

Possible role of bradykinin on stimulus-secretion coupling in adrenal chromaffin cells

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Abstract : Nonapeptide bradykinin is known to be a central nervous system neurotransmitter and to play a role in regulation of neuronal function. However, few details are known of the function of its peptide on stimulus-secretion coupling in neuronal cells. In this article, the role of bradykinin on catecholamine biosynthesis, secretion and Ca^{2+} movement in adrenal chromaffin cells as a model for catecholamine-containing neurons are examined. Bradykinin receptors are classified as B_1 and B_2 receptor subtypes. These receptors are present on the adrenal chromaffin cell membrane. Bradykinin increases the influx of Ca^{2+} and the turnover of phosphoinositide through the stimulation of bradykinin B_2 receptor. The secretion of catecholamine from the cells is initiated by the raise of $[\text{Ca}^{2+}]_i$. An increase in $[\text{Ca}^{2+}]_i$ and production of diacylglycerol stimulate the activation of calcium-dependent protein kinases. These kinases stimulate the activation of tyrosine hydroxylase, a rate-limiting enzyme in the biosynthesis of catecholamine. Otherwise, bradykinin increases Ca^{2+} efflux from the cells through the stimulation of the bradykinin- B_2 receptor. This action may be explained by an extracellular Na^+ -dependent mechanism, probably through acceleration of $\text{Na}^+/\text{Ca}^{2+}$ exchange. It is interesting that bradykinin, which stimulates the biosynthesis and secretion of catecholamine in adrenal chromaffin cells, plays a role in the termination of calcium-signal transduction through the stimulation of Ca^{2+} efflux from the cells. *J. Med. Invest.* **46** : 1-9, 1999

Key words : *Bradykinin, Catecholamine, Biosynthesis, Secretion, Calcium*

INTRODUCTION

The nonapeptide bradykinin has been found to influence several physiological processes including pain generation (1), blood pressure (2) and cardiovascular regulation (3). It has also been suggested to be a central nervous system neurotransmitter and to play a role in the regulation of neuronal function (4, 5).

Adrenal chromaffin cells and their cancer cell line, pheochromocytoma PC-12 cells, are useful for studying the mechanism of the stimulus-secretion coupling, and

are regarded as a model for catecholamine-containing neurons. Physiological stimulations of the cells cause an increase in the levels of intracellular free Ca^{2+} ($[\text{Ca}^{2+}]_i$) which comes from both the intracellular and extracellular pools. The increase in $[\text{Ca}^{2+}]_i$ causes exocytosis including the stimulation of catecholamine secretion and activation of its biosynthesis (6, 7).

In this article, the role of bradykinin on catecholamine biosynthesis, secretion and Ca^{2+} movement in adrenal chromaffin cells and pheochromocytoma PC-12 cells are examined.

General stimulus-secretion coupling in adrenal chromaffin cells

Chromaffin cells synthesize, store and secrete catecholamines (dopamine, noradrenaline and adrenaline) in nerve stimulations. The cells have an excitable action in response to acetylcholine or to electrical

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stimulation of the splanchnic nerve. Following activation of acetylcholine nicotinic receptors, acetylcholine causes the opening of the receptor-mediated ion channel, allowing the influx of Na^+ , and to a lesser extent Ca^{2+} (8, 9). This influx into the chromaffin cells of Na^+ results in a mild depolarization of the cell membrane sufficient to activate voltage-dependent Na^+ channels (10). The opening of Na^+ channels induces the activation of voltage-dependent Ca^{2+} channels (11). The opening of Na^+ and Ca^{2+} channels causes the firing of action potentials and the entry of Ca^{2+} from extracellular spaces (12). An increase in $[\text{Ca}^{2+}]_i$ is the trigger for exocytosis of chromaffin granules (secretion of catecholamine) and stimulation of biosynthesis of catecholamine. Acetylcholine nicotinic and muscarinic receptors are present in chromaffin cells, and these receptors in most species stimulate secretion of catecholamine. In bovine adrenal chromaffin cells, however, only nicotinic receptor stimulation evoke secretion of catecholamine. Catecholamine secretion can also be evoked by high K^+ which directly activate the voltage-dependent channels. The depolarization induced by high K^+ directly opens the Ca^{2+} channels without the contribution of the Na^+ channel. The role of bradykinin, a putative neuropeptide, on stimulus-secretion coupling including biosynthesis and secretion of catecholamine in adrenal chromaffin cells and pheochromocytoma PC-12 are discussed below.

