	154±24
Isoprenaline	156±14	158±30
$LaCl_3$	165±12	103±15*
Hoe 140	163±15	2.1±1.1*
D-Arg[Hyp³, Thi⁵,⁸, D-Phe⁷]-BK	156±11	10±2.9*

Pretreatment of cells with forskolin (1 μM, 10 min) or isoprenaline (1 μM, 10 min) did not affect bradykinin (1 μM)-induced Ca^{2+} metabolism. Blocking Ca^{2+} entry by pretreatment of cells with $LaCl_3$ (50 μM, 2 min) reduced the bradykinin-induced evoked rise in $[Ca^{2+}]_i$. The bradykinin-induced maximal increase in cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) was abolished by the bradykinin B_2 receptor antagonist Hoe 140 (D-Arg[Hyp³, Thi⁵, D-Tic⁷, Oic⁸]-BK, 1 μM, 2 min) and D-Arg[Hyp³, Thi⁵,⁸, D-Phe⁷]-BK (10 μM, 2 min). *Different from stimulation with bradykinin, $P<0.01$. Data are presented as mean ± s.e.mean of at least 4 experiments.

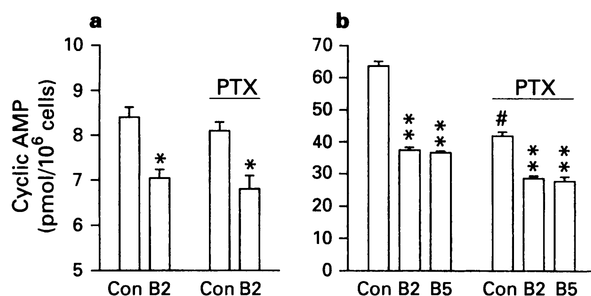

Figure 3 The effect of pertussis toxin on the bradykinin induced decline in cyclic AMP. Cells were treated in the presence or absence of pertussis toxin (PTX, 100 ng ml⁻¹, 24 h, horizontal bar). (a) The bradykinin (B2, 1 μM, 2 min)-induced reduction in forskolin pretreated (1 μM, 10 min) cells. (b) The bradykinin (1 μM, B2:2 min, B5:5 min)-induced reduction in isoprenaline pretreated (1 μM, 10 min) cells. *Different from respective control stimulation in the absence of bradykinin, $P<0.05$; **$P<0.01$. #Different from values obtained in the absence of PTX, $P<0.01$. Data are expressed as mean ± s.e.mean of 8 experiments.

