CHARACTERISTICS OF NICOTINIC AND MUSCARINIC SECRETORY RESPONSES IN THE RAT ADRENAL MEDULLA STUDIED BY REAL-TIME MONITORING OF CATECHOLAMINE RELEASE

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ABSTRACT

Secretory responses in the perfused rat adrenal medulla stimulated by cholinergic agonists were studied by real-time monitoring of catecholamine (CA) release. Nicotinic response evoked by 10 μ M nicotine (or 100 μ M acetylcholine (ACh) in the presence of 1 μ M atropine) was distinct in various aspects from muscarinic response evoked by 100 μ M muscarine (or ACh in the presence of 1 mM hexamethonium). During a continuous nicotinic stimulation, CA release rapidly increased to a peak and declined exponentially with a time constant of about 80 sec close to a basal level. The nicotinic response was inhibited by Ca^{2+} channel blocker, either nifedipine or Cd^{2+} . The muscarinic response consisted of a brief, initial phase of CA release followed by a maintained secretion. Both phases of the muscarinic response were not suppressed by 30 μ M nifedipine but inhibited by 200 μ M Cd^{2+} . Furthermore, adrenaline and noradrenaline were nonselectively secreted in response to the nicotinic stimulation whereas adrenaline was selectively released to the muscarinic stimulation. During the initial 4 min of stimulation with 100 μ M ACh, the amount of CA (adrenaline plus noradrenaline) secretion mediated by nicotinic ACh receptors was comparable with that mediated by muscarinic ACh receptors and the addition of both contributions roughly reconstituted the total CA secretion caused by a simultaneous stimulation of both types of ACh receptors. When adrenal medullae were stimulated through the splanchnic nerve, only the nicotinic secretory response took place predominately.

A number of studies have been carried out to clarify pharmacologic properties of acetylcholine (ACh) receptors on adrenal medulla cells. On this subject, however, there exists considerable confusion in the literature partly because types of ACh receptors and their contributions to the catecholamine (CA) secretion seem to be different in different animal species from which adrenal medulla cells under study are derived. For instance, bovine

adrenal medulla cells secreted little CA by application of muscarinic agonists (16, 21) while muscarinic ACh receptors (in addition to nicotinic ACh receptors) played a substantial role for the CA secretion in the cat (5), dog (17, 18), rat (19, 23) and guinea pig (11, 14). In the chicken adrenal medulla (8), on the other hand, the secretory response was mediated only through muscarinic ACh receptors.

Another kind of confusion may arise on

comparing results obtained from *in vivo* secretion experiments with those from *in vitro* experiments since relative contributions of nicotinic and muscarinic ACh receptors to adrenal CA secretion are known to depend in some case on whether ACh was applied exogenously through perfusion medium or derived endogenously by splanchnic nerve stimulation (19).

In this study, properties of nicotinic and muscarinic ACh receptors in the perfused rat adrenal medulla are characterized in detail using a real-time CA monitoring method. The type of ACh receptors involved in the CA secretion that is evoked by electric stimulation of the splanchnic nerve is also examined.

MATERIALS AND METHODS

Perfusion of the rat adrenal gland was carried out with a modification of the method described previously (20). In brief, the left adrenal gland of male Wistar rats (300–500 g) was removed under sodium pentobarbital anesthesia and perfused retrogradely at a rate of 0.15 ml/min through a cannula inserted into the adrenal vein. The solution with which the gland was perfused was selected according to stimulus protocol with a 6-way rotary valve (Rheodyne) and introduced by a roller pump into the gland. The 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 embedded. The active and reference electrodes of the cell were connected to an electric controller (LC-4B; Bioanalytical Systems) for a continuous electrochemical detection of catecholamine in the perfusate. In some experiments, adrenals were dissected out together with a subdiaphragmatic part of the splanchnic nerve. In these preparations, the secretory response could be evoked by electric stimulation of the nerve with a pair of Ag-AgCl electrodes set in the chamber.

In usual measurements, the applied voltage to the carbon electrodes in the detector cell was adjusted to 0.4–0.45 V to obtain 10–20 nA of the oxidation current when a standard solution containing 1 μ M adrenaline was passed at a rate of 0.15 ml/min. Under this condition,

less than 6% of the total catecholamine in the perfusate was oxidized in the detector cell during measurement. The detector output, after I-V conversion, was sampled at a frequency of 0.5 Hz with an 8-bit digital memory and stored in a magnetic disk. Records presented in this paper were drawn, by D-A conversion of the digitalized data, with an X-Y recorder.