Catecholamine biosynthesis regulated by bradykinin

Tyrosine hydroxylase, which is a rate-limiting enzyme in the biosynthesis of catecholamine, is phosphorylated and activated by cAMP-dependent protein kinase and calcium-dependent protein kinases (calcium/calmodulin-dependent protein kinase and protein kinase C) (13-16) : it is activated on incubation of cells with cAMP analogs or forskolin (an activator of adenylate cyclase), phorbol esters (activators of protein kinase C) or compounds that elevate the $[\text{Ca}^{2+}]_i$ concentration (compounds causing nicotinic receptor stimulation or K^+ -induced depolarization or calcium ionophores). Increased activation and phosphorylation of tyrosine hydroxylase on depolarization with high K^+ or treatments with cholinergic agonists, dibutyryl cAMP, phorbol esters and bioactive neuropeptides (vasoactive intestinal polypeptide and pituitary adenylate cyclase-activating polypeptide) in adrenal chromaffin cells were reported (17-32).

Fig. 1 compares the activation and phosphorylation of tyrosine hydroxylase produced by bradykinin

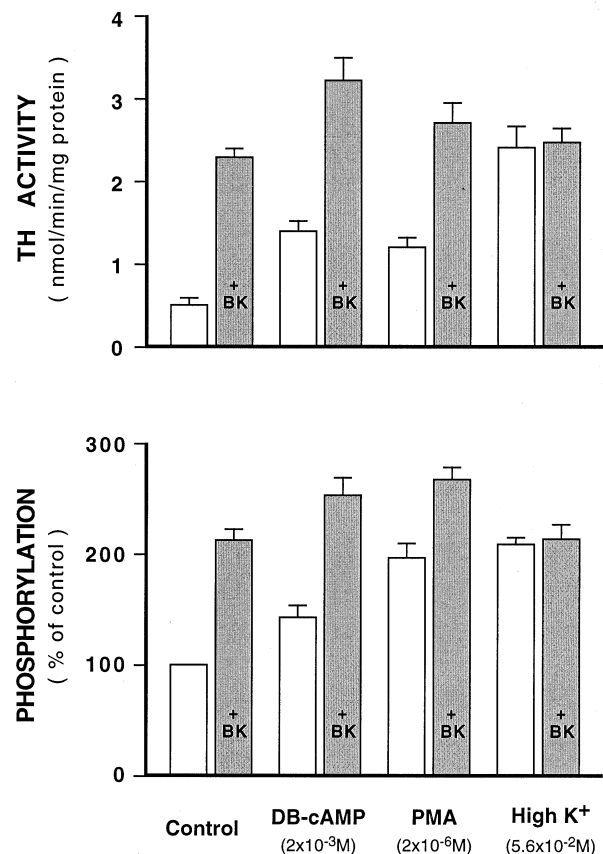


Fig.1. Effects of bradykinin on dibutyryl cAMP-, PMA- and high K^+ -stimulated activation and phosphorylation of tyrosine hydroxylase in PC-12 cells. The PC-12 cells were incubated for 15 min with concentrations of dibutyryl cAMP ($2 \times 10^{-3} \text{M}$), PMA ($2 \times 10^{-6} \text{M}$) or high K^+ ($5.6 \times 10^{-2} \text{M}$). These concentrations had previously been shown to produce maximal activation and phosphorylation of tyrosine hydroxylase. The PC-12 cells were incubated with bradykinin (10^{-5}M) for 5 min. (28)

with the activation and phosphorylation of tyrosine hydroxylase produced by treatment with dibutyryl cAMP, phorbol ester phorbol myristate acetate (PMA) and high K^+ in pheochromocytoma PC-12. Concentrations of these compounds were chosen to produce maximal activation and phosphorylation of tyrosine hydroxylase and the additivity of this activation and phosphorylation with that produced by bradykinin was evaluated. The increase in tyrosine hydroxylase activity produced by bradykinin (356%) and dibutyryl cAMP (181%) were additive (544%). Likewise, the enhanced phosphorylation of tyrosine hydroxylase produced by bradykinin (114%) and dibutyryl cAMP (44%) were additive (153%). PMA increased by 19% the activation of tyrosine hydroxylase produced by bradykinin. Likewise, the phosphorylation of tyrosine hydroxylase was increased to a similar degree. High K^+ had no effect on the increase in tyrosine hydroxylase activity or

phosphorylation produced by bradykinin. These data suggest that the mechanism of activation and phosphorylation of tyrosine hydroxylase produced by bradykinin is similar to that produced by high K^+ and to a minor extent by PMA.

