BRADYKININ-INDUCED CALCIUM MOBILIZATION AND CATECHOLAMINE SECRETION IN RAT ADRENAL MEDULLARY CELLS

AKIRA WARASHINA¹, NAOSHI FUJIWARA² and KOKI SHIMOJI²

Departments of ¹Physiology and ²Anesthesiology, Niigata University School of Medicine, Niigata 951, Japan

ABSTRACT

Secretory responses in the perfused rat adrenal medula during stimulation with bradykinin and nicotine were studied by fluorometric monitoring of intracellular free Ca^{2+} concentration, $[\text{Ca}^{2+}]_i$, and electrochemical detection of catecholamine release. Bradykinin elicited the cellular response through bradykinin B_2 receptor. The time course of catecholamine secretion evoked by bradykinin ($>100 \text{ nM}$) was very similar to that of change in $[\text{Ca}^{2+}]_i$, showing a prominent, initial transient followed by a maintained response. An intracellular Ca^{2+} antagonist, TMB-8, suppressed the initial component of bradykinin-induced response, both in $[\text{Ca}^{2+}]_i$ rise and in secretion, without affecting the maintained response. Nifedipine, which inhibited nicotine-evoked secretion, did not apparently affect bradykinin-evoked secretion in its either phase. Both bradykinin- and nicotine-evoked secretions were blocked by Cd^{2+} . When the extracellular Ca^{2+} concentration, $[\text{Ca}^{2+}]_o$, was $16 \mu\text{M}$, the secretion induced by a 30-sec of stimulation with bradykinin was 45% of that when $[\text{Ca}^{2+}]_o$ was 2 mM. When $[\text{Ca}^{2+}]_o$ was 10 mM, the secretion occurred at a level slightly lower than that of the control (2 mM Ca^{2+}). The nicotine-evoked secretion, on the other hand, was very weak at $[\text{Ca}^{2+}]_o$ below $80 \mu\text{M}$ but increased in proportion to the log of $[\text{Ca}^{2+}]_o$ in the range from $80 \mu\text{M}$ to 10 mM. The results suggest that the initial transient phase of catecholamine secretion following bradykinin stimulation is induced partly by Ca^{2+} released from intracellular stores while the maintained secretion is associated with Ca^{2+} -entry activated by internal messenger(s) produced after bradykinin receptor activation.

Abbreviations: BK, bradykinin; CA, catecholamine; EGTA, ethylene glycol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid; fluo-3AM, 1-[2-amino-5-(2,7-dichloro-6-hydroxy-3-oxy-9-xanthonyl)phenoxy]-2-(2'-amino-5'-methylphenoxy)ethane- N,N,N',N' -tetraacetic acid, acetoxymethyl ester; HEPES, N -(2-hydroxyethyl)piperazine- N' -ethanesulfonic acid; TMB-8, 3,4,5-trimethoxybenzoic acid 8-(N,N -diethylamino)octyl ester

The stimulant effect of bradykinin (BK) on adrenal medullary catecholamine (CA) secretion was first shown by Feldberg and Lewis (4). Some pharmacological properties of BK-evoked CA secretion were investigated by Yoshizaki (21). As for the mechanism by which BK induces the secretory response, possible role of an intracellular Ca^{2+} store was suggested in previous studies (9, 18) which

demonstrated that a part of BK-evoked secretion in adrenal medullary cells remained when Ca^{2+} in the external medium was omitted. Release of Ca^{2+} from the intracellular store in BK-stimulated single bovine adrenal chromaffin cells has recently been visualized using fluorescent Ca^{2+} indicator by O'Sullivan *et al.* (10). The cellular response elicited by BK receptor stimulation is thought to be mediated, at least partly, through phosphatidylinositol metabolism. In fact, Stoehr *et al.* (14) showed that inositol trisphosphate (IP_3) was capable of mobilizing intracellular Ca^{2+} in permeabilized chromaffin cells.