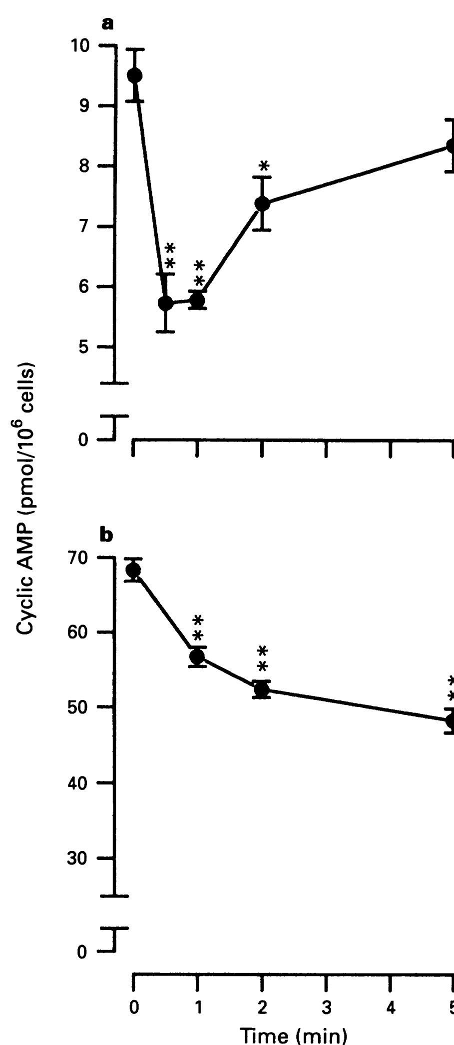

Figure 4 The effects of ionomycin on prestimulated cyclic AMP levels. (a) Ionomycin (5 μM) caused a transient decline in the forskolin (1 μM, 10 min) induced cyclic AMP level and (b) a sustained reduction in the isoprenaline (1 μM, 10 min)-enhanced cyclic AMP level in Ca^{2+} (1.8 mM) containing buffer. *Different from values obtained in the absence of ionomycin, $P<0.05$; **$P<0.01$. Each point represents the mean ± s.e.mean of 4 experiments.

lium (10 μM, 10 min) did not affect the forskolin- or isoprenaline-induced cyclic AMP level. Furthermore, the bradykinin-induced reduction in the forskolin and isoprenaline-stimulated cyclic AMP level was not affected by calmidazolium (Table 4).

The effect of bradykinin on cyclic AMP-phosphodiesterase activity

Cyclic AMP phosphodiesterase activity was measured after inhibition of *de novo* synthesis of cyclic AMP. The cyclic AMP level of unstimulated cells decreased time-dependently under these conditions (Figure 6). This decrease in the cyclic AMP level was completely inhibited in the presense of the non-specific phosphodiesterase inhibitor 3-isobutyl-1-methyl xanthine (IBMX, 200 μM), which showed that the reduction in the cyclic AMP level was completely due to cyclic AMP phosphodiesterase activity. Bradykinin (1 μM), added immediately after removal of isoprenaline, enhanced the rate of cyclic AMP breakdown, an effect which was also completely inhibited by IBMX. Ionomycin (5 μM) did not mimic the effect of bradykinin on phosphodiesterase activity (not shown).

Bradykinin receptor subtype

The bradykinin B_1 receptor agonist, des-Arg⁹-bradykinin did not affect isoprenaline induced cyclic AMP formation (Table 5). The bradykinin B_2 receptor antagonist, D-Arg[Hyp³, Thi⁵, D-Tic⁷, Oic⁸]-BK (Hoe 140) completely blocked the bradykinin-induced reduction of the cyclic AMP level in forskolin and isoprenaline prestimulated cells. Hoe 140 concentration-dependently blocked the inhibitory response of bradykinin (1 μM) on isoprenaline-stimulated cyclic AMP accumulation (IC_{50} value: 34.0 ± 1.5 nM, Figure 7). The bradykinin B_2 receptor antagonist, D-Arg[Hyp³, Thi⁵,⁸, D-Phe⁷]-BK, likewise abolished the effect of bradykinin on the isoprenaline-induced cyclic AMP level (Table 5).

The bradykinin (1 μM)-induced rise in $[Ca^{2+}]_i$ was completely abolished by Hoe 140 (1 μM) and for 93% by D-Arg-[Hyp³, Thi⁵,⁸, D-Phe⁷]-BK (10 μM) (Table 1). Exposure of cells to desArg⁹-bradykinin (0.3 μM) did not affect $[Ca^{2+}]_i$ (increase $[Ca^{2+}]_i$: 1.3 ± 1.1 nM, $n=4$).

Discussion

This study shows that bradykinin caused a transient or sustained reduction in prestimulated cellular cyclic AMP levels in