In order to evaluate this further, the sites on tyrosine hydroxylase that were phosphorylated by bradykinin were determined. PC-12 cells were incubated with ^{32}P for 1 hr to label the cellular ATP stores. After exposure of the cells to bradykinin, tyrosine hydroxylase was isolated, subjected to SDS-polyacrylamide gel electrophoresis, eluted from the gels, and digested with trypsin for 12 to 18 hr at 37 °C. The ^{32}P -labeled phosphopeptides derived from tyrosine hydroxylase were separated by HPLC. Fig. 2 illustrates the HPLC elution pattern of free ^{32}P -labeled peptide peaks that were produced by bradykinin, peptide peak A (retention time, 16min), peak B (19min) and peak C (27min). The phosphorylation of peak A and B was increased markedly when PC-12 cells were treated with bradykinin, whereas peak C was unaffected. It is known that peak B is phosphorylated by activators of protein kinase C (PMA or 1-oleyl-2-acetyl-glycerol), peak C is phosphorylated by activators of cAMP-dependent protein kinase (dibutyryl cAMP or forskolin), and peak A is phosphorylated by compounds that increase $[Ca^{2+}]_i$ concentration and possibly activate calcium/calmodulin-dependent protein kinase (high K^+ or ionomycin). As bradykinin selectively stimulated the phosphorylation of peaks A and B, the possibility exists that bradykinin may activate tyrosine hydroxylase

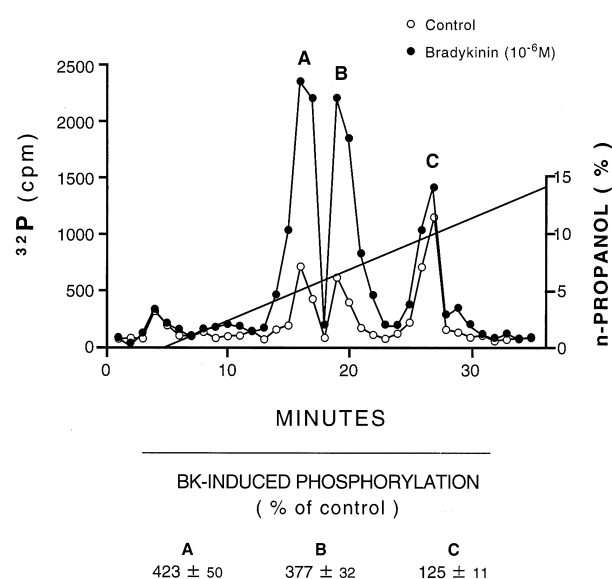


Fig. 2. HPLC analysis of tryptic phosphopeptide isolated from phosphorylated tyrosine hydroxylase obtained from PC-12 cells after treatment with bradykinin. ^{32}P -labeled tryptic peptides from tyrosine hydroxylase were separated by HPLC. (28)

by an action on both calcium/calmodulin-dependent protein kinase and protein kinase C.

Catecholamine secretion regulated by bradykinin

Activation of acetylcholine nicotinic receptors with cholinergic agents or membrane depolarization with high K^+ is known to initiate transient rapid catecholamine secretion accompanied by an increase in Ca^{2+} influx into the cells (6, 33, 34).