In this study, BK-evoked secretion in the perfused rat adrenal medulla is investigated in detail. We studied its dependence on the extracellular Ca^{2+} concentration, on its sensitivities to Ca^{2+} channel blockers (nifedipine and Cd^{2+}), and on an intracellular Ca^{2+} antagonist (TMB-8). The role of intracellular Ca^{2+} store and the pathway through which adrenal medullary cells take up extracellular Ca^{2+} are discussed based on the characteristics of BK-evoked secretory response.

EXPERIMENTAL PROCEDURES

Real-time Monitoring of Catecholamine Secretion

Perfusion of the rat adrenal gland was carried out by the method described previously (19). In brief, the left adrenal glands of male Wistar rats (280–480 g) were removed under sodium pentobarbital anesthesia and perfused retrogradely at a rate of 0.15 ml/min through a cannula inserted into the adrenal vein. Perfusion solutions were selected with a 6-way rotary valve (type 50; Rheodyne) and introduced into the gland by a roller pump. The perfused gland was sealed in an airtight chamber made of a disposable tip of a 5-ml hand pipette. The perfusate was led into a flow cell (TL-5; Bioanalytical Systems) in which glassy carbon electrodes were installed. The electrodes were connected to a control unit (LC-4B; Biochemical Systems) for an electrochemical detection of CA (adrenaline plus noradrenaline) in the perfusate. The voltage applied to the carbon electrodes was 0.35–0.4 V. When a standard solution containing 1 μM adrenaline was passed through the flow cell at a rate of 0.15 ml/min, oxidation current of 20–30 nA

was generated. Deterioration of the electrode surface was tested with the standard solution. The decrease in the sensitivity during a 65-min experiment was usually less than 15%. Records shown in this paper were not corrected for the sensitivity change.

The detector output was sampled at 2-sec intervals with an 8-bit analog-to-digital (A/D) converter and the digitized data were stored on a magnetic disk. Records presented in this paper were drawn with an X-Y pen recorder after retrieving the stored data through a D/A conversion.

For quantitative analyses of CA output, response areas were calculated using the digitized data and compared with the size of calibration response. Our previous study (19) has shown that for analyzing adrenal medulla perfusate the method described above gives a result consistent with that obtained by a more elaborate CA detection method in which a high performance liquid chromatography (HPLC) was used.

Monitoring of Intracellular Ca^{2+} Concentration

Adrenal medullary cells were loaded with a Ca^{2+} indicator dye, fluo-3 (6), by perfusing the gland with a modified Krebs (MK) solution that contained 10 μM fluo-3 AM and 0.1% pluronic F-127. After a 1-h perfusion for fluo-3 loading, about half of the adrenal cortex was removed by dissection. Then, the gland under perfusion was placed in a chamber that was set on the stage of an inverted fluorescence microscope (IMT-2; Olympus). A 430–480 nm light from a 100 W tungsten/halogen lamp was selected with a filter assembly (type B with EL435; Olympus) and focused onto a small portion (about 100 μm in diameter) of the medulla. The fluo-3 fluorescence with the wavelength longer than 510 nm was measured with a photomultiplier.

Data obtained were recorded in the same way as described above for the secretion experiment. The resting fluorescence decreased during measurement due to photobleaching and leakage of fluo-3. The record presented was corrected each for this attenuation by a computer calculation, assuming an exponential decay curve through the resting fluorescence levels at the beginning and at the end

of experiment. The time constant of the attenuation was 220 ± 59 min (mean $\pm$ SD; $n=22$).

Data Presentation

Records presented in this paper are representative of more than three independent experiments unless otherwise mentioned.

Solutions

A modified Krebs solution (MK solution) was used as the standard perfusion medium. The MK solution consisted of (mM) 150 NaCl, 5.6 KCl, 2 CaCl₂, 1 MgCl₂, 5.6 glucose, 1 NaH₂PO₄-10 HEPES buffered to pH 7.3 with NaOH. Ca²⁺-free-Krebs solution denotes MK solution from which Ca²⁺ was omitted (nominally Ca²⁺-free). Ca²⁺-free-Krebs solution supplemented with 1 mM EGTA is referred to as EGTA-Krebs. When Cd²⁺ was added, phosphate in MK solution was removed to prevent the precipitation. Perfusion solutions were bubbled with 100% O₂ at room temperature (23–27°C).

