$[Ca^{2+}]_i$ CHANGES ARISING IN INDIVIDUAL GUINEA-PIG CHROMAFFIN CELLS IN RESPONSE TO VARIOUS RECEPTOR AGONISTS AND THEIR RELATION TO CATECHOLAMINE SECRETION IN THE PERFUSED ADRENAL GLAND

AKIRA WARASHINA¹, YOSHIKI HABARA², KEN'ICHI YAMAGUCHI¹ and TOMIO KANNO^{2,3}

¹Department of Physiology, Niigata University School of Medicine, Niigata 951, ²Laboratory of Cellular Metabolism, Department of Molecular Physiology, National Institute for Physiological Sciences, Okazaki 444, and ³Department of Physiology, Faculty of Veterinary Medicine, Hokkaido University, Sapporo 060, Japan

ABSTRACT

Changes in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) arising in isolated guinea-pig adrenal chromaffin cells stimulated by various receptor agonists were analyzed by fura-2 imaging method. Nicotine (10 μ M) and angiotensin II (100 nM; ANG II) induced rises in $[Ca^{2+}]_i$ in all cells tested. A muscarinic agonist, carbamyl- β -methylcholine (10 μ M; BCh) and bradykinin (100 nM; BK) elicited $[Ca^{2+}]_i$ rises in 60% and 74% of cells tested, respectively. Cells responsive and nonresponsive to BCh or BK co-existed even among cells within a cluster, suggesting that receptors for these agonists may be distributed on individual cells in a considerably inhomogeneous fashion. None of cells showed $[Ca^{2+}]_i$ response to histamine (100 μ M). The agonists which induced $[Ca^{2+}]_i$ rises consistently evoked catecholamine (CA) secretion from perfused adrenal glands of the guinea-pig. A small transient $[Ca^{2+}]_i$ rise induced by ANG II, BCh or BK in Ca^{2+} -deficient medium indicated a release of Ca^{2+} from intracellular Ca^{2+} stores. However, a major portion of $[Ca^{2+}]_i$ rise induced by these agonists was derived from Ca^{2+} entry, since a blocker for agonist-mediated Ca^{2+} entry, 1- β -[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole hydrochloride (SK&F 96365) (30 μ M) extensively inhibited both the agonist-induced $[Ca^{2+}]_i$ rise and CA secretion. The agonist-induced Ca^{2+} entry consisted in part of Ca^{2+} influx through voltage-gated Ca^{2+} channels, which was sensitive to nifedipine.

In addition to nicotinic and muscarinic agonists, various receptor agonists such as histamine, bradykinin and angiotensin are known to act as potent stimulants in adrenal chromaffin cells (9, 19, 20, 28). However, there are considerable differences in the

mode of Ca^{2+} mobilization and catecholamine (CA) secretory response when chromaffin cells from different animal species are stimulated by these agonists. For instance, muscarinic agonists induced an intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) rise which was originated by a Ca^{2+} release from intracellular Ca^{2+} stores but evoked no substantial CA secretion in bovine adrenal chromaffin cells (6, 13, 18). On the other hand, in chromaffin cells of the rat or the cat, muscarinic agonists not only induced Ca^{2+} release from intracellular Ca^{2+} stores but also promoted Ca^{2+} entry, leading to effective CA secretion (2, 24, 26, 27). Thus, modes of $[Ca^{2+}]_i$ rises and secretory responses during stimulation with various receptor agonists should be

Abbreviations: ANG II, angiotensin II; BCh, carbamyl- β -methylcholine chloride (bethanecol); BK, bradykinin; BSA, bovine serum albumin; CA, catecholamine; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; fura-2 AM, acetoxyethylmethyl ester of fura-2; GABA, γ -aminobutyric acid; HEPES, N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid; MEM, minimal Eagle's medium; SK&F 96365, 1- β -[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole hydrochloride

investigated in detail using cells from different origins in order to obtain a comprehensive view on the relationship between agonist-induced Ca^{2+} dynamics and CA secretion in chromaffin cells.

In this study, $[\text{Ca}^{2+}]_i$ changes arising in isolated guinea-pig adrenal medullary cells in response to stimulation with receptor agonists, including angiotensin II (ANG II), bradykinin (BK) and histamine as well as nicotine and carbamyl- β -methylcholine chloride (bethanecol) (BCh), were monitored by a digital imaging technique with fura-2. The image records suggested that the number of receptors for BCh or BK on individual cells may vary to a large extent from one cell to another. The pathways for Ca^{2+} mobilization in agonist-stimulated cells were also investigated.