The time courses of catecholamine secretion induced by $10^{-6}M$ bradykinin and $10^{-5}M$ ouabain, an Na^+/K^+ ATPase inhibitor, separately or in combination were carried out in adrenal chromaffin cells. Bradykinin alone induced slight, transient secretion of catecholamine within 2 min, followed by slower, sustained secretion for at least 30 min. Ouabain alone also induced slight secretion for at least 30 min. The secretion was markedly higher with bradykinin plus ouabain, about 15% of the total intracellular catecholamine being secreted in 30 min. Stimulation of catecholamine secretion in either the presence or absence of $10^{-5}M$ of ouabain was observed with more than $10^{-8}M$ bradykinin and was maximal with $10^{-5}M$ bradykinin. The concentration of ouabain necessary to potentiate the bradykinin-induced catecholamine secretion was $10^{-6}M$ to $10^{-4}M$. Catecholamine secretion stimulated by bradykinin in the presence of ouabain was inhibited by $10^{-6}M$ D-Arg-[Hyp³, Thi^{5,8}, D-Phe⁷]-bradykinin, a bradykinin-B₂ receptor antagonist (35), but not by $10^{-6}M$ Des-Arg⁹-[Leu⁸]-bradykinin, a bradykinin-B₁ receptor antagonist (3), and was reduced in medium without Ca^{2+} . These results indicate that the stimulatory effect of bradykinin plus ouabain on catecholamine secretion was mediated via the bradykinin-B₂ receptor and dependent on the presence of Ca^{2+} in the medium. It is further indicated that stimulation of the bradykinin-B₂ receptor may stimulate the influx of Ca^{2+} into the cells.

We examined the $^{45}Ca^{2+}$ influx into the cells stimulated by bradykinin and ouabain separately or together. Bradykinin or ouabain alone increased $^{45}Ca^{2+}$ influx into the cells slightly, whereas bradykinin plus ouabain increased $^{45}Ca^{2+}$ influx markedly. The time courses of $^{45}Ca^{2+}$ influx were similar to those of catecholamine secretion, indicating that continuous $^{45}Ca^{2+}$ influx is associated with catecholamine secretion.

To determine whether Na^+ , as well as Ca^{2+} , is involved in the secretion of catecholamine induced by bradykinin and bradykinin plus ouabain, we examined their effects in Na^+ -depleted sucrose medium. We found that Na^+ -depleted sucrose medium containing Ca^{2+} itself induced secretion about 5-6% of

the total catecholamine by increasing Ca^{2+} influx into the cells. Therefore, we incubated the cells with bradykinin for 30 min in Ca^{2+} -free medium in the presence or absence of ouabain and then stimulated them for 15 min with Ca^{2+} -medium (containing 0.55-2.2mM Ca^{2+}) without bradykinin and ouabain. As shown in Fig. 3, prior stimulation of the cells with bradykinin increased the catecholamine secretion induced by each concentration of Ca^{2+} tested, and this stimulatory effect of bradykinin was greatly potentiated by the presence of ouabain. The stimulatory effect of bradykinin plus ouabain on catecholamine secretion in Na^+ -free sucrose medium was much lower. During the first stimulation of the cells with bradykinin plus ouabain in Ca^{2+} -free medium, $^{22}\text{Na}^+$ accumulation in the cells was significantly higher than that with bradykinin or ouabain alone, the levels induced by 10^{-6}M bradykinin, 10^{-5}M ouabain and 10^{-6}M bradykinin plus 10^{-5}M ouabain being 25 ± 4 , 210 ± 24 and 285 ± 31 (nmol/dish in 30 min), respectively. These results indicated that activation of the bradykinin- B_2 receptor and inhibition of the Na^+ pump by ouabain both increase the accumulation of Na^+ in the cells, resulting in increases in Ca^{2+} influx, the $[\text{Ca}^{2+}]_i$ level and catecholamine secretion (36).

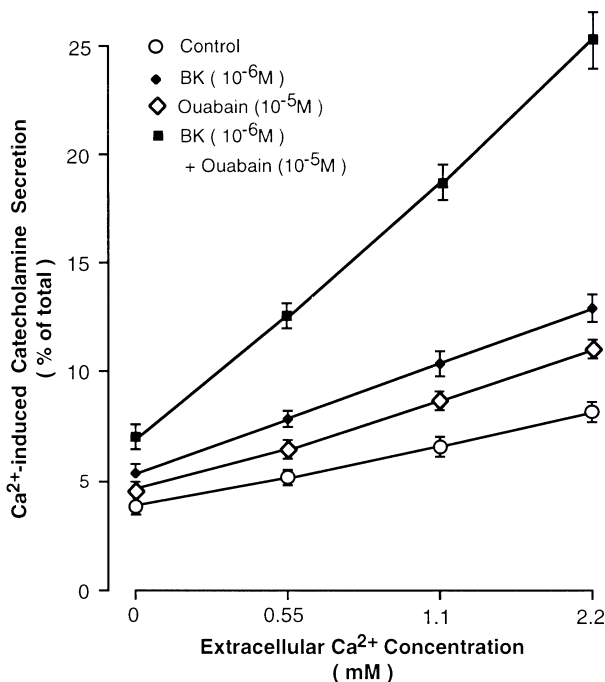


Fig. 3. Effects of extracellular Ca^{2+} on catecholamine secretion from bradykinin-and ouabain-pretreated adrenal chromaffin cells. Cells were incubated with 10^{-6}M bradykinin for 30 min in Ca^{2+} -free medium in the presence or absence of 10^{-5}M ouabain and then stimulated for 15 min with Ca^{2+} -medium (Ca^{2+} -concentration : 0.55 to 2.2mM) without bradykinin and ouabain. (36)