Chemicals

Bradykinin and bradykinin analogs were purchased from Protein Research Institute (Osaka), fluo-3, fluo-3AM and HEPES from Dojin Chemicals (Kumamoto), nifedipine and TMB-8 from Sigma Chemicals (St. Louis, MO), pluronic F-127 from Calbiochem (La Jolla, CA).

RESULTS

Catecholamine Secretion Evoked by Bradykinin at Various Concentrations

Fig. 1A shows the time course of the oxidation current produced by CA in the perfusate from a rat adrenal gland stimulated for 13.5 min with various doses of BK. At higher concentrations of BK, the secretory response consisted of an initial, transient response followed by a maintained response that lasted as long as BK stimulation was present as shown by the records for 100 nM and 500 nM BK in the figure.

The amount of CA (adrenaline plus noradrenaline) released during the initial 12 min after the onset of stimulation with various

Fig. 1 A: Catecholamine (CA) secretion elicited in a perfused rat adrenal medulla by bradykinin (BK) at various concentrations. Changes in the oxidation current produced by CA in the perfusate are shown. The period (13.5 min) in which the medulla was exposed to BK was indicated by the horizontal line along with the concentration of BK at each record. Due to a dead volume in the perfusion line, the exposure period was delayed by 80 sec from the onset- (the downward blip) and offset-manipulation (the upward blip) to change the perfusion solutions. The record for 3.3 nM BK was taken with a sensitivity 2.5 times higher than the others. The vertical line represents the current (14 nA) produced by a standard solution (1 μ M adrenaline), which is equivalent to CA secretion rate at 150 pmol/min. B: Dose-response curve for BK-evoked CA secretion. The amount of secretion during the initial 12 min following stimulation with BK at various concentrations is normalized for each gland to that evoked by 100 nM BK. Unity on the ordinate corresponds to CA secretion (for the 12-min period) of 1.6 ± 0.6 nmol/gland ($n=10$). The data are expressed by mean $\pm$ SD ($n=4$ or 5).

doses of BK was evaluated using response curves such as shown in Fig. 1A. For each adrenal gland, three to four measurements were performed at 30-min intervals with

Fig. 2 BK- or nicotine-evoked CA secretion at a low extracellular Ca^{2+} concentration. Aa: The change in CA secretion in response to 6-successive, 30-sec stimulations at 10-min intervals with 200 nM BK. Ab: The secretion profile elicited by 200 nM BK in the same way as in *a* except that the perfusion medium was changed 5 min ahead of the fourth stimulation from MK solution (2 mM Ca^{2+}) to 16- μM - Ca^{2+} -Krebs solution. B, *a* and *b*: The secretion profiles elicited by 10 μM nicotine with the same experimental protocol as in A. The vertical line at each record corresponds to CA secretion rate at 150 pmol/min.

increasing doses of BK, including always a measurement with 100 nM BK. The results from each gland were normalized to the CA secretion evoked by 100 nM BK and are plotted in Fig. 1B. The half-maximal secretion occurs at a concentration of BK around 100 nM.

Dependence of Bradykinin- and Nicotine-evoked Catecholamine Secretion on Extracellular Ca^{2+} Concentration

The record *a* in Fig. 2A illustrates the pattern of CA secretion associated with 6-successive, 30-sec stimulations with 200 nM BK at 10-min intervals. In the experiment of the record *b*, the Ca^{2+} concentration of the perfusion solution, $[\text{Ca}^{2+}]_o$, was lowered from normal (2 mM) to 16 μM . The records *a* and *b* in Fig. 2B were similarly obtained with 10 μM nicotine as a stimulant.