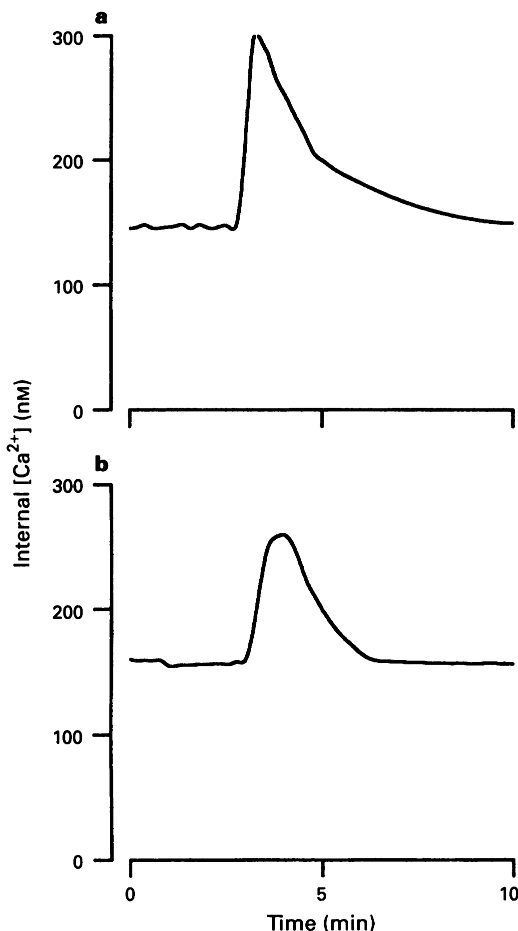

Figure 5 The effect of inhibition of Ca^{2+} entry on the bradykinin-induced increase in $[Ca^{2+}]_i$. (a) The bradykinin (1 μM)-induced rise in $[Ca^{2+}]_i$ in Ca^{2+} containing buffer. (b) $LaCl_3$ (50 μM) added 2 min in advance reduced the bradykinin-evoked maximal increase in $[Ca^{2+}]_i$ and completely inhibited the slowly declining phase of $[Ca^{2+}]_i$ representing Ca^{2+} entry. Each tracing is representative of at least 4 experiments.

DDT₁ MF-2 smooth muscle cells. A transient decline in the cyclic AMP level was observed after stimulation of the catalytic subunit of adenylyl cyclase with forskolin. After stimulation of β-adrenoceptors with isoprenaline, the bradykinin-induced reduction in the cyclic AMP level was pronounced and sustained in nature. These different characteristics of the response to bradykinin cannot be explained by a difference in the cyclic AMP level elicited by forskolin and isoprenaline, since the bradykinin-induced reduction in the cyclic AMP level was also pronounced and sustained in cells pretreated with a low concentration of isoprenaline.

Bradykinin activates phospholipase C, resulting in formation of $Ins(1,4,5)P_3$, mobilization of Ca^{2+} from internal stores and Ca^{2+} entry across the plasma membrane (Gerwins & Fredholm, 1992; Dickenson & Hill, 1992; this paper). We showed that an increase in $[Ca^{2+}]_i$ evoked by ionomycin mimicked the effects of bradykinin on the forskolin- and isoprenaline-induced cyclic AMP level. These observations demonstrate, that an increase in intracellular Ca^{2+} is probably involved in the bradykinin-induced reduction of cellular cyclic AMP in DDT₁ MF-2 cells. Since the bradykinin-induced increase in $[Ca^{2+}]_i$ is not changed by either forskolin or isoprenaline, the differential effect of bradykinin on cyclic AMP cannot be explained by discrete regulation of bradykinin-induced Ca^{2+} metabolism. Bradykinin and P_{2U} purinoceptor-mediated transient inhibition of prostaglandin E_1-induced cyclic AMP formation in NCB-20 hybrid neuronal cells has been reported previously (Garritsen *et al.*, 1992a,b). Likewise, sustained inhibition of agonist-induced cyclic AMP elevation caused by stimulation of endogenous bradykinin receptors and stably transfected substance K receptors was observed in C6-2B rat glioma cells (Debernardi *et al.*, 1991). Release of Ca^{2+} from internal stores was supposed to be responsible for the

Table 2 Ca^{2+} entry and the bradykinin induced reduction in cellular cyclic AMP