Calcium efflux from chromaffin cells regulated by bradykinin

After stimulation of catecholamine secretion from adrenal chromaffin cells induced by various secretagogues, the increase in $[\text{Ca}^{2+}]_i$ should rapidly return to the resting level to enable response to a subsequent stimulation (37-43). In this part, the mechanism of decrease in elevated $[\text{Ca}^{2+}]_i$ and the effect of bradykinin on Ca^{2+} efflux from adrenal chromaffin cells are discussed.

Fig. 4 shows the effluxes of $^{45}\text{Ca}^{2+}$ from adrenal chromaffin cells in culture induced by various concentrations of bradykinin. The stimulatory effect of bradykinin on $^{45}\text{Ca}^{2+}$ efflux was dose-dependent at concentrations of 10^{-9} - 10^{-6}M bradykinin. The efflux of $^{45}\text{Ca}^{2+}$ increased to a peak value within about 1 min after bradykinin addition. The peak value with 10^{-6}M bradykinin was $8.2 \pm 0.7\%$ of the total $^{45}\text{Ca}^{2+}$ in the cells. After the peak, the efflux decreased rapidly for the next 5 min. The effects of the bradykinin-receptor antagonists Des-Arg⁹-[Leu⁸]-bradykinin (B_1 -receptor antagonist (3)) and D-Arg-[Hyp³, Thi^{5,8}, D-Phe⁷]-bradykinin (B_2 -receptor antagonist (35)) on the submaximal $^{45}\text{Ca}^{2+}$ efflux from the cells induced by 10^{-7}M bradykinin were evaluated. This efflux was inhibited 81% by 10^{-6}M D-Arg-[Hyp³, Thi^{5,8}, D-Phe⁷]-bradykinin, but was not inhibited by Des-Arg⁹-[Leu⁸]-bradykinin. This result suggests that the $^{45}\text{Ca}^{2+}$ efflux

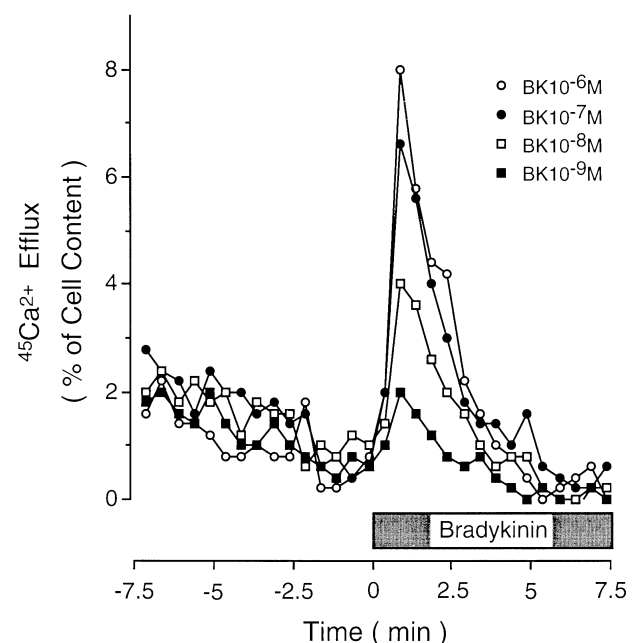


Fig. 4. Effects of different concentrations of bradykinin on $^{45}\text{Ca}^{2+}$ efflux from cultured bovine adrenal chromaffin cells. Cells were preloaded with $^{45}\text{Ca}^{2+}$ for 1 hr, and were washed 30 times at intervals of 30 sec. They were then incubated 15 times with 1 ml volumes of reaction mixture. (53)

induced by bradykinin was mediated through the bradykinin B₂-receptor.