The catecholamine (adrenaline plus noradrenaline) output in its necessary part of response was numerically calculated from the digitalized record by referring to the size of response elicited with the standard adrenaline solution.

In order to measure adrenaline and noradrenaline secretions separately, high performance liquid chromatography with electro-chemical detection (HPLC-ECD) was employed. For this analysis, the perfusate from the outlet of the detector cell was collected into tubes which contained perchloric acid and EDTA (ethylenediaminetetraacetic acid) at final concentrations of 0.4 M and 5 mM, respectively. After perfusion experiments, catecholamines of each gland were extracted by 0.8 M perchloric acid and 10 mM EDTA and assayed by HPLC-ECD.

A modified Krebs solution (MK solution) was used as the standard medium of the gland. The MK solution consisted of (mM) 150 NaCl, 5.6 KCl, 2 CaCl₂, 1 MgCl₂, 5.6 glucose, 1 NaH₂PO₄·10 HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffered to pH 7.3 with NaOH. Perfusion solutions were bubbled with 100% O₂. The perfusion experiment was performed at temperatures of 23–26°C.

RESULTS

Real-time Monitoring of ACh-induced Catecholamine Secretion

The change in oxidation current presented in Fig. 1a is associated with the CA secretion from a rat adrenal gland stimulated 3 times by 100 μ M ACh at 20.5 min intervals. During each stimulation lasting for 5.5 min, the CA output increases rapidly, attaining the maximum within 80 sec, and then declines due to desensitization. The amount of catecholamine (adrenaline plus noradrenaline) which was released during the initial 4 min of

Fig. 1 Effects of hexamethonium (C6), atropine (atro.) and D600 on CA secretion from rat adrenal medullae stimulated by ACh. The traces show changes in the oxidation current associated with the CA secretion under the following conditions. a: The adrenal medulla was exposed for 5.5 min to 100 μ M ACh at 20.5-min intervals (3 times) as marked with the horizontal bars underneath the trace. b: As indicated above the trace, the second stimulation with 100 μ M ACh was performed in the presence of 1 mM C6, and 1 μ M atropine was further added to the perfusion medium prior to the third stimulation. c: The second stimulation with 100 μ M ACh was performed in the presence of 1 μ M atropine, and 100 μ M D600 was further added prior to the third stimulation. After a 40-min washout, the fourth stimulation was carried out to show the recovery of the response. The downward and upward blips beside the traces indicate, respectively, the moments of ON and OFF switching of the valve to introduce ACh-containing Krebs solution. The vertical bar in each frame denotes the intensity of the oxidation current (20–28 nA) obtained with standard solution (2 μ M adrenaline), corresponding to CA output at a rate of 300 pmol/min. Adrenaline and noradrenaline outputs during the shaded portions of the responses were assayed by HPLC-ECD and the results are shown in Fig. 2.

the first stimulation was $1,820 \pm 550$ pmol (mean $\pm$ SD) when perfusates from 18 experiments were analyzed by HPLC-ECD. In the

corresponding period for the second stimulation (the shaded portion in Fig. 1a), the CA output was 84% of that for the first stimulation. Since the CA output due to the first stimulation amounted to only 4% of the total CA in the gland, a major portion of the decrease is due to the desensitization that developed during the first exposure to ACh and still remains after the 15-min washout. The similar is shown for the third stimulation.

It was shown in previous works (19, 23) that both nicotinic and muscarinic ACh receptors contributed to the catecholamine secretion in the perfused rat adrenal medulla. To select CA secretion mediated by muscarinic ACh receptors, in this study, the second stimulation with ACh is applied in the presence of a nicotinic ACh blocker, hexamethonium (C6) as shown in Fig. 1b. This C6-resistive response solely consists of the muscarinic partition of the CA secretion since addition of atropine in the C6-containing medium completely abolishes the response as shown in the third stimulation with ACh (Fig. 1b).