Treatment	*Cyclic AMP* (pmol/10⁶ cells) *Isoprenaline*	*Forskolin*
None	64.9 ± 1.0	9.3 ± 0.5
Bradykinin (2 min)	42.7 ± 0.9**	7.2 ± 0.3*
Bradykinin (5 min)	41.2 ± 1.2**	
$LaCl_3$	66.2 ± 1.8	9.1 ± 0.6
$LaCl_3$, bradykinin (2 min)	46.4 ± 0.7**	7.2 ± 0.4*
$LaCl_3$, bradykinin (5 min)	46.3 ± 1.0**	

Inhibition of Ca^{2+}-entry by pre-exposure of cells to $LaCl_3$ (50 μM, 2 min) did not affect the bradykinin (1 μM)-induced reduction in the forskolin (1 μM, 10 min) or isoprenaline (1 μM, 10 min)-stimulated cyclic AMP level. *Different from values obtained in the absence of bradykinin $P<0.05$; **$P<0.01$. Data are expressed as mean ± s.e.mean of 6 experiments.

Table 3 Protein kinase C and the bradykinin induced reduction in the cyclic AMP level

Treatment	*Cyclic AMP* (pmol/10⁶ cells) *Isoprenaline*	*Forskolin*
None	64.4 ± 2.5	9.3 ± 0.5
Bradykinin (2 min)	44.9 ± 2.6**	7.2 ± 0.3*
Bradykinin (5 min)	43.3 ± 0.7**	
Staurosporine	68.7 ± 2.0	9.6 ± 0.9
Staurosporine, bradykinin (2 min)	46.1 ± 0.9**	7.5 ± 0.4*
Staurosporine, bradykinin (5 min)	48.4 ± 1.3**	

The bradykinin (1 μM)-evoked decline in the forskolin (1 μM, 10 min) and isoprenaline (1 μM, 10 min)-stimulated cyclic AMP level was not affected by pretreatment of cells with staurosporine (0.5 μM, 5 min). *Different from values obtained in the absence of bradykinin $P<0.05$, **$P<0.01$. Data are expressed as mean ± s.e.mean of 6 experiments.

Table 4 Calmodulin and the bradykinin induced reduction in the cyclic AMP level

Treatment	*Cyclic AMP* (pmol/10⁶ cells) *Isoprenaline*	*Forskolin*
None	63.7 ± 1.8	9.1 ± 0.4
Bradykinin (2 min)	36.0 ± 1.6**	6.5 ± 1.7*
Bradykinin (5 min)	41.0 ± 1.4**	
Calmidazolium	62.0 ± 1.6	9.0 ± 0.3
Calmidazolium, bradykinin (2 min)	41.1 ± 1.9**	6.6 ± 0.4*
Calmidazolium, bradykinin (5 min)	40.9 ± 1.1**	

Pretreatment of cells with the calmodulin antagonist, calmidazolium (10 μM, 10 min), did not influence the bradykinin (1 μM)-evoked decline in the forskolin (1 μM, 10 min) or isoprenaline (1 μM, 10 min)-stimulated cyclic AMP level. *Different from values obtained in the absence of bradykinin $P<0.05$, **$P<0.01$. Data are expressed as mean ± s.e.mean of 4 experiments.