To examine the dependence of BK-evoked secretion on $[\text{Ca}^{2+}]_o$, a series of experiments with the same stimulus protocol as in the record *b* in Fig. 2 were performed by changing $[\text{Ca}^{2+}]_o$ to various levels. The result is shown

Fig. 3 Dependence of BK- or nicotine-evoked CA secretion on the extracellular Ca^{2+} concentration, $[\text{Ca}^{2+}]_o$. The relative size (peak area) of the fourth response elicited, such as shown in Fig. 2, Ab and Bb, by BK (200 nM) or nicotine (10 μM) at various levels of $[\text{Ca}^{2+}]_o$ is plotted by the filled and open circles, respectively. The data were normalized to S4-values, the presumptive sizes of the fourth responses in 2 mM Ca^{2+} medium (for detail, see the text). The CA secretions evaluated from the means of S4-values were 450 ± 100 pmol/gland ($n=16$) for BK and 490 ± 120 pmol/gland ($n=16$) for nicotine. The data are expressed by mean $\pm$ SD ($n=4$).

by filled circles in Fig. 3, in which the peak area (P4) of the fourth response at the indicated level of $[Ca^{2+}]_o$ on the abscissa was normalized to S4, the fourth response area which would have been obtained if it had been elicited at 2 mM Ca^{2+} without changing Ca^{2+} level. In practice, S4 in each determination was evaluated by a proportional extrapolation from P2 and P3, peak areas of the second and third responses. This analysis assumes that waning of the response during the successive stimulations always occurs in a proportional manner, despite a large variation in its extent for each gland. This was valid in seven control experiments (such as shown in Fig. 2Aa) in which 6-successive responses were all elicited at 2 mM Ca^{2+} . In these measurements, P2, P3 and P4 satisfied the relation, $(P4/P3) = (P3/P2)$.

The same normalization procedure was used to plot the dependence on $[Ca^{2+}]_o$ of CA secretion evoked by 10 μ M nicotine. The result was illustrated by the open circles in Fig. 3.

As seen in Fig. 3, the dependence of BK-evoked secretion on $[Ca^{2+}]_o$ differed from that of nicotine-evoked secretion. At $[Ca^{2+}]_o$ as low as 16 μ M, nicotine-evoked secretion was inhibited to 11% of control, whereas the secretion by BK remained at 45%. BK-evoked secretion at 10 mM Ca^{2+} was slightly less than the control (2 mM Ca^{2+}). This contrasts with the nicotine-evoked secretion which increased in proportion to the log of $[Ca^{2+}]_o$, reaching 150% of the control at 10 mM.

These differences suggest that the secretory responses induced by BK and nicotine are mediated by distinct mechanisms. The release of Ca^{2+} from intracellular stores may partially contribute to CA secretion evoked by stimulation with BK as judged from the remaining response at low Ca^{2+} concentrations.

Effect of TMB-8 on Bradykinin-evoked Secretion

Fig. 4 depicts the influence of an intracellular Ca^{2+} antagonist, TMB-8, on BK-evoked secretion. In the presence of TMB-8 at 50 μ M, the secretion elicited by 30-sec stimulation with 200 nM BK in MK solution was inhibited by $58 \pm 6\%$ ($n=5$) of the control (Fig. 4Aa). This inhibition occurred in the initial phase of the

Fig. 4 The effect of TMB-8 on BK-evoked CA secretion. Aa: The change in CA secretion in response to 6-successive, 30-sec stimulations at 10-min intervals with 200 nM BK. The perfusion medium was changed 5 min ahead of the fourth stimulation from MK solution to TMB-8 (50 μ M)-containing MK solution. Ab: The secretion profile associated with 12-min stimulation with 200 nM BK in the presence of 50 μ M TMB-8. Prior to the stimulation, the medulla was washed for 30 min with TMB-8-containing MK solution. Ac: The secretion associated with 12-min stimulation with 200 nM BK in the absence of TMB-8. Prior to the stimulation, the medulla was washed for 10 min with TMB-8-containing MK solution and subsequently for 20 min with TMB-8-free MK solution. B: The secretion profiles elicited by 200 nM BK with the same experimental protocol as in A except that 16- μ M- Ca^{2+} -Krebs solution was used during the period in which 50 μ M TMB-8 was present. The vertical lines in A and B correspond to CA secretion rate at 150 pmol/min.

secretory response, leaving the maintained phase little affected (Fig. 4Ab). When 50 μ M TMB-8 was applied to a gland perfused with 16- μ M- Ca^{2+} -Krebs solution, both the initial and maintained phases of BK-evoked secre-

tion were strongly suppressed (Fig. 4B, *a* and *b*).