On the other hand, the nicotinic partition of the ACh-evoked secretion is shown as the second response in Fig. 1c, where an adrenal medulla was stimulated by ACh in the presence of atropine. This nicotinic response is completely suppressed by 100 μ M D600 added to the medium, suggesting that the secretion is triggered by a calcium entry through voltage-dependent Ca^{2+} channels as has been shown in previous studies (6, 15–17).

Nicotinic and Muscarinic Contributions in ACh-evoked CA Secretion

The nicotinic and muscarinic secretory responses are quantitatively compared using data obtained from experiments such as shown in Fig. 1. Namely, the amounts of adrenaline (open columns in Fig. 2) and noradrenaline (hatched columns) released during the second stimulation (initial 4 min) are illustrated after normalization of them to the total CA output during the first stimulation. The addition of the nicotinic CA secretion (0.41 as denoted by the column b) to the muscarinic CA secretion (0.35 by the column c) roughly reconstitutes the total CA secretion (0.81 by the column a) which was obtained in the medium containing no ACh-blocking agents.

Fig. 2 Relative amounts of adrenaline (the open columns) and noradrenaline (the hatched columns) released from adrenal medulla stimulated by 100 μ M ACh under the following conditions; a, in the absence of ACh-receptor blocking agents; b, in the presence of 1 μ M atropine; c, in the presence of 1 mM C6; d, in the presence of both 1 μ M atropine and 1 mM C6. The sample perfusates to assay for adrenaline (A) and noradrenaline (NA) were collected during the initial 4 min of the first and second responses in the experimental protocol as shown in Fig. 1. In each determination, the amounts of A and NA released by the second stimulation were normalized to the catecholamine (A+NA) released by the first stimulation. Vertical lines denote standard deviations from 4 independent experiments.

Fig. 2 also shows that the molar ratio of noradrenaline to adrenaline (NA/A ratio) in the muscarinic secretion is 0.025 ± 0.020 ($n=5$), which is apparently smaller than 0.25 ± 0.07 , the NA/A ratio found in the nicotinic secretion. The latter figure is close to the mean NA/A ratio (0.28 ± 0.04 , $n=5$) in the adrenal glands used in the experiments for the nicotinic stimulation. The NA/A ratio in the ACh-evoked secretion with no ACh-blocking agents lies between the above two figures.

CA Secretions Evaluated by the Real-time Measurement

The validity of the real-time measurement of CA secretion may be assessed using the results obtained from experiments illustrated in Figs. 1 and 2. For the purpose, areas under response curves of the real-time measure-

ment, in portions where perfusates were sampled for CA assay, were evaluated by numerical integration using data stored in magnetic disks, and they were converted into the amounts of CA in the perfusates by referring to the size of response area associated with the standard adrenaline solution. The values thus obtained were highly proportional to the amounts of CA detected by HPLC-ECD in the sample perfusates.

However, absolute amounts of CA calculated from the response curves were found to be systematically larger than those determined by HPLC-ECD by a factor of 1.25 ± 0.23 ($n=18$), even after the decrease in CA concentration due to the electrochemical reaction which should have taken place on the detector electrode (less than 6% of the total CA released) was corrected. Although the secretion of ascorbic acid along with CA produces an additional oxidation current, this is estimated to be only 1% of the total current since ascorbic acid content is about 4% of total CA in rat chromaffin granules (22) while the current generated by ascorbic acid under the condition of our real-time measurement was one-fifth of that produced by equimolar adrenaline. In this study, we could not locate the source of the disagreement between the two different CA determinations. However, any descriptions in the present paper are not affected by this slight, systematic discrepancy.

Effects of Ca^{2+} Channel Blockers on Nicotinic and Muscarinic Secretions

The time course of CA secretion evoked by 100 μ M ACh in the presence of 1 μ M atropine is shown in Fig. 3Aa. The secretion is attenuated exponentially with a time constant of 78 ± 19 sec ($n=5$) close to the basal level during the continuous stimulation. A comparable amount of CA is released by 10 μ M nicotine as shown in Fig. 3Ab. When exposed to nicotine in prolonged periods, the time course of the secretion was very similar to that in Fig. 3Aa (data not shown here). The nicotine-evoked secretion (and also ACh-evoked secretion in the presence of atropine) is suppressed with 30 μ M nifedipine by $95 \pm 3\%$ ($n=3$) of that with no nifedipine (Fig. 3Bb). Cd^{2+} also suppressed the nicotinic secretory response,