MATERIALS AND METHODS

Chemicals and Solutions

1- $\{\beta$ -[3-(4-Methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole hydrochloride (SK&F 96365) was kindly provided by Dr J. E. Merritt (Smith Kline & French Research Ltd.). Bovine serum albumin (fraction V, BSA), BCh, muscarine chloride, nifedipine, soybean trypsin inhibitor (type 1-S) were from Sigma Chemicals (St. Louis, MO). Acetoxymethyl ester of fura-2 (fura-2 AM) and *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid (HEPES) were from Dojindo Laboratories (Kumamoto). Histamine chloride, ($\pm$)Bay K 8644 and inorganic chemicals were from Wako Pure Chemical (Osaka). Collagenase (Yakult product) was from Sankou Chemicals (Tokyo). Purified collagenase (CLSPA) was from Worthington (Freehold, NJ). Other chemicals used were bradykinin (Peptide Institute; Minoh), Cell-Tak (Collaborative Research; Bedford, MA) and minimal Eagle's medium essential amino acids (Flow Laboratories; Irvine).

A modified Krebs (MK) solution was used in experiments to measure CA secretion from perfused adrenal glands. MK solution consisted of (mM): 150 NaCl, 5.6 KCl, 2.0 CaCl_2 , 1.0 MgCl_2 , 1.0 NaH_2PO_4 , 10 HEPES and 5.6 glucose, titrated with NaOH to pH 7.4. MK solution was supplemented with 0.1% BSA when used in preparation of isolated chromaffin cells and in measurement of $[\text{Ca}^{2+}]_i$ changes in these cells. A low- Ca^{2+} MK solution containing 100 μM CaCl_2 was employed in treating adrenal medulla with collagenase. To examine effect of a deprivation of extracellular Ca^{2+} , a Ca^{2+} -deficient solution was prepared by

omitting CaCl_2 from MK solution without adding Ca^{2+} chelating agents.

In order to confirm that observed properties of cells were not influenced by the composition of medium and the method of cell preparation, some experiments concerning $[\text{Ca}^{2+}]_i$ measurements were carried out using HR solution. HR solution contained (mM): 118 NaCl, 4.7 KCl, 2.5 CaCl_2 , 1.13 MgCl_2 , 1.0 Na_2HPO_4 , 10 HEPES, and 5.5 glucose, supplemented with 0.2% BSA, 0.01% trypsin inhibitor, 2 mM L-glutamine and minimal Eagle's medium (MEM) amino acids, and titrated to pH 7.4 with NaOH. All solutions were gassed with 100% O_2 .

Isolation of Chromaffin Cells

Chromaffin cells were obtained from adrenal glands which was removed from the guinea pig (400–500 g) after head blow followed by exsanguination. Cells were prepared by the following two different procedures (A and B). In the procedure A, adrenal medullae were isolated from the cortex and were torn into small pieces. The pieces were treated for 40–60 min at room temperature with 1 mg/ml collagenase (Yakult) in the low- Ca^{2+} MK solution. In the procedure B, parenchyma of the adrenal gland was infused for 30 min at 37°C with HR solution containing 75 U/ml collagenase (CLSPA, Worthington), then the gland was cut into two pieces and the medullary tissue was sucked and blown several times with a pipette containing HR solution and resuspended in HR solution. Isolated cells were used for experiments within 10 h.

Fura-2 Loading and Digital Image Analysis

Chromaffin cells were incubated in MK solution containing 10 μM fura-2 AM for 60 min at room temperature in the procedure A or in HR solution containing 10 μM fura-2 AM for 30 min at 37°C in the procedure B. Fura-2-loaded cells were transferred onto a Cell-Tak-coated nonfluorescent glass cover slide mounted in a chamber of Sykus-Moore type. The chamber was then placed on the stage of an inverted microscope (TMD-2, Nikon, Tokyo) and perfused with MK solution or HR solution depending on the procedure (A or B) for cell preparation. Fluorescence images formed by irradiation of cells with 340 nm and 380 nm lights were alternately focused on a SIT (silicon intensified target) camera. The signal from the camera was analyzed with a digital image processor (Argus-50/Ca,

Hamamatsu Photonics, Hamamatsu). The temporal change in $[Ca^{2+}]_i$ was calculated from the ratio of 340 nm to 380 nm fluorescence intensity on a certain area in individual cells by referring to a calibrating relation for fura-2 emission in Ca^{2+} -EGTA solutions (25).