To determine whether bradykinin-stimulated ⁴⁵Ca²⁺ efflux is mediated by activation of Ca²⁺ channels, we examined whether it was inhibited by Ca²⁺ channel blockers. Nifedipine, an organic voltage-dependent Ca²⁺ channel blocker, had no effect on ⁴⁵Ca²⁺ efflux from the cells induced by bradykinin (Fig. 5). Nor did the inorganic Ca²⁺ channel blockers Co²⁺ and Cd²⁺, which inhibit voltage-dependent and receptor-operated Ca²⁺ channels, inhibit bradykinin-stimulated ⁴⁵Ca²⁺ efflux (Fig. 5). Thus stimulation of ⁴⁵Ca²⁺ efflux by bradykinin is probably not due to increased Ca²⁺ flux through Ca²⁺ channels.

The stimulations of bradykinin, histamine and muscarinic acetylcholine receptors are reported to induce breakdown of phosphatidyl inositol 4,5-bisphosphate (PIP₂) in bovine adrenal chromaffin cells (44, 45). The breakdown products of PIP₂ (inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol) increase the [Ca₂₊]_i level and stimulate protein kinase C (24, 46, 47). The effects of bradykinin, histamine, acetylcholine and PMA (an activator of protein kinase C) on ⁴⁵Ca²⁺ efflux from the cells were examined. Bradykinin (10⁻⁶M) and histamine (10⁻⁵M) increased the ⁴⁵Ca²⁺ efflux to about 460% and 350% of the control level, respectively. Acetylcholine (10⁻⁴M) increased ⁴⁵Ca²⁺ efflux from the cells slightly, but significantly, whereas PMA (10⁻⁶M) had no effect on ⁴⁵Ca²⁺ efflux. These results suggest that bradykinin-stimulated ⁴⁵Ca²⁺ efflux from the cells is related to the forma-

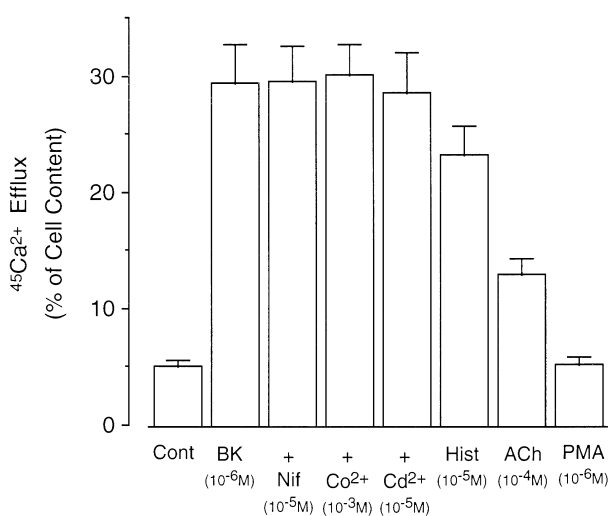


Fig. 5. Effects of various agents on ⁴⁵Ca²⁺ efflux from cultured bovine adrenal chromaffin cells. Cells were incubated in the presence or absence of bradykinin (BK; 10⁻⁶M), histamine (Hist; 10⁻⁵M), acetylcholine (ACh; 10⁻⁴M) or PMA (10⁻⁶M). Nifedipine (Nif; 10⁻⁵M), Co²⁺ (10⁻³M) or Cd²⁺ (10⁻⁵M) was added 150 sec before bradykinin (10⁻⁶M). ⁴⁵Ca²⁺ efflux 5 min after addition of agents was calculated. (53)

tion of IP₃, but not to activation of protein kinase C.

To determine whether the increased ⁴⁵Ca²⁺ efflux induced by bradykinin is dependent on the elevation of [Ca²⁺]_i level, the effects of bradykinin, histamine and acetylcholine on [Ca²⁺]_i concentration were examined. Bradykinin (10⁻⁶M) and histamine (10⁻⁵M) increased the [Ca²⁺]_i to approximately 430nM and 360nM from 100nM. Acetylcholine (10⁻⁴M) increased it to about 890nM. However, acetylcholine-stimulated ⁴⁵Ca²⁺ efflux from the cells was less than bradykinin- or histamine-stimulated ⁴⁵Ca²⁺ efflux (Fig.5). Therefore, bradykinin-stimulated ⁴⁵Ca²⁺ efflux from the cells may not be dependent on the elevation of the intracellular [Ca²⁺]_i level in the cells.