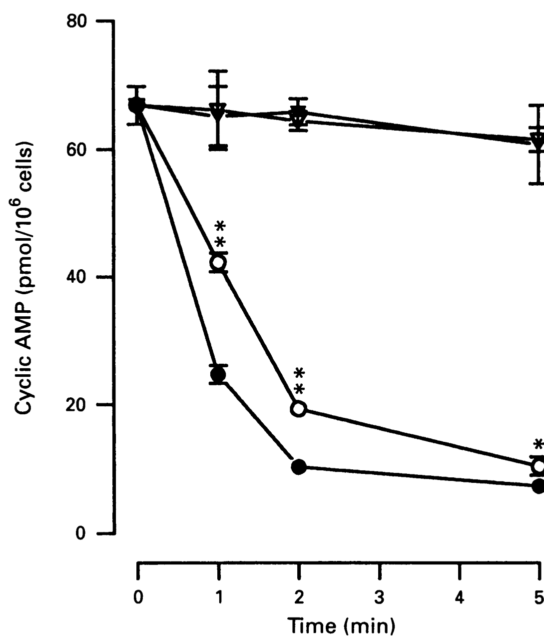

Figure 6 Bradykinin enhanced the rate of cyclic AMP breakdown. DDT_1 MF-2 cells were pretreated with isoprenaline (1 μM, 10 min) and after removal of isoprenaline, cyclic AMP was measured. Control (○); in the presence of isobutyl methylxanthine (▽, IBMX, 200 μM); bradykinin (●, 1 μM); or IBMX and bradykinin (▼). *Different from cyclic AMP level in the presence of bradykinin, $P<0.05$; ** $P<0.01$. Data are expressed as mean ± s.e.mean of 5 experiments.

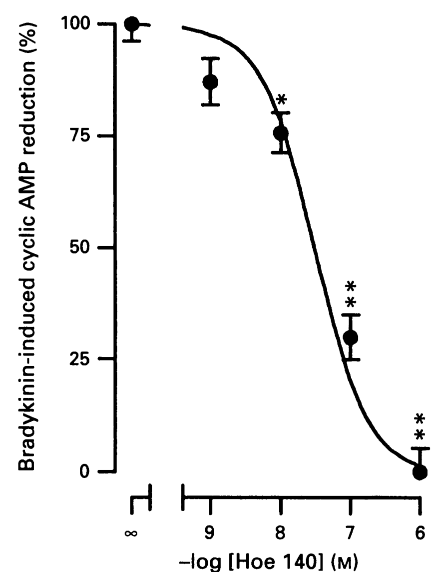

Figure 7 Inhibition of bradykinin-induced reduction of the cyclic AMP level by Hoe 140. The B_2 receptor antagonist, Hoe 140 (D-Arg[Hyp³, Thi⁵, D-Tic⁷, Oic⁸]-BK), added to the cells 2 min in advance, concentration-dependently inhibited the bradykinin (1 μM, 2 min)-induced reduction of the cyclic AMP level in isoprenaline (1 μM, 10 min) prestimulated cells. The control levels of isoprenaline enhanced cyclic AMP and of isoprenaline given together with Hoe 140 (1 μM, 2 min) were 64.5 ± 2.8 and 68.9 ± 1.9 pmol/10^6 cells, respectively. *Different from values obtained in the absence of Hoe 140, $P<0.05$; ** $P<0.01$. Each point represents the mean ± s.e.mean of 4 experiments.

Table 5 The effect of bradykinin receptor agonists and antagonists on cellular cyclic AMP in DDT_1 MF-2 cells

	Cyclic AMP (pmol/10^6 cells)	
Treatment	*Isoprenaline*	*Forskolin*
None	64.5 ± 2.0	8.7 ± 0.2
Bradykinin	40.9 ± 0.4**	7.1 ± 0.3*
desArg⁹-bradykinin (0.3 μM)	69.6 ± 1.7	
Hoe 140	65.0 ± 1.8	8.8 ± 0.2
Hoe 140 + bradykinin	69.1 ± 1.1	8.3 ± 0.3
Hoe 140 + bradykinin (5 min)	61.1 ± 1.7	
D-Arg[Hyp³, Thi⁵˒⁸, D-Phe⁷]-BK	62.1 ± 4.0	
D-Arg[Hyp³, Thi⁵˒⁸, D-Phe⁷]-BK + bradykinin	63.0 ± 1.5	

Bradykinin (1 μM, 2 min, unless otherwise stated) was used to decrease the prestimulated cyclic AMP level. Enhanced cyclic AMP levels were obtained by pretreatment of cells with isoprenaline (1 μM, 10 min) or forskolin (1 μM, 10 min). Concentrations used of Hoe 140 (D-Arg[Hyp³, Thi⁵˒⁸, D-Tic⁷, Oic⁸]-BK) and D-Arg[Hyp³, Thi⁵˒⁸, D-Phe⁷]-BK were 1 μM and 10, respectively. *Different from values obtained in the absence of bradykinin $P<0.05$; ** $P<0.01$. Data are expressed as mean ± s.e.mean of at least 4 experiments.