Measurement of CA Secretion from Perfused Adrenal Gland

A real-time monitoring of CA secreted from perfused adrenal medulla was performed in a manner similar to that described previously for the rat adrenal gland (24). Either side of the adrenal gland of male guinea pig (400–500 g) immediately after removal from the body was cannulated through the adrenal vein and was perfused with MK solution at a rate of 0.2 ml/min. The perfusate was led into a flow cell (Bioanalytical Systems, TL-5) in which glassy carbon electrodes were embedded. The amount of CA in the perfusate was measured as oxidation current with an amperometric detector (Yanako, VMD-101A) at an applied electrode voltage of 0.4 V. The detector output was sampled every 4 s with an analog to digital (A/D) converter and stored on magnetic disks using a personal computer. The deflection on the record can be read as the mole of CA secreted per min, based on the calibration taken at each measurement with $1 \mu M$ adrenalin standard solution.

RESULTS

$[Ca^{2+}]_i$ Responses Differentially Induced in Individual Cells by Stimulation with Various Receptor Agonists

Most of $[Ca^{2+}]_i$ measurements were carried out using clusters consisting of 2–5 cells rather than single cells. Properties of $[Ca^{2+}]_i$ responses were not appreciably different among cells obtained by the procedure A or B. In the experiment of Fig. 1a, two cells in the same cluster were successively stimulated each for 4 min with 100 nM BK, 10 μM BCh and 10 μM nicotine with 3-min intermissions. The agonists induced similar $[Ca^{2+}]_i$ responses in the two cells. However, $[Ca^{2+}]_i$ responses to these agonists were not always alike. For instance, as shown in Fig. 1b, three cells in the same cluster failed to respond to BCh but they increased $[Ca^{2+}]_i$ by BK and nicotine. Another example is displayed in Fig. 1c; BK induced the response in only one of three cells, whereas two cells showed the $[Ca^{2+}]_i$ response to BCh, though one response occurred with a con-

Fig. 1 Various patterns in $[Ca^{2+}]_i$ changes occurring in fura-2-loaded guinea-pig adrenal chromaffin cells in response to different stimulants. The records obtained from two or three individual cells in the same cluster are displayed in each frame. The 4-min periods of stimulation with 100 nM BK, 10 μM BCh and 10 μM nicotine (Nic) are indicated by the horizontal lines underneath the records. The ordinate indicates the ratio of 340 nm to 380 nm fura-2 fluorescence intensity.

siderable delay. Nicotine elicited the $[Ca^{2+}]_i$ response in all three cells, which were larger than those by the other stimulants.

According to the differential sensitivity of cells to BCh (10 μM), BK (100 nM) or nicotine (10 μM), forty-four individual cells tested showed one of the following four response types: twenty-three cells

Table 1 $[Ca^{2+}]_i$ Changes Evoked by Various Stimulants in Guinea-Pig Adrenal Chromaffin Cells

Stimulants	(conc.)	Number of clusters	Responsiveness			Response size peak $[Ca^{2+}]_i$
			(+)	(-)	(uc)	
Nicotine	(10 μ M)	47	177	0	0	1,160 $\pm$ 230 nM
BCh	(10 μ M)	23	50	33	3	430 $\pm$ 80
	(100 μ M)	13	44	1	3	600 $\pm$ 120
BK	(100 nM)	28	73	25	1	400 $\pm$ 130
	(200 nM)	6	13	6	0	550 $\pm$ 170
	(600 nM)	1	0	3	0	
ANG II	(100 nM)	16	49	0	1	640 $\pm$ 110
Histamine	(100 μ M)	9	0	29	7	

BCh, BK and ANG II stand for bethanecol, bradykinin and angiotensin II, respectively. The number of cells responsive (+) and nonresponsive (–) to the respective agonists are listed. The cases in which changes in $[Ca^{2+}]_i$ were close to the noise level or slow fluctuation of base line, are classified as uncertain response (uc). For the calculation of the mean response size, data from the responsive cells were used. The resting level of $[Ca^{2+}]_i$ was 110 $\pm$ 40 nM ($n = 133$).

responded to the three agonists, eight cells to BK and nicotine but not to BCh, five cells to BCh and nicotine but not to BK, and eight cells to nicotine alone.

$[Ca^{2+}]_i$ responses to the respective agonists in individual cells obtained from experiments such as shown in Fig. 1 were summarized in Table 1. The resting $[Ca^{2+}]_i$ level was 110 $\pm$ 40 nM (mean $\pm$ SD; $n = 133$). Nicotine at 10 μ M elicited large elevations in $[Ca^{2+}]_i$ without exception, which reached 1,160 $\pm$ 230 nM at the peak on average of 177 cells. ANG II also induced the response in all the cells tested, except one uncertain case, with the averaged peak magnitude of 640 $\pm$ 110 nM ($n = 49$). On the other hand, 10 μ M BCh elicited the response in 60% of cells. The population of responsive cells was increased to 98% when 100 μ M BCh was applied. The $[Ca^{2+}]_i$ response to 100 nM BK was observed in 74% of cells. The elevation of BK concentration up to 600 nM did not obviously increase the fraction of responsive cells. None of cells showed appreciable $[Ca^{2+}]_i$ response to 100 μ M histamine.

