CHARACTERISTICS OF BRADYKININ-EVOKED SECRETORY RESPONSE IN THE PERFUSED RAT ADRENAL MEDULLA

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ABSTRACT

Catecholamine secretion in the perfused rat adrenal medulla stimulated by bradykinin was characterized. The mean adrenaline secretion rate over the first 4 min after exposure to 100 nM bradykinin was 280 pmol/min. The secretion decreased by desensitization to 40% of the initial level within 20 min. The stimulant effect of bradykinin was removed by a 10-min washout but the desensitization persisted for longer periods. Although noradrenaline secretion was also evoked by bradykinin, it amounted to only 4% of the adrenaline secretion, which was markedly smaller as compared with 24% for acetylcholine (ACh)-evoked noradrenaline secretion. In a Ca^{2+} -deficient medium, the bradykinin-evoked catecholamine secretion was reduced by 49% of the control (2 mM Ca^{2+}), whereas the ACh-evoked secretion by 87%. The bradykinin-evoked secretion was almost completely inhibited in 1 mM EGTA-containing, Ca^{2+} -free medium. The presence of a Ca^{2+} channel blocker, methoxyverapamil (D600) (100 μM) did not affect the bradykinin-evoked secretion but inhibited the ACh-evoked secretion by 80%. These results suggest that bradykinin leads to the catecholamine secretion by a pathway different from that for ACh.

Although bradykinin is known as a potent stimulant for adrenal medullary catecholamine secretion (2, 10, 15), the secretory response has not been characterized in detail. Accordingly, the mechanism of action of bradykinin resulting in the secretion remains unelucidated.

In the present study, it is demonstrated that catecholamine secretion evoked by bradykinin is different from that evoked by acetyl-

choline in its requirement for extracellular calcium and in its sensitivity to a Ca channel-blocking agent.

MATERIALS AND METHODS

Perfusion of the adrenal gland was carried out by a method similar to that described by Kidokoro and Ritchie (5). The left adrenal gland of male Wistar rats (250-450 g) was cannulated under sodium pentobarbital anesthesia through the adrenal vein and perfused retrogradely with a standard medium (modified Krebs solution). The gland was then removed and stabilized by a 50-min perfusion with the standard medium before initiation of secretory stimulation. The solutions with which the adrenal gland was perfused at a rate of

Abbreviations: ACh, acetylcholine; D600, methoxyverapamil; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography

0.15 ml/min were changed according to experimental protocols by switching a teflon rotary valve (Rheodyne) which was set between a 4-channel roller pump and the gland. Perfusate was collected at 4-min intervals into test tubes which contained perchloric acid and ethylenediaminetetraacetic acid (EDTA) at final concentrations of 0.4 N and 5 mM, respectively. Experiments were performed at room temperature (22–28°C). After the perfusion experiment, catecholamines in each adrenal gland were extracted by 0.8 N perchloric acid in 10 mM EDTA in order to determine the total catecholamine content in the gland.

Catecholamines in the samples were assayed by an electrochemical detector (LC-4B, Bioanalytical System) after separation by high performance liquid chromatography (HPLC).

Modified Krebs solution (MK solution) consists of (mM) 150 NaCl, 5.6 KCl, 2 CaCl₂, 1 MgCl₂, 1 NaH₂PO₄, 10 HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffered to pH 7.3 with NaOH and 5 glucose. Ca²⁺-Deficient-Ringer denotes MK solution from which 2 mM CaCl₂ was omitted. When Ca²⁺-deficient-Ringer was supplemented by 1 mM ethylene glycol bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), it is referred to as EGTA-Ringer. Perfusion solutions were bubbled with 100% O₂.

Bradykinin was purchased from Protein Research Institute (Osaka, Japan). Methoxyverapamil (D600) was obtained from Knolls Pharmaceutical.

RESULTS

Characteristics of Bradykinin-evoked Catecholamine Secretion

The secretion of catecholamines (adrenaline and noradrenaline) from rat adrenal medullae in response to a stimulation by 100 nM bradykinin is shown in Fig. 1. Open and hatched columns in the figure represent mean secretion rates over 4 min for adrenaline and noradrenaline, respectively. Basal rate of the catecholamine secretion shortly before the bradykinin stimulation was as low as 0.3 pmol/min (indicated by *bs*). After application of bradykinin at time zero the catecholamine secretion rate markedly increases to

295 $\pm$ 86 (mean $\pm$ SD; *n*=4) pmol/min (a 1-min lag required for the solutions to reach the gland is deleted in the figure.) The secretion decreases with time; the adrenaline output between 20 and 24 min after the onset of the bradykinin application becomes 37% of the output during the initial 4 min, while the noradrenaline output is reduced to 32% during the same period.