inhibitory action on adenylyl cyclase under these conditions. The bradykinin-evoked slowly declining phase of the Ca^{2+} response is caused by Ca^{2+} entry from the extracellular environment in DDT_1 MF-2 cells. Our results showed that inhibition of Ca^{2+} entry by $LaCl_3$ did not affect the bradykinin-induced decline in the cyclic AMP level. Therefore, Ca^{2+} entry does not play a substantial role in the effect of bradykinin on cyclic AMP formation. Thus, the bradykinin-induced response is solely mediated by Ca^{2+} release from internal compartments. In contrast, it was reported that inhibition of the forskolin, dopamine and 5′-N-ethyl-carboxamidoadenosine-induced cyclic AMP level by bradykinin as observed in D384-human astrocytoma cells was dependent on Ca^{2+} entry (Balmforth *et al.*, 1992; Altiok & Fredholm, 1993).

Reduction in cyclic AMP levels by the activation of protein kinase C was reported previously (Fleming *et al.*, 1992; Bascands *et al.*, 1993). Recently, Assender *et al.* (1994), reported the presence of 3 protein kinase C isoenzymes, among which was the Ca^{2+}-dependent α-isoform, in DDT_1 MF-2 cells. The bradykinin-induced response in DDT_1 MF-2 cells was not affected by the protein kinase C inhibitor, staurosporine, a feature also observed after stimulation of P_{2U} purinoceptors in DDT_1 MF-2 cells (Sipma *et al.*, 1994). Moreover, it was shown that the protein kinase C activator, phorbol 12-myristate 13-acetate, did not inhibit cyclic AMP formation (Sipma *et al.*, 1994). Thus, protein kinase C does not play a role in the regulation of bradykinin-evoked cyclic AMP metabolism in DDT_1 MF-2 cells.

The calmodulin inhibitor, calmidazolium, did not affect the bradykinin-induced reduction in the forskolin and isoprenaline-enhanced cyclic AMP level, which showed that calmodulin-dependent enzymes, like protein kinases and phosphodiesterases were not involved in the response. Moreover, it has been shown that Ca^{2+} ions directly inhibited adenylyl cyclase activity in a biphasic and cooperative manner in membrane preparations of D384-human astrocytoma cells (Altiok & Fredholm, 1993), NCB-20 hybrid neuroblastoma cells (Boyanjian *et al.*, 1991), platelets and GH_3 pituitary derived cells (Caldwell *et al.*, 1992) and of cardiac cells (Colvin *et al.*, 1991). These observations strongly suggest that Ca^{2+} ions exert a direct effect on the mechanism leading to adenylyl cyclase stimulation in DDT_1 MF-2 cells.

Besides the Ca^{2+}-dependent mechanism of cyclic AMP reduction, bradykinin was found to enhance the rate of cyclic AMP breakdown in a protocol where *de novo* synthesis of cyclic AMP was prevented. Activation of the phosphodiesterase activity by bradykinin was not dependent on Ca^{2+} since ionomycin did not mimic the effect of bradykinin. Increased phosphodiesterase activity was found after stimulation of

muscarinic receptors in 1321N1 human astrocytoma cells (Tanner *et al.*, 1986) and in the pregnant-rat myometrium (Goureau *et al.*, 1990). However, these activations appeared to be secondary to the simultaneous increase in $[Ca^{2+}]_i$.

The bradykinin-induced reduction in cyclic AMP levels was not inhibited by PTX, which demonstrated that an inhibitory G-protein was not involved. In contrast, stimulation of P_{2U} purinoceptors in DDT_1 MF-2 cells (Sipma *et al.*, 1994) and stimulation of P_2 purinoceptors in rat hepatocytes (Okajima *et al.*, 1987) caused inhibition of adenylyl cyclase via a G_i protein. Remarkably, PTX decreased the isoprenaline-induced formation of cyclic AMP. How PTX induced this effect remains to be established.