Fig. 3 Effects of Ca^{2+} -channel blocking agents on the CA secretion mediated by nicotinic and muscarinic ACh receptors. A: Nicotinic secretory response was induced by $100\ \mu\text{M}$ ACh in the presence of $1\ \mu\text{M}$ atropine in a and c, or by $10\ \mu\text{M}$ nicotine in b. The second stimulations in b and c were performed in the presence of $30\ \mu\text{M}$ nifedipine and $200\ \mu\text{M}$ Cd^{2+} , respectively. B: Muscarinic secretory response was induced by $100\ \mu\text{M}$ ACh in the presence of $1\ \text{mM}$ hexamethonium (C6) in a and c, or by $100\ \mu\text{M}$ muscarine in b. The second stimulations in b and c were performed in the presence of $10\ \mu\text{M}$ nifedipine and $200\ \mu\text{M}$ Cd^{2+} , respectively. The periods in which the adrenal medullae were exposed to the stimulants are indicated underneath the traces, and the periods perfused with nifedipine- or Cd^{2+} -containing Krebs solution are shown above the traces. The vertical bars represent the oxidation current obtained with $1\ \mu\text{M}$ adrenaline.

showing a $91 \pm 4\%$ ($n=3$) inhibition at $200\ \mu\text{M}$ (Fig. 3c) and a complete inhibition at $500\ \mu\text{M}$.

The time course of muscarinic secretory response evoked by ACh in the presence of $1\ \text{mM}$ C6 (Fig. 3Ba) is dissimilar to that of the nicotinic response (Fig. 3Aa). The initial phase of the muscarinic response consisted of a very sharp peak with a duration of $80 \pm 11\ \text{sec}$ ($n=5$) at half-height, which is significantly shorter than $114 \pm 14\ \text{sec}$ ($n=5$), that for the nicotinic secretory response. The muscarinic response is also characterized by a high level of the maintained secretion which lasts as long as the stimulation is present (Fig. 3Ba).

It is noted that the secretion evoked by $100\ \mu\text{M}$ muscarine (Fig. 3Bb) or $100\ \mu\text{M}$ ACh in the presence of C6 is not affected by $30\ \mu\text{M}$ nifedipine. The CA secretion during the initial 4 min of the second stimulation with these drugs (2 experiments with muscarine and 3 with ACh plus C6) in the presence of

nifedipine was $82 \pm 13\%$ of that during the first stimulation. The same quantity in the absence of nifedipine was $85 \pm 6\%$. There is no significant difference between the above two figures (t -test, $P > 0.05$).

On the other hand, Cd^{2+} suppressed the muscarinic response completely at $500\ \mu\text{M}$, by $96 \pm 3\%$ ($n=3$) at $200\ \mu\text{M}$ (Fig. 3Bc) and $72 \pm 5\%$ ($n=3$) at $50\ \mu\text{M}$.

Catecholamine Secretion Induced by Nerve Stimulation

A marked CA release is elicited by an electrical stimulation to the splanchnic nerve with 1-msec pulses to 10 V at 20 Hz. The secretion declines rapidly over the period of continuous stimulations as shown in Fig. 4 (the left and right peaks). In the presence of $1\ \text{mM}$ C6 (middle), however, the same electrical stimulation to the nerve induces only a trace

Fig. 4 Effect of hexamethonium (C6) on the catecholamine secretion evoked by electric stimulation of the splanchnic nerve. The nerve was stimulated, during the periods indicated by the horizontal bars labelled NS, with 1-msec pulses to 10 V at a frequency of 20 Hz. C6 at 1 mM was presented during the second period of stimulation. Four calibration responses before and after the nerve stimulations were elicited with standard solution of 1 μ M adrenaline.

amount of CA secretion ($5.7 \pm 3.4\%$ ($n=8$) of the first response). After a washout of C6, the secretory response to the nerve stimulation recovers (right peak). The result suggests that ACh released from splanchnic nerve terminals effectively stimulates nicotinic ACh receptors but a very limited fraction of the released ACh reaches muscarinic ACh receptors on rat adrenal medulla cells.