Agonist-Induced $[Ca^{2+}]_i$ Responses in Ca^{2+} -Free Medium

In the experiment shown in Fig. 2, the receptor agonists were applied to cells that had been superfused with the Ca^{2+} -deficient MK solution to examine the contribution of Ca^{2+} released from intracellular Ca^{2+} stores to the agonist-induced $[Ca^{2+}]_i$ change. BCh, ANG II and BK elicited small transient $[Ca^{2+}]_i$ rises as shown in Fig. 2 (a, b and c,

respectively). A large increase in $[Ca^{2+}]_i$ was shown when the perfused medium was switched back to MK solution, which is known as 'Ca²⁺ reintroduction response' that causes a massive CA secretion in adrenal chromaffin cells (8).

Effects of Nifedipine and SK&F 96365 on Agonist-Induced $[Ca^{2+}]_i$ Changes

It is shown in Fig. 2 (d, e and f) that 30 μ M nifedipine, a blocker for L-type Ca^{2+} channels, inhibited the $[Ca^{2+}]_i$ increase induced by BCh, ANG II or BK. This suggests that the receptor stimulation with these agonists may promote Ca^{2+} entry through voltage-gated Ca^{2+} channels.

The effect of SK&F 96365 on the agonist-induced $[Ca^{2+}]_i$ increase was examined since this agent was reported to inhibit receptor-mediated Ca^{2+} entry (4, 5, 14). In Fig. 3 (a, b and c), 30 μ M SK&F 96365 effectively suppressed the $[Ca^{2+}]_i$ increase induced by BCh, ANG II or BK. However, Fig. 3 (d and e) shows that SK&F 96365 also inhibited the $[Ca^{2+}]_i$ increase induced by 10 μ M nicotine or 56 mM extracellular potassium. This supports the result described by Merritt *et al.* (14) in which SK&F 96365 inhibited not only receptor-mediated Ca^{2+} entry but also Ca^{2+} influx through voltage-gated Ca^{2+} channels.

CA Secretory Responses Evoked by the Receptor Agonists

Efficacy of the receptor agonists in evoking CA secretion was investigated using perfused adrenal

Fig. 2 Records a, b and c: Agonist-induced $[Ca^{2+}]_i$ changes in Ca^{2+} -deficient Krebs solution. Cells were stimulated by (a) $100 \mu M$ BCh, (b) $100 nM$ ANG II or (c) $100 nM$ BK. The periods during which the Ca^{2+} -deficient medium was perfused and the 3-min stimulation with the agonists were indicated by the horizontal lines above and underneath the records, respectively. Records d, e and f: Effect of $30 \mu M$ nifedipine on agonist-induced $[Ca^{2+}]_i$ rises. The presence of (d) $100 \mu M$ BCh, (e) $100 nM$ ANG II or (f) $100 nM$ BK is designated by the horizontal line underneath the records, and the 4-min application of $30 \mu M$ nifedipine is indicated by the line above the records. The $[Ca^{2+}]_i$ changes in two or three cells are superimposed in each frame. The ordinate indicates $[Ca^{2+}]_i$ in μM .

glands of the guinea-pig. The profiles of CA secretory response evoked by 10-min stimulation with the agonists are shown in Fig. 4. It is evident that the agonists which induced $[Ca^{2+}]_i$ changes also elicited CA secretion. Histamine failed to evoke CA secretion to an appreciable extent, being consistent with the result of the $[Ca^{2+}]_i$ measurement. In addition to the receptor agonists whose effects were tested in the $[Ca^{2+}]_i$ measurements, effect of γ -aminobutyric acid (GABA) was also examined. GABA at $200 \mu M$ evoked a sharp transient secretion to give 126 ± 62 pmol/min ($n = 4$) at the peak rate (Fig. 4e).

The time course of CA secretion evoked by BCh or nicotine was more or less similar to that of BCh-induced $[Ca^{2+}]_i$ change. On the other hand, ANG II-evoked CA secretion showed a rapid increase to the peak and quickly fell to a level close to the basal level despite the continued presence of ANG II.