The above result is compatible with the notion that a part of the initial phase of BK-evoked secretion may be associated with Ca^{2+} release from intracellular stores while the rest part of the secretion may be sustained by Ca^{2+} -entry through the plasma membrane.

Effects of Nifedipine and Cd^{2+} on Bradykinin- and Nicotine-evoked Catecholamine Secretions

Effect of a Ca^{2+} channel blocker, nifedipine on BK-evoked CA secretion is shown in Fig. 5A. Nifedipine at $30\ \mu\text{M}$ did not apparently affect the secretory response, either in its initial or in maintained phase (Fig. 5A, *a* and *b*). Nicotine-evoked CA secretion was, on the other hand, extensively inhibited by nifedipine (Fig. 5B, *a* and *b*).

This characteristic difference between BK- and nicotine-evoked secretory responses to nifedipine sensitivity is more precisely illustrated in Fig. 6A. In the figure, the relative response sizes as a function of nifedipine dose were plotted by the filled circles for BK and the open circles for nicotine after evaluated from records such as shown in Fig. 5, *Aa* and *Ba* by the same procedure as used to obtain Fig. 3.

It is evident from Fig. 6A that nifedipine-sensitive Ca^{2+} channels plays an essential role in the secretory pathway activated by nicotine but that is not the case with BK.

Effects of an inorganic Ca^{2+} channel blocker, Cd^{2+} on the secretory responses were examined by the same procedure and are presented in Fig. 6B. Both BK- and nicotine-evoked secretions were extensively inhibited, showing the half-block concentrations of Cd^{2+} around 50 and $130\ \mu\text{M}$, respectively.

Changes in Intracellular Free Ca^{2+} Concentration in Response to Stimulation with Bradykinin

Changes in intracellular free calcium concentration, $[\text{Ca}^{2+}]_i$, during secretory responses elicited by BK were observed using perfused adrenal medullae loaded with fluo-3 and some of records are displayed in Fig. 7. The time course of rise in $[\text{Ca}^{2+}]_i$ induced by a 12-min stimulation with 200 nM BK (the record *a* in

Fig. 5 Influence of nifedipine on BK- or nicotine-evoked CA secretion. *Aa*: The change in CA secretion associated with 6 successive, 30-sec stimulations with 200 nM BK at 10-min intervals. The perfusion medium was changed 5 min ahead of the fourth stimulation from MK solution to nifedipine ($30\ \mu\text{M}$)-containing MK solution as illustrated by the horizontal line. *Ab*: The secretion profile associated with 12-min stimulation with 200 nM BK in the presence of $30\ \mu\text{M}$ nifedipine. Prior to the stimulation, adrenal medulla was washed for 30 min with nifedipine-containing MK solution. *Ac*: The secretion with 12-min stimulation with 200 nM BK in the absence of nifedipine. Prior to the stimulation, adrenal medulla was washed for 10 min with nifedipine-containing MK solution and subsequently for 20 min with nifedipine-free MK solution. *B*: An adrenal medulla was stimulated by $10\ \mu\text{M}$ nicotine with the same protocol as in *A*. The period in which $30\ \mu\text{M}$ nifedipine was added to the perfusion solution is indicated. The vertical lines in *A* and *B* correspond to CA secretion rate at 150 pmol/min.

Fig. 7) resembled that of CA secretion elicited by BK (the records in Fig. 1A).