It has been established that bradykinin increases cyclic GMP (cGMP) in adrenal chromaffin cells through the activation of guanylate cyclase (48). Therefore, the role of cAMP and cGMP on Ca²⁺ efflux from cultured bovine adrenal chromaffin cells was evaluated to determine whether these intracellular messengers are involved in regulation of the Ca²⁺ efflux mechanism. The effects of dibutyryl cAMP (10⁻³M), forskolin (10⁻⁶M : an activator of adenylate cyclase (18)), dibutyryl cGMP (10⁻³M) and nitroprusside (10⁻³M : an activator of guanylate cyclase (49, 50)) on ⁴⁵Ca²⁺ efflux from cultured bovine adrenal chromaffin cells preloaded with ⁴⁵Ca²⁺ were examined. All these agents stimulated efflux of ⁴⁵Ca²⁺ from the cells. These stimulations of ⁴⁵Ca²⁺ efflux were clearly observed at concentrations of more than 2x10⁻⁴M of dibutyryl cAMP and dibutyryl cGMP, 10⁻⁷M of forskolin and 10⁻⁴M of nitroprusside. Since these agents did not affect the [Ca²⁺]_i level measured using the Ca²⁺ indicator fura-2 (control 98 ± 8; 10⁻³M dibutyryl cAMP 103 ± 10; 10⁻⁶M forskolin 101 ± 9; 10⁻³M dibutyryl cGMP 97 ± 9; 10⁻³M nitroprusside 102 ± 11 nM, respectively), this stimulation of Ca²⁺ efflux was not the result of increase in the [Ca²⁺]_i level. These results suggest that both cAMP and cGMP are involved in stimulation of Ca²⁺ efflux from the cells, through these nucleotide-dependent protein kinases (51).

To determine whether the increased ⁴⁵Ca²⁺ efflux induced by bradykinin is Na⁺-dependent, we carried out a series of experiments in the absence of extracellular Na⁺. As shown in Fig. 6, complete replacement of Na⁺ by sucrose significantly blocked the enhanced ⁴⁵Ca²⁺ efflux from the cells induced by bradykinin. Amiloride, an inhibitor of the Na⁺/Ca²⁺ exchanger (52), also significantly inhibited bradykinin-stimulated ⁴⁵Ca²⁺ efflux from the cells. Therefore, the effect of bradykinin in stimulating Ca²⁺ efflux across the plasma membrane

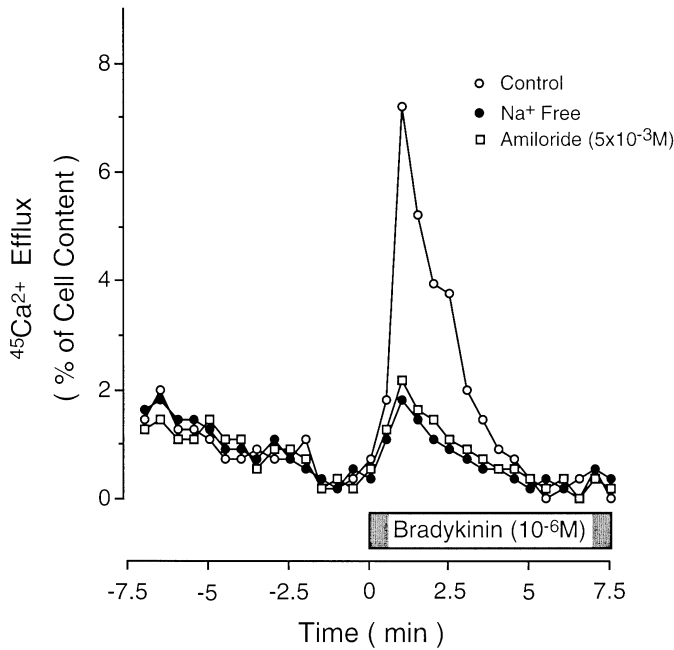


Fig. 6. Effects of Na⁺-free medium and amiloride on bradykinin-induced ⁴⁵Ca²⁺ efflux from cultured bovine adrenal chromaffin cells. The medium was changed to Na⁺-free medium (with sucrose instead of all Na⁺) 150 sec before adding bradykinin (10⁻⁶M). In the case of the amiloride experiment, amiloride (5 × 10⁻³M) was added 150 sec before bradykinin (10⁻⁶M) in normal medium. (53)

may be mediated in part by a Na⁺/Ca²⁺ exchange mechanism (53).