This profile contrasted to that of the ANG II-induced $[Ca^{2+}]_i$ response which was maintained as long as ANG II was present. The peak rate of CA secretion evoked by ANG II was 351 ± 93 ($n = 8$) pmol/min, which was 1.6-fold of that in BCh ($100 \mu M$)-evoked secretion, whereas the size of ANG II-induced $[Ca^{2+}]_i$ change was comparable with that of BCh ($100 \mu M$)-induced $[Ca^{2+}]_i$ change (Table 1). The BK-evoked CA secretion was very weak and transient. In fact, BK-evoked CA secretion was practically absent in two of eight glands tested and the averaged rate of secretion at the peak was 14 ± 12 ($n = 8$) pmol/min.

Effects of Dihydropyridines and SK&F 96365 on the Agonist-Evoked Secretion

The inhibitory effect of nifedipine on BCh-evoked CA secretion was shown in Fig. 5a. The record

Fig. 3 Effect of 30 μM SK&F 96365 on $[\text{Ca}^{2+}]_i$ rises induced by (a) 100 μM BCh, (b) 100 nM ANG II, (c) 100 nM BK, (d) 10 μM nicotine (Nic), or (e) 56 mM extracellular K^+ . The periods during which the respective stimulants were applied are indicated by the horizontal lines underneath the records, and the 4-min applications of SK&F 96365 are indicated by the horizontal lines above the records. The $[\text{Ca}^{2+}]_i$ changes in three individual cells are shown in each frame. The ordinate indicates $[\text{Ca}^{2+}]_i$ in μM .

plotted with the dotted line represents the control by which time course of CA secretion evoked by a 12-min stimulation with 100 μM BCh is shown. The record drawn with the solid line shows the effect of 30 μM nifedipine which was applied in the later 6-min period during 12-min stimulation with BCh. Nifedipine inhibited BCh-evoked secretion to $45 \pm 7\%$ ($n = 5$) at the end of the 12-min stimulation. Conversely, Bay K 8644, an activator of L-type Ca^{2+} channel increased BCh-evoked secretion to $146 \pm 13\%$ ($n = 4$) as shown in Fig. 5b. Bay K 8644 effectively increased the nicotine-induced CA secretion to $220 \pm 27\%$ ($n = 4$) of the control (Fig. 5c).

By applying 30 μM SK&F 96365 between 6th and 12th min in a continuous 18-min stimulation with 10 μM BCh, the secretory response was inhibited to $22 \pm 6\%$ ($n = 4$) of the control at the end of the SK&F 96365 application (Fig. 5d). Similarly, CA secretion evoked by 56 mM extracellular K^+

was suppressed to $58 \pm 11\%$ ($n = 5$) of the control (Fig. 4e). The inhibitory effect of SK&F 96365 in the above cases was not readily removed by a washout carried out during the period between 12th and 18th min.

DISCUSSION

Inhomogeneous Receptor Distribution on Guinea-Pig Chromaffin Cells

The various receptor agonists were used in this study to investigate their efficacy to induce $[\text{Ca}^{2+}]_i$ change and CA secretion in adrenal chromaffin cells of the guinea-pig. Nicotine at 10 μM or ANG II at 100 nM invariably induced the $[\text{Ca}^{2+}]_i$ response in all the cells tested. Histamine which is a potent stimulant for chromaffin cells in various animal species (3, 21, 23) was found to be completely ineffective to induce $[\text{Ca}^{2+}]_i$ response in guinea-pig chromaffin cells. The distribution of

Fig. 4 CA secretion from perfused guinea-pig adrenal gland in response to stimulation with various receptor agonists. The oxidation current due to CA in the perfusate was recorded. The period in which (a) 100 μ M BCh, (b) 100 nM ANG II, (c) 10 μ M nicotine (Nic), (d) 100 nM BK, (e) 200 μ M GABA or (f) 100 μ M histamine (His) is delivered is indicated by the horizontal line underneath each record.

receptors for BCh or BK on individual cells was revealed to be considerably inhomogeneous, as suggested from the co-existence of responsive and nonresponsive cells even among cells in the same cluster in the digital imaging analysis (Fig. 1). The stimulation with 10 μ M BCh or 100 nM BK caused the response in 60% or 74% of cells, respectively. Since almost all cells exhibited the $[Ca^{2+}]_i$ response when the BCh concentration was increased from 10 μ M to 100 μ M, it is evident that every cell possesses enough number of muscarinic receptors to produce the $[Ca^{2+}]_i$ response if they are simultaneously stimulated by higher concentrations of BCh. By 10 μ M BCh, however, only a subfraction of the receptors is activated, which may be critical to produce the $[Ca^{2+}]_i$ response. Thus, the response may be exhibited in some cells but not in other

cells.