It is demonstrated in the record *b* of Fig. 7 that the $[\text{Ca}^{2+}]_i$ response elicited by 200 nM BK was blocked by $[\text{Thi}^{5,8}, \text{D-Phe}^7]$ -bradykinin at $8\ \mu\text{M}$. BK-evoked CA secretion was also inhibited by this bradykinin- B_2 -receptor

Fig. 6 Dependence of BK- or nicotine-evoked CA secretion on doses of nifedipine and Cd²⁺. A: The relative size (peak area) of the fourth response, such as shown in Fig. 5, Aa and Ba, elicited by BK (200 nM) or nicotine (10 μM) at various concentrations of nifedipine is plotted by the filled and open circles, respectively. The data were normalized to S4-values, the presumptive sizes of the fourth responses in nifedipine-free MK solution. The CA secretions evaluated from the means of S4-values were 400±90 pmol/gland (n=16) for BK and 430±240 pmol/gland (n=16) for nicotine. B: The dose-dependent inhibition by Cd²⁺ of BK- or nicotine-evoked secretion is shown by the filled and the open circles. The method for analysis was the same as used for A. The CA secretion evaluated from the means of S4-values were 420±120 pmol/gland (n=16) for BK and 450±190 pmol/gland (n=16) for nicotine. The data are expressed by mean±SD (n=4).

Fig. 7 Changes in the fluorescence intensity in adrenal medullae loaded with fluo-3. a: The medulla was stimulated for 12 min with BK at 200 nM. b: Bradykinin B₂ receptor blocker, [Thi^{5,8}, D-Phe⁷]-bradykinin (8 μM), was applied in a period between 8 and 13 min during 21-min stimulation with 200 nM BK. c: A large increase in the resting fluorescence of fluo-3-loaded medullary cells during an exposure to 500 μM Cd²⁺. The upward blips on the traces are the artifact created at the time when the perfusion was stopped for a few seconds to change the solutions. The record was corrected for the decline of the fluorescence intensity due to photobleaching and leakage of fluo-3. The arrow at each record indicates a 20% change of the fluo-3 fluorescence.

Fig. 8 Influences of the extracellular Ca^{2+} concentration and TMB-8 on $[\text{Ca}^{2+}]_i$ response in fluo-3-loaded adrenal medullary cells during stimulation with BK. *a*: The change in $[\text{Ca}^{2+}]_i$ in response to 4-min stimulations with 200 nM BK repeated 3 times at 19-min intervals in the normal medium. *b*: The $[\text{Ca}^{2+}]_i$ in responses induced by 200 nM BK with the same protocol as in *a* except that the second response was elicited in Ca^{2+} -free-Krebs solution. *c*: The second response in EGTA-Krebs solution. *d*: The second response in TMB-8 (50 μM)-containing MK solution. *e*: The second response was elicited in Ca^{2+} -free-Krebs solution in the presence of 50 μM TMB-8. The arrow indicates a 20% change of the fluo-3 fluorescence.

blocker (16) (records not shown). Furthermore, a bradykinin- B_1 -receptor blocker, des-Arg⁹-[Leu⁸]-bradykinin had no effect on the secretory response elicited by 200 nM BK.

When an adrenal medulla loaded with fluo-3 was perfused with a Cd^{2+} -containing MK solution (with no stimulant), a large increase in fluo-3 fluorescence preceded by a small, transient change was observed (Fig. 7c). The initial, small change may be due to a brief rise in $[\text{Ca}^{2+}]_i$ and probably has a relation to a brief secretion arising at the commencement of perfusion with Cd^{2+} -containing MK solution (records not shown). On the other hand, the large increase in fluo-3 fluorescence is not likely associated with an increase in $[\text{Ca}^{2+}]_i$ since the secretory response is strongly inhibited during this period. Rather, it is likely that Cd^{2+} enters medullary cells and binds to fluo-3. In fact, our test-tube experiment showed that properties of the Cd^{2+} -bound fluo-3 fluorescence was little distinguished from that of Ca^{2+} -bound fluo-3.