By washing the adrenal medulla with bradykinin-free MK solution from 24 to 36 min, the bradykinin effect is almost completely reversed. Rechallenge of the medulla with 100 nM bradykinin from 40 min induces the secretion again but the output reaches only 39% of that during the initial 4 min of the first challenge.

In Fig. 2A, adrenal medullae were intermittently stimulated 4 times by 100 nM bradykinin. Between the stimulation periods, the adrenal medulla was washed with bradykinin-free MK solution for 15 min. The mean adrenaline and noradrenaline secretion rates over

Fig. 1 Time course of catecholamine secretion from rat adrenal medulla stimulated by 100 nM bradykinin. Open and hatched parts of each column represent mean adrenaline and noradrenaline secretion rates over a 4-min perfusate collection. The mean basal level (before bradykinin stimulation) was labeled *bs*. Time after the bradykinin application was indicated by the abscissa. Between 24 to 36 min, the adrenal medulla was perfused with bradykinin-free MK solution (the perfusate was not sampled for analysis). At 40 min, the medulla was stimulated again by 100 nM bradykinin. The vertical lines indicate $\pm$ SD of 4 determinations.

Fig. 2 A: Catecholamine secretions evoked by four intermittent stimulations with 100 nM bradykinin. The responses to the respective stimulations are represented by the numbered columns, in which the open and hatched parts indicate mean adrenaline and noradrenaline secretion rates over 4-min collection periods, respectively. Between the stimulations, adrenal medulla was perfused with bradykinin-free MK solution for 15 min. The basal secretion rate before bradykinin secretion was shown above the mark *bs*. The vertical lines indicate $\pm$ SD of 4 determinations. B: Catecholamine secretion evoked by four intermittent stimulations with 100 μ M ACh. Experimental design was the same as in A.

a 4-min perfusate collection during 4 respective stimulations are represented by the numbered columns. Similarly, the catecholamine secretion evoked by 100 μ M acetylcholine (ACh) was determined and presented in Fig. 2B.

The results presented in Figs. 2A and 2B served as controls for experiments such as exemplified in Fig. 4, in which effects of various agents on the bradykinin- and ACh-evoked catecholamine secretions were evaluated.

As shown in Figs. 2A and 2B, the ratio of noradrenaline to adrenaline secretion (NA/A ratio) largely differs depending on whether the secretion is evoked by bradykinin or ACh. Namely, the NA/A ratio (on a molar basis) for bradykinin during the initial 4-min after stimulation was calculated to be 0.039 ± 0.023 ($n=10$). On the other hand, the corresponding value for ACh is 0.24 ± 0.10 ($n=11$). The latter figure is more or less close to a ratio of NA to A in the adrenal gland, 0.29 ± 0.12 ($n=21$), which was determined with the gland extract.

Effect of Various Agents on the Bradykinin-evoked Secretion

Using the same experimental protocol as in Fig. 2, influences of various agents on bradykinin- and ACh-evoked catecholamine secretions were examined. In Fig. 3A, for instance, the effect of a complete omission of calcium ions from the external medium by the use of EGTA-Ringer on the bradykinin-evoked catecholamine secretion was shown in the columns 2 and 4 in comparison with the secretion in MK solution (columns 1 and 3). In these experiments, adrenal medullae were perfused with EGTA-Ringer for 15 min prior to the second and fourth stimulations with 100 nM bradykinin in EGTA-Ringer. The catecholamine output in each experiment was normalized to that of the first stimulation (column 1) in order to eliminate a large variation in the absolute level of the evoked secretion in individual glands and to facilitate comparison among results obtained from similar experiments.

The effect of a calcium channel blocker,

Fig. 3 A: The influence of Ca^{2+} -free, EGTA (1 mM) containing medium (EGTA-Ringer) on bradykinin-evoked catecholamine secretion. The columns represent adrenaline (open parts) and noradrenaline (hatched parts) secretion rates in response to four intermittent stimulations with 100 nM bradykinin either in MK solution (columns 1 and 3) or in EGTA-Ringer (columns 2 and 4). The catecholamine output are normalized to the level of first stimulation. The same experimental protocol as in Fig. 2 was used. B: The influence of D600 on ACh-evoked catecholamine secretion. D600 at 100 μM was presented during second and fourth stimulations with 100 μM ACh.