On the other hand, the increase in BK concentration did not increase the population of responsive cells, suggesting that BK receptors in some fraction of cells are too few to produce the $[Ca^{2+}]_i$ response even if they are stimulated by BK at high concentrations.

Whether the inhomogeneous distribution of receptors is inherent in individual cells or derived artificially from a loss of receptors during the cell preparation can not readily be asserted. However, the interpretation of the phenomenon based on cell damage seems to be less likely since the use of cells prepared by the distinct procedures (A and B) with different sources and concentrations of collagenase did not alter the situation.

Comparison between the Agonist-Induced $[Ca^{2+}]_i$ and CA Secretory Responses

CA secretion from the guinea-pig adrenal medulla was evoked by nicotine, ANG II, BCh or BK but not by histamine. This is consistent with the result shown in $[Ca^{2+}]_i$ responses to these agonists. In a quantitative aspect, it was predicted that BK was less potent in evoking CA secretion as compared with other agonists in view of the smaller elevation in $[Ca^{2+}]_i$ and more fraction of nonresponsive cells with BK (Table 1). However, the extent of small secretion evoked by BK, that was less than one tenth of those by the other agonists, would not have been expected. This result suggests a non-linear relation between $[Ca^{2+}]_i$ increase and CA secretion.

The time course of ANG II-evoked CA secretion was transient, which contrasted with the sustained $[Ca^{2+}]_i$ rise induced by ANG II. This may result from one of the following two reasons. First, ANG II receptor activation might produce an inhibitory factor which terminates CA secretion even in cells with elevated $[Ca^{2+}]_i$. Second, the desensitization of activated ANG II receptors may take place in adrenal medulla but this property may be lost for some reason in isolated chromaffin cells.

Ca^{2+} Dynamics in Agonist-Stimulated Chromaffin Cells of the Guinea Pig

It was shown previously (12) that stimulation of guinea-pig chromaffin cells with 100 μ M carbachol induced Ca^{2+} release from intracellular stores. It also occurred in cells stimulated by BCh, ANG II or BK, because these agonists induced small tran-

Fig. 5 Effects of various agents on CA secretion evoked by different stimulants. (a) (Solid line): A perfused adrenal gland was stimulated during the first 6 min by 100 μ M BCh and during the later 6 min by 100 μ M BCh in the presence of 30 μ M nifedipine. (Dotted line): The response profile obtained by 12-min stimulation with 100 μ M BCh. (b): The effect of 1 μ M Bay K 8644 on BCh (100 μ M)-evoked secretion was examined with the same protocol as used in (a). (c): The effect of 1 μ M Bay K 8644 on nicotine (10 μ M)-evoked secretion was examined with the same protocol as used in (a). (d) (Solid line): A gland was stimulated for 18 min by 100 μ M BCh, during which 30 μ M SK&F 96365 was applied between 5th and 11th min. (Dotted line): The response profile obtained by a 12-min stimulation with 100 μ M BCh. (e) (Solid line): A gland was stimulated for 18 min by 56 mM extracellular K^+ , during which 30 μ M SK&F 96365 was applied between 6th and 12th min. In each experiment, the test measurement (the solid line) was preceded by the control measurement (the dotted line) with 30 min intermission. The test and control records are normalized at the peak. The vertical line beside each record indicates CA secretion rate at 200 pmol/min.

sient increases in $[Ca^{2+}]_i$ in the Ca^{2+} -deficient solution. At present, very limited information is available concerning properties of intracellular Ca^{2+} stores in guinea-pig chromaffin cells. The presence of caffeine-sensitive Ca^{2+} stores is suggested since caffeine evoked CA secretion in the guinea-pig adrenal gland in Ca^{2+} -free medium (15). It may be speculated that inositol trisphosphate (IP_3)-sensitive Ca^{2+} stores may play a role in the $[Ca^{2+}]_i$ rise, since muscarinic agonists, ANG II or BK, promote inositol metabolism in chromaffin cells obtained from other animal species (10, 17, 22). The mus-

carinic agonist-induced Ca^{2+} release from intracellular stores is thought to lead to CA secretion in guinea-pig chromaffin cells (16). This contrasts with the results in bovine chromaffin cells where muscarinic agonist-induced Ca^{2+} release did not trigger CA secretion (6, 18).