Effect of TMB-8 on Bradykinin-induced Change in Intracellular Free Ca^{2+} Concentration

BK-induced $[\text{Ca}^{2+}]_i$ changes under various conditions are examined in Fig. 8. The record *a* shows three $[\text{Ca}^{2+}]_i$ responses, at 19-min intervals, elicited each by a 4-min stimulation with 200 nM BK in MK solution. In the experiment of record *b* or *c*, the perfusion solution was changed, at 10 min prior to the second stimulation with BK, from the normal to Ca^{2+} -free- or EGTA-Krebs solutions, respectively. During perfusion of medullae with these Ca^{2+} -deprived solutions, the resting $[\text{Ca}^{2+}]_i$ is lowered as shown in the record *b* and *c*. BK-induced $[\text{Ca}^{2+}]_i$ change partially remained in Ca^{2+} -free-Krebs solution (record *b*) whereas only a small, brief response (record *c*) was observed in EGTA-Krebs solution.

Effects of TMB-8 on BK-induced $[\text{Ca}^{2+}]_i$ response are also shown in Fig. 8. In the presence of TMB-8 at 50 μM , only the initial rise in $[\text{Ca}^{2+}]_i$ is significantly reduced in MK solu-

tion (the second response in the record *d*) whereas the extensive inhibition of the response, leaving only a small brief rise, occurred in Ca^{2+} -free-Krebs solution (the second response in the record *e*).

Results very similar to those shown by the records *b* and *e* were obtained from experiments in which $16\text{-}\mu\text{M-Ca}^{2+}$ -Krebs solution was used instead of nominally Ca^{2+} -free MK solution without changing other conditions. The decrease in resting level of $[\text{Ca}^{2+}]_i$ also took place when medullae were perfused with $16\text{-}\mu\text{M-Ca}^{2+}$ -Krebs solution.

DISCUSSION

Real-time Observation of $[\text{Ca}^{2+}]_i$ Change and CA Secretion in the Rat Adrenal Medulla

Real-time monitoring of CA secretion from the rat adrenal gland, which has been established by various investigators (1, 5, 19), was carried out in this study. In addition, we developed the method to monitor $[\text{Ca}^{2+}]_i$ change in the perfused rat adrenal medulla. This combination allowed us to examine detailed relations between $[\text{Ca}^{2+}]_i$ change and CA secretion under various stimulus conditions. It was shown that CA secretion faithfully tracked the rise in $[\text{Ca}^{2+}]_i$ during BK stimulation, indicating that the level of $[\text{Ca}^{2+}]_i$ is the essential determinant for BK-evoked CA secretion.

Intracellular Ca^{2+} Mobilization in BK-stimulated Adrenal Medullary Cells

BK-evoked secretory response in the rat adrenal medullary cell is mediated through bradykinin B_2 receptor (Fig. 7*b*). The properties of this secretory response were compared with those of nicotine-evoked response. Nicotinic secretory response in adrenal medullary cells has been ascribed to Ca^{2+} -entry through voltage-dependent Ca^{2+} channels (13, 15). The extensive inhibition of the nicotine-evoked secretion at low $[\text{Ca}^{2+}]_o$ (Figs. 2*Bb* and 3) and in the presence of nifedipine and Cd^{2+} (Fig. 6) is consistent with this view.

Nifedipine, on the other hand, failed to affect BK-evoked secretory response either in the initial, transient phase or in the maintained phase. Thus, no substantial part of the secretion evoked by BK is mediated by Ca^{2+} -influx through nifedipine-sensitive Ca^{2+} chan-

nels.

The secretion elicited by BK was more sustainable at low $[\text{Ca}^{2+}]_o$ than that by nicotine (Fig. 3). This suggests that a part of CA secretion evoked by BK may be derived from a Ca^{2+} release from intracellular stores. TMB-8, supposed to inhibit Ca^{2+} release from intracellular Ca^{2+} stores (3), reduced rather specifically the initial phase both in $[\text{Ca}^{2+}]_i$ rise (Fig. 8*d*) and in CA secretion (Fig. 4*Ab*) in adrenal medullae stimulated by BK in the normal medium. Thus, the release of Ca^{2+} from intracellular stores may contribute to the secretory response in this portion.