Three mechanisms of cyclic AMP reduction have been identified in DDT_1 MF-2 cells. Stimulation of P_{2U} purinoceptors leads to activation of a G_1 protein and effects a pronounced reduction in cyclic AMP levels. This response is sustained in nature in both forskolin and isoprenaline pretreated cells (Sipma *et al.*, 1994). Two other processes thought to decrease cellular cyclic AMP are described in this paper and concern bradykinin-induced activation of a cyclic AMP phosphodiesterase in a Ca^{2+}/calmodulin-independent manner and a Ca^{2+}-dependent mechanism. The obtained transient reduction in the forskolin-elevated cyclic AMP level implies that both the activation of the phosphodiesterase and the Ca^{2+}-dependent inhibition of adenylyl cyclase catalytic activity are short-lived. Since a sustained inhibition of the cyclic AMP level was observed in isoprenaline pretreated cells, this effect was most likely mediated by Ca^{2+} acting at the level of the β-adrenoceptor and/or the stimulatory G-protein. The results obtained with $LaCl_3$ showed that a transient increase in $[Ca^{2+}]_i$ apparently can induce events leading to a sustained inhibition of adenylyl cyclase as suggested previously by De-Bernardi *et al.* (1991). Histamine H_1 receptors were also reported to mediate a modest transient reduction in cyclic AMP in DDT_1 MF-2 cells. This effect was most probably due to a histamine-induced increase in $[Ca^{2+}]_i$ (Sipma *et al.*, 1994). Pretreatment of DDT_1 MF-2 cells with cyclic AMP-enhancing agents reduced the histamine H_1 receptor-mediated formation of inositol phosphates and the release of Ca^{2+} from internal stores (Dickenson *et al.*, 1993; Sipma *et al.*, 1995) but not the rise in $[Ca^{2+}]_i$ induced by bradykinin. This may explain that bradykinin is more effective in reducing cellular cyclic AMP than histamine.

The effect of bradykinin on the cellular cyclic AMP level and the increase in $[Ca^{2+}]_i$ was abolished in the presence of the bradykinin B_2 receptor antagonists, Hoe 140 and D-Arg[Hyp^3, Thi^{5,8}, D-Phe^7]-BK in DDT_1 MF-2 cells. It was reported that D-Arg[Hyp^3, Thi^{5,8}, D-Phe^7]-BK antagonized bradykinin B_2 receptor-mediated total inositol phosphate formation in canine tracheal smooth muscle cells (Yang *et al.*, 1994) and $Ins(1,4,5)P_3$ formation in guinea-pig cultured tracheal smooth muscle (Pyne & Pyne, 1993). Hoe 140 has been identified as a highly potent B_2 receptor antagonist (Lembeck *et al.*, 1991), with IC_{50} values from 0.1 nM – 40 nM for several contractile responses. The IC_{50} value (34 nM) found by us for reduction of the cyclic AMP level is in the same range. It is proposed that bradykinin B_3 receptors are insensitive to bradykinin B_2 receptor antagonists (Pyne & Pyne, 1993; 1994). Thus, since both the Ca^{2+}-dependent and Ca^{2+}-independent mechanism resulting in the bradykinin-induced decline in the cyclic AMP level as well as activation of the phospholipase C pathway were blocked by bradykinin B_2 receptor antagonists and not elicited by a bradykinin B_1 receptor agonist, desArg^9-bradykinin, the responses described are mediated by bradykinin B_2 receptors.

In summary, bradykinin B_2 receptors mediate a reduction in the prestimulated cyclic AMP level in DDT_1 MF-2 cells. Besides activation of a Ca^{2+}/calmodulin-independent phosphodiesterase, the effect of bradykinin on cyclic AMP was mediated by Ca^{2+} acting on different components of the adenylyl cyclase signal transduction pathway.

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