It seems certain that a major portion of the agonist-induced $[Ca^{2+}]_i$ rise and that of CA secretion are supported by Ca^{2+} influx. In adrenal chromaffin cells, at least two different pathways of Ca^{2+} entry are known, one through voltage-gated Ca^{2+} channels and the other mediated by intracel-

lular messenger(s) following receptor stimulation (24). The involvement of L-type Ca^{2+} channels in $[\text{Ca}^{2+}]_i$ increase as well as in CA secretion evoked by BCH, ANG II or BK is suggested by the inhibition of these responses by nifedipine (Fig. 2, d, e, f and Fig. 5a). The enhancement of BCH-evoked CA secretion with Bay K 8644 (Fig. 5b) further supports this idea. However, the extent of this enhancement was smaller compared with that obtained when Bay K 8644 was applied to nicotine-evoked CA secretion (Fig. 5c). This may be compatible with the idea that nicotine-evoked CA secretion depends exclusively on Ca^{2+} influx through voltage-gated Ca^{2+} channels, whereas this Ca^{2+} influx may only partially contribute to the agonist-evoked secretion.

The link between the receptor stimulation and the activation of L-type Ca^{2+} channels might be mediated through the membrane depolarization which occurs following the receptor stimulation (1, 7) due either to a close of some type of K^+ channels (1) or to an increase in conductance of cation non-selective channels (11). However, this point apparently remains to be studied.

SK&F 96365 effectively inhibited the agonist-induced $[\text{Ca}^{2+}]_i$ rise (Fig. 3, a, b and c) and CA secretion (Fig. 5d). Although SK&F 96365 is known as a blocker for receptor-mediated Ca^{2+} entry (4, 5), it also inhibits voltage-gated Ca^{2+} channels (14). The latter effect of SK&F 96365 was implicated in this study by the fact that it inhibited nicotine- and high- K^+ -induced $[\text{Ca}^{2+}]_i$ increases (Fig. 3, d and e), and high- K^+ -evoked CA secretion (Fig. 5e). The blockade by SK&F 96365 of Ca^{2+} entry through these two different pathways may result in very extensive inhibition of BCH-induced CA secretion (Fig. 5d).

We thank Ms Y. Abe for her technical assistance. A part of this work was performed at the National Institute for Physiological Sciences in Okazaki with financial support. This study was supported in part by a grant-in-aid (06670050) for Scientific Research from the Ministry of Education, Science and Culture of Japan.

Received 20 June 1994; and accepted 1 July 1994

REFERENCES

- AKAIKE A., MINE Y., SASA M. and TAKAORI S. (1990) Voltage and current clamp studies of muscarinic and nicotinic excitation of the rat adrenal chromaffin cells. *J. Pharmacol. Exp. Ther.* **255**, 333–339
- BALLESTA J. J., BORGES R., GARCÍA A. G. and HIDALGO M. J. (1989) Secretory and radioligand binding studies on muscarinic receptors in bovine and feline chromaffin cells. *J. Physiol.* **418**, 411–426
- BORGES R. (1993) Ionic mechanisms involved in the secretory effects of histamine in the rat adrenal medulla. *Eur. J. Pharmacol.* **241**, 189–194
- BUSIK J., HABARA Y., WARASHINA A. and KANNO T. (1994) Effect of SK&F 96365 on nicotinic or muscarinic agonist-induced and spontaneous Ca^{2+} dynamics in rat adrenal chromaffin cells. *Biomedical Res.* **15**, 155–163
- CHAN J. and GREENBERG D. A. (1991) SK&F 96365, a receptor-mediated calcium entry inhibitor, inhibits calcium responses to endothelin-1 in NG108-15 cells. *Biochem. Biophys. Res. Commun.* **177**, 1141–1146
- CHEEK T. R. and BURGOYNE R. D. (1985) Effect of activation of muscarinic receptors on intracellular free calcium and secretion in bovine adrenal chromaffin cells. *Biochem. Biophys. Acta* **846**, 167–173
- DOUGLAS W. W., KANNO T. and SAMPSON S. R. (1967) Effects of acetylcholine and other medullary secretagogues and antagonists on the membrane potential of adrenal chromaffin cells: An analysis employing techniques of tissue culture. *J. Physiol.* **188**, 107–120
- DOUGLAS W. W. and RUBIN R. P. (1961) The role of calcium in the secretory response of the adrenal medulla to acetylcholine. *J. Physiol.* **159**, 40–57
- FELDBERG W. and LEWIS G. P. (1964) The action of peptides on the adrenal medulla. Release of adrenaline by bradykinin and angiotensin. *J. Physiol.* **171**, 98–108
- FORSBERG E. J., ROJAS E. and POLLARD H. B. (1986) Muscarinic receptor enhancement of nicotine-induced catecholamine secretion may be mediated by phosphoinositide metabolism in bovine adrenal chromaffin cells. *J. Biol. Chem.* **261**, 4915–4920
- INOUE M. and KURIYAMA H. (1991) Muscarinic receptor is coupled with a cation channel through a GTP-binding protein in guinea-pig chromaffin cells. *J. Physiol.* **436**, 511–529
- KANNO T. and HABARA Y. (1991) Dose-dependency in spatial dynamics of $[\text{Ca}^{2+}]_i$ in adrenal chromaffin cells. *Cell Calcium* **12**, 523–531
- KAO L.-S. and SCHNEIDER A. S. (1985) Muscarinic receptors on bovine chromaffin cells mediate a rise in cytosolic calcium that is independent of extracellular calcium. *J. Biol. Chem.* **260**, 2019–2022
- MERRITT J. E., ARMSTRONG W. P., BENHAM C. D., HALLAN T. J., JACOB R., JAXA-CHAMIEC A., LEIGH B. K., MCCARTHY S. A., MOORES K. E. and RINK T. J. (1990) SK&F 96365, a novel inhibitor of receptor-mediated calcium entry. *Biochem. J.* **271**, 515–522
- NAKAZATO Y., HAYASHI H. and TERAOKA H. (1992) Comparisons of the effects of ryanodine on catecholamine secretion evoked by caffeine and acetylcholine in perfused adrenal glands of the guinea-pig. *Brit. J. Pharmacol.* **105**, 597–602
- NAKAZATO Y., OHGA A., OLESHANSKY M., TOMITA U. and YAMADA Y. (1988) Voltage-independent catecholamine release mediated by the activation of muscarinic receptors in guinea pig adrenal glands. *Brit. J. Pharmacol.* **93**, 101–109
- NOBLE E. P., BOMMER M., SINCINI E., COSTA T. and HERZ A. (1986) H_1 -histamine activation stimulates inositol-1-phosphate accumulation in chromaffin cells. *Biochem. Biophys. Res. Commun.* **135**, 566–573
- O'SULLIVAN A. J. and BURGOYNE R. D. (1989) A comparison of bradykinin, angiotensin II and muscarinic stimula-