The Ca^{2+} store may be depleted rapidly at extremely low $[\text{Ca}^{2+}]_o$ since only a small, brief rise in $[\text{Ca}^{2+}]_i$ remained after a short period of perfusion of medulla with EGTA-Krebs solution (Fig. 8*c*).

Recently, O'Sullivan *et al.* (10) demonstrated by video imaging of fura-2 that Ca^{2+} mobilization from intracellular Ca^{2+} stores actually took place in single bovine adrenal medullary cells following BK stimulation. Since BK-stimulated accumulation of inositol phosphates occurred in adrenal medullary cells (8) and in pheochromocytoma cells (2), inositol trisphosphate (IP_3) is the putative messenger that acts on Ca^{2+} stores to release Ca^{2+} . In fact, IP_3 caused a release of Ca^{2+} in permeabilized chromaffin cells (14).

Requirement for Extracellular Ca^{2+} in BK-evoked Secretory Response

The mechanism of introducing extracellular Ca^{2+} is considered to operate during the maintained response elicited by BK. By this, the cell can sustain the elevated $[\text{Ca}^{2+}]_i$ level against a continuous extrusion of Ca^{2+} through the Ca^{2+} pump and $\text{Na}^+/\text{Ca}^{2+}$ exchange mechanism. This view is consistent with the observation that the maintained phase of response, both in $[\text{Ca}^{2+}]_i$ rise and in CA secretion, was not affected by TMB-8 in the normal medium (Figs. 8*d* and 4*Ab*) but abolished in the low Ca^{2+} medium (Figs. 8*e* and 4*Bb*).

On the other hand, the occurrence of active Ca^{2+} extrusion is suggested from the observation that the resting fluorescence from fluo-3-loaded medullary cells decreased in $16\text{-}\mu\text{M-Ca}^{2+}$ -Krebs solution (Fig. 8*b*) despite the fact

that $16 \mu\text{M}$ Ca^{2+} was a low but much higher concentration than the resting level of $[\text{Ca}^{2+}]_i$.

A novel type of Ca^{2+} channel which is controlled by intracellular mediators such as GTP-binding protein (12) has recently been found in various cell types. In addition to voltage- and nifedipine-sensitive Ca^{2+} channels, such non-traditional Ca^{2+} channels may reside on rat adrenal medullary cells and they may be activated during stimulation with BK.

Alternatively, extracellular Ca^{2+} can be introduced by a Ca^{2+} transport mechanism that exists either on the plasma membrane or at particular junctional sites (11, 17) between the plasma membrane and intracellular Ca^{2+} stores.

These Ca^{2+} channels or Ca^{2+} transport mechanism may be blocked by Cd^{2+} . However, Cd^{2+} inhibited BK-evoked secretion not only in its maintained phase but also in its initial phase (records not shown). Furthermore, Cd^{2+} enters adrenal medullary cells (Fig. 7c). Therefore, the possibility that Cd^{2+} inhibits some intracellular secretory process is not ignored.

The Mode of CA Secretion Evoked by Receptor Agonists

We studied previously the characteristics of CA secretion evoked by muscarine (19). Muscarine-stimulated rat adrenal medulla selectively released adrenaline with a time course very similar to that shown with BK in Fig. 1A. Muscarine-evoked secretion was not affected by nifedipine but inhibited by Cd^{2+} . The rise in $[\text{Ca}^{2+}]_i$ in adrenal medullary cells after muscarinic receptor activation in the absence of external Ca^{2+} was inhibited by TMB-8 (7, 20). These characteristics of muscarinic responses in adrenal medullary cells agree with those of BK-induced responses.

Moreover, it was shown in our preliminary study that histamine induced the secretory response whose characteristics were very similar to those of BK- or muscarine-evoked secretion.

Thus, it is likely that common intracellular processes are activated to lead to CA secretion in rat adrenal medullary cells following stimulation of these different receptors.

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