Bradykinin-stimulated ⁴⁵Ca²⁺ efflux from the cells may be regulated by nitric oxide (NO)/ cGMP pathway because 1) Bradykinin increases the formation of cGMP (48), 2) an increase in cGMP formation stimulates the calcium efflux from adrenal chromaffin cells (51), and 3) the efflux of calcium is regulated by NO production (54). However, we have no data on whether bradykinin stimulates NO production in adrenal chromaffin cells.

CONCLUSION

The role of bradykinin on stimulus-secretion coupling in adrenal chromaffin cells is illustrated in Fig 7. Bradykinin receptors are classified as B₁ and B₂ receptor subtypes. These receptors are present on the adrenal chromaffin cell membrane. Bradykinin increases the influx of calcium and the activity of phospholipase C through the stimulation of bradykinin B₂ receptor on the cell membrane. Diacylglycerol is produced concurrently with IP₃ on breakdown of PIP₂ by phospholipase C and is thought to activate protein kinase C by increasing the affinity of the enzyme for calcium (46). In the

pathway of catecholamine biosynthesis, an increase in [Ca²⁺]_i and production of diacylglycerol stimulate the activation of calcium-dependent protein kinases (calcium/calmodulin-dependent protein kinase and protein kinase C). These kinases phosphorylate and activate tyrosine hydroxylase, a rate-limiting enzyme in the biosynthesis of catecholamine. The catecholamine which is formed by tyrosine hydroxylase is stored in chromaffin granules. In the pathway of catecholamine secretion, the stimulation of the bradykinin-B₂ receptor elevates the accumulation of Na⁺ in the cells. An increase in intracellular Na⁺ concentration leads to Ca²⁺ influx into the cells through the depolarization of the cell membrane and/or the reverse mode of Na⁺/Ca²⁺ exchange mechanism. The secretion of catecholamine from the cells is initiated by the raise of [Ca²⁺]_i. However, the detailed mechanism of the calcium-induced catecholamine secretion pathway remains unclear.

Physiological stimulation of adrenal chromaffin cells cause an increase in [Ca²⁺]_i, leading to initiation of stimulus-secretion coupling. However, this increased [Ca²⁺]_i should be restored to a physiological level for response to subsequent stimulation. In the pathway of Ca²⁺ efflux, bradykinin increases Ca²⁺ efflux from the cells through the stimulation of the bradykinin-B₂ receptor. Bradykinin-induced Ca²⁺ efflux may be explained by an extracellular Na⁺-dependent mechanism, probably through acceleration of Na⁺/Ca²⁺ exchange. It is interesting

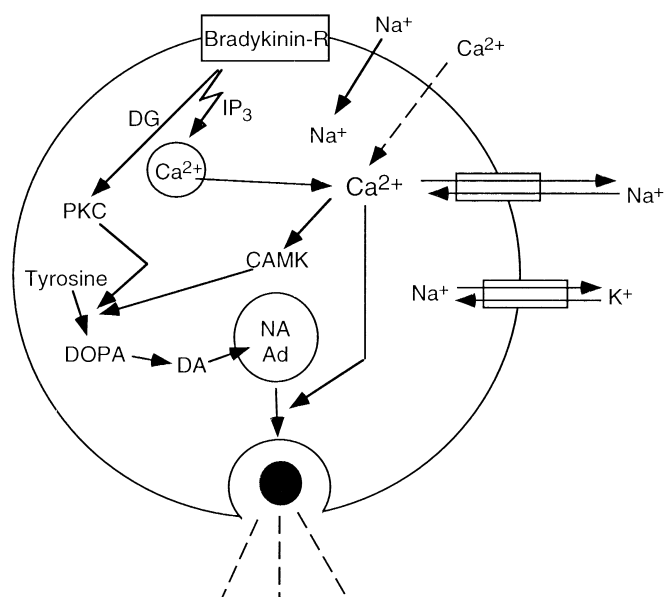


Fig. 7. Schematic stimulus-secretion coupling in adrenal chromaffin cells.

that bradykinin, which stimulates the biosynthesis and secretion of catecholamine in adrenal chromaffin cells, plays a role in the termination of Ca^{2+} -signal transduction through the stimulation of Ca^{2+} efflux from the cells.

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