- tion of cultured bovine adrenal chromaffin cells. *Biosci. Rep.* **9**, 243-252
19. POISNER A. M. and DOUGLAS W. W. (1966) The need for calcium in adrenomedullary secretion evoked by biogenic amines, polypeptides, and muscarinic agents. *Proc. Soc. Exp. Biol. Med.* **123**, 62-64
 20. STASZEWSKA-BARCZAK J. and VANE J. R. (1965) The release of catechol amines from the adrenal medulla by histamine. *Brit. J. Pharmacol.* **25**, 728-742
 21. STAUDERMAN K. A., MURAWSKY M. M. and PRUSS R. M. (1990) Agonist-dependent patterns of cytosolic Ca^{2+} changes in single bovine adrenal chromaffin cells: Relationship to catecholamine release. *Cell Regulation* **1**, 683-691
 22. STAUDERMAN K. A. and PRUSS R. M. (1990) Different patterns of agonist-stimulated increases of ^3H -inositol phosphate isomers and cytosolic Ca^{2+} in bovine adrenal chromaffin cells: Comparison of the effects of histamine and angiotensin II. *J. Neurochem.* **54**, 946-953
 23. WARASHINA A. (1992) Calcium mobilization and catecholamine secretion in histamine-stimulated rat adrenal medullary cells. *Biomedical Res.* **13**, 415-421
 24. WARASHINA A., FUJIWARA N. and SHIMOJI K. (1989) Characteristics of nicotinic and muscarinic secretory responses in the rat adrenal medulla studied by real-time monitoring of catecholamine release. *Biomedical Res.* **10**, 157-164
 25. WILLIAMS D. A. and FAY F. S. (1990) Intracellular calibration of the fluorescent calcium indicator Fura-2. *Cell Calcium* **11**, 75-83
 26. XU Y., DUARTE E. P. and FORSBERG E. J. (1991) Calcium dependency of muscarinic and nicotinic agonist-induced ATP and catecholamine secretion from porcine adrenal chromaffin cells. *J. Neurochem.* **56**, 1889-1896
 27. YAMAGAMI K., NISHIMURA S. and SORIMACHI M. (1991) Internal Ca^{2+} mobilization by muscarinic stimulation increases secretion from adrenal chromaffin cells only in the presence of Ca^{2+} influx. *J. Neurochem.* **57**, 1681-1689
 28. YOSHIZAKI T. (1973) Effect of histamine, bradykinin and morphine on adrenaline release from rat adrenal gland. *Jap. J. Pharmacol.* **23**, 695-699