D600, on ACh-evoked catecholamine secretion is shown in Fig. 3B. The second and fourth stimulations were carried out in the presence of 100 μM D600.

Results are summarized in Figs. 4A (for ACh) and 4B (for bradykinin) by displaying the second columns from experiments such as exemplified by Figs. 3A and 3B. The catecholamine secretion evoked by bradykinin in Ca^{2+} -deficient-Ringer is reduced by 49% of the control and further inhibited by 92% in EGTA-Ringer. The ACh-evoked secretion is more extensively suppressed (by 87%) in Ca^{2+} -deficient-Ringer as compared with the bradykinin-evoked secretion.

It is noted that D600 has no significant effect (*t*-test, $P > 0.05$) on the bradykinin-evoked secretion whereas it suppresses the ACh-evoked secretion by 80%. On the other hand, the addition of 2.5 mM CoCl_2 to MK solution inhibits bradykinin-evoked secretion by 85%. A complete inhibition of ACh-evoked secretion in the rat adrenal medulla by CoCl_2 was reported by Kidokoro and Ritchie (4).

In addition to those presented in Fig. 4, three other agents were examined by two experiments for each. Atropine (50 μM) did not affect the bradykinin-evoked secretion whereas it caused a 78% inhibition of the ACh-evoked secretion. Anesthetics, ketamine (100 μM) and lidocaine (1 mM) did not suppress the bradykinin evoked secretion but reduced the ACh-evoked secretion by 60% and 80%, respectively.

In the experiments such as shown in Fig. 3, the response to the third stimulation (third column) indicates whether or not the influence of the presence or absence of the agents (second column) was thoroughly removed after a 15-min wash. In the cases examined here, only the inhibitory effect of D600 exhibited during the second stimulation still remained to the significant degree (*t*-test, $P < 0.05$) during the third stimulation (*cf.* Figs. 2B and 3B). When the fourth columns were compared, the effects of the agents on the secretory responses were essentially the same as shown in Fig. 4.

Fig. 4 Comparison of the influence of various agents on ACh (100 μ M)-evoked (A) and bradykinin (100 nM)-evoked (B) catecholamine secretions. Adrenaline (open columns) and noradrenaline (hatched columns) secretion rates during second stimulation in the protocol as in Fig. 2 are presented after normalization to the catecholamine output during first stimulation. The labels denote that adrenal medullae were stimulated in normal MK solution (cont); in Ca^{2+} -deficient-Ringer ($-Ca^{2+}$); in EGTA-Ringer (EGTA); in 100 μ M D600-containing MK solution (D600); and in 2.5 mM $CoCl_2$ -containing MK solution ($+Co^{2+}$).

DISCUSSION

Characteristics of Bradykinin-evoked Catecholamine Secretion

In this study, various aspects of the bradykinin-evoked catecholamine secretion from the rat adrenal medulla were investigated. It was shown that 50 μ M atropine did not affect the bradykinin-evoked secretion. On the other hand, the ACh-evoked catecholamine secretion was largely inhibited by 50 μ M atropine as was reported by Kidokoro and Ritchie (5). Therefore, either nerve terminals which innervate adrenal medulla cells or postsynaptic

ACh receptors are not the site for bradykinin to evoke catecholamine secretion.

During a continuous stimulation by 100 nM bradykinin, the catecholamine secretion rate decreased, showing about a 40% reduction within 20 min (Fig. 1). The total secretion in this period amounted to about 5% of the total catecholamine content of the medulla. Thus, the secretory response to bradykinin was desensitized before the catecholamine store in the medulla was depleted. While the stimulant effect of bradykinin was reversed quickly (within 12 min) by washout, the desensitizing effect remained in a longer period as indicated from the low efficiency of the second stimulation (Fig. 1).

It was found that the NA/A ratio in catecholamine secretion with bradykinin was markedly lower than that with ACh. The lower potency of bradykinin for the noradrenaline secretion may result from one of two possibilities. First, bradykinin receptors on noradrenaline secreting-cells are fewer in number than those on adrenaline-secreting cells. Second, bradykinin can activate secretory processes more effectively in adrenaline-secreting cells.

Role of Extracellular Ca^{2+} for the Bradykinin-evoked Secretion

The requirement for extracellular Ca^{2+} in adrenomedullary secretion evoked by bradykinin was shown by Poisner and Douglas (10). Their result was confirmed in this study when the adrenal medulla was exposed to bradykinin in EGTA-Ringer (Fig. 4B). In Ca^{2+} -deficient-Ringer, on the other hand, the bradykinin-evoked secretion was only partially suppressed whereas the ACh-evoked secretion was extensively inhibited (Fig. 4B).

The dependence of the bradykinin-evoked secretory response on the extracellular calcium concentration, $[Ca^{2+}]_o$, may be correlated with the observation made by Osugi *et al.* (9) in neuroblastoma $\times$ glioma cells. They reported that bradykinin induced two phases of increase in the intracellular free calcium concentration, $[Ca^{2+}]_i$. On lowering $[Ca^{2+}]_o$ to a micromolar range, the transient (first) phase of $[Ca^{2+}]_i$ increase was sustained whereas the second phase was diminished. Both phases of $[Ca^{2+}]_i$ change were totally abolished in

EGTA-containing, Ca^{2+} -free medium. Since submicromolar Ca^{2+} was likely contaminated in our Ca^{2+} -deficient-Ringer due to mixing of the solutions in the perfusion circuit and a release of Ca^{2+} from the tissue, it may be concluded that the $[\text{Ca}^{2+}]_o$ dependence of the bradykinin-evoked catecholamine secretion mimics that of the bradykinin-induced transient phase of $[\text{Ca}^{2+}]_i$ increase exhibited in the cell line.

We found that the bradykinin-evoked catecholamine secretion was insensitive to D600 (Fig. 4A). Osugi *et al.* (9) also showed that the bradykinin-induced transient phase of $[\text{Ca}^{2+}]_i$ increase was unaffected by verapamil and nifedipine.

Mechanism of Catecholamine Secretion by Bradykinin

In a recent study by Harish *et al.* (3), properties of muscarinic receptor-mediated catecholamine secretion from the rat adrenal medulla were clarified. The muscarine-evoked secretion was sustained either in a Ca^{2+} -deficient medium or in the presence of verapamil, but abolished during a short-term exposure of the medulla to EGTA-containing, Ca^{2+} -free medium. Thus, these characteristics of the muscarine-evoked catecholamine secretion is strikingly similar to those of the bradykinin-evoked secretion shown in this study.

As to the mechanism by which muscarine evokes the catecholamine secretion, Harish *et al.* (3) suggested that muscarinic receptors mediated the breakdown of phosphatidylinositol bisphosphate (PIP_2) to produce inositol trisphosphate (IP_3), which in turn induced calcium release from intracellular pools (1) and led to the catecholamine secretion. This pathway might be involved in the bradykinin-evoked catecholamine secretion since the occurrence of bradykinin-induced PIP_2 breakdown was reported in neuroblastoma $\times$ glioma cells (12, 13) and bovine adrenal medulla cells in culture (8).

The above speculation raises an obvious question about why the use of EGTA-Ringer abolished the bradykinin-evoked secretion if the secretion was mediated by IP_3 -induced intracellular Ca^{2+} mobilization. Two possibilities may be considered. First, EGTA-containing medium may cause a removal of tightly

bound calcium from the external cell surface, which might inhibit the IP_3 production. However, it was demonstrated by Yano *et al.* (14) that bradykinin-induced PIP_2 hydrolysis took place in EGTA-containing medium. Second, a prolonged exposure of cells to Ca^{2+} -free medium may cause the depletion of intracellular Ca^{2+} stores, which causes a gradual reduction of IP_3 -induced Ca^{2+} release. In fact, Harish *et al.* (3) demonstrated that muscarine-evoked catecholamine secretion was diminished in EGTA-containing medium within 6 min whereas it maintained a 70–60% secretion after a 20-min exposure to Ca^{2+} -deficient medium.

Although IP_3 is a probable second messenger for bradykinin-mediated catecholamine secretion from adrenal medulla, the result obtained by Swilem *et al.* (11) argues against this view. They described that muscarinic stimulation caused IP_3 production in bovine adrenal medulla cells but did not either induced $[\text{Ca}^{2+}]_i$ increase or evoked catecholamine secretion. Furthermore, the cause of inhibition of bradykinin-evoked catecholamine secretion in the presence of Co^{2+} (Fig. 4B) is not yet understood.

Under the circumstances, alternative possibilities are not ruled out. For instance, bradykinin might activate some type of Ca^{2+} channels which is sensitive to Co^{2+} but insensitive to D600. Also, phospholipid breakdown might induce, by some mechanism, a Ca^{2+} -influx as proposed previously by Michell (6). The latter idea was recently fortified with the result obtained by Morris *et al.* (7). They suggested that inositol tetrakisphosphate which is formed from IP_3 may act, synergistically with IP_3 , to stimulate Ca^{2+} uptake from the extracellular medium.

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