DISCUSSION

Real-time Monitoring of CA Secretion from the Rat Adrenal Gland

In this study, real-time monitoring of CA secretion from the rat adrenal gland was performed by electrochemical detection of CA. The method similar to this has been developed with the isolated cat adrenal gland by Borges *et al.* (2) and with the rat adrenal gland *in situ* by Kumakura *et al.* (9). The method is particularly useful for studying precise time courses of CA secretions in response to various chemical stimuli as well as electric nerve stimulation. The validity of the method was already described by the above authors. We also confirmed an excellent proportional relation between the intensity of oxidation current in the real-time measurement and the amount of CA detected by HPLC-ECD in sample perfusates.

Nicotinic and Muscarinic Contributions in ACh-evoked CA Secretion

Involvement of both nicotinic and muscarinic ACh receptors in ACh-evoked CA secretion from perfused rat adrenal medulla has been described in previous papers (10, 23). The present study confirmed this fact and further documented distinct characteristics of the secretory responses mediated by the two classes of ACh receptors. The different time courses of the secretory responses elicited by nicotine (or ACh in the presence of atropine) and muscarine (or ACh in the presence of C6) were found by means of the real-time measurement. The nicotinic secretory response during a continuous stimulation decreased exponentially, probably due to the desensitization of nicotinic ACh receptors, until the CA output went down close to the basal level whereas the muscarinic response exhibited a maintained secretion that lasted as long as the stimulation was continued. The maintained secretion as well as the initial, spiky output increased with increasing concentration of ACh up to 1 mM in the presence of C6 (data not shown). This suggests that unlike nicotinic receptors, muscarinic ACh receptors are not desensitized by an exposure to concentrated ACh.

Recently, Malhotra *et al.* (10) reported the time course of CA secretion, with a 5-min time resolution, from the perfused rat adrenal stimulated by nicotine or muscarine. As compared their results with ours, the basic features of the time courses of the nicotinic and muscarinic responses are commonly shown. In detail, however, some differences are recognized. The initial, spiky phase of the muscarinic response (Fig. 3Ba) is absent in their measurement. The nicotinic response in our measurement declined faster due to desensitization than that in theirs, showing about 1 min and 15 min for half-decay, respectively. These discrepancies, however, seem to be derived, at least partially, by the low time-resolution of CA detection in their experiments, with which transient changes, particularly the initial peak phases, may be smoothed out in the detection.

It was shown that the muscarinic stimulation selectively evoked adrenaline release while the nicotinic stimulation caused both

adrenaline and noradrenaline secretions non-selectively. The preferential secretion of adrenaline over noradrenaline on stimulating muscarinic receptors was already reported with the cat adrenal medulla (3). Wakade and Wakade (19) also reported that muscarine preferentially evoked adrenaline secretion in the rat adrenal medulla, showing that the NA/A ratio was 0.212 with muscarine while it was 0.289 with nicotine. In this study, the NA/A ratio with muscarine was found to be 0.025, which is far smaller than their figure. The reason for this discrepancy is not clear.

Concerning the impotency of muscarine to secrete noradrenaline, the following two cases are conceivable; noradrenaline-secreting cells in those adrenal medullae may either have fewer number of muscarinic receptors or they induce weaker intracellular secretory processes after stimulation of muscarinic ACh receptors.

Mechanisms of the CA Secretion through Nicotinic and Muscarinic ACh Receptors

It was shown that Ca^{2+} channel blockers could be used to characterize the difference between nicotinic and muscarinic responses. The blockade of Ca^{2+} channels either by nifedipine or Cd^{2+} inhibited the CA secretion evoked by nicotinic stimulation (Fig. 3A, b and c). This suggests that the nicotinic secretory response in chromaffin cells is associated with an increased Ca^{2+} -influx through voltage-dependent Ca^{2+} channels (presumably classified by L-type based on nifedipine and Cd^{2+} sensitivities; 12, 13) which are activated by nicotine-receptor-mediated depolarization.

In the muscarinic response, on the other hand, the CA secretion was not affected by nifedipine (Fig. 3Bb). In rat adrenal chromaffin cells, a poor sensitivity of the muscarinic secretory response to an organic Ca^{2+} channel blocker (verapamil) was reported by Harish *et al.* (6). They further showed that the CA secretion evoked by muscarine persisted in a low Ca^{2+} medium. They suggested from these observations that muscarine induced the production of a second messenger (such as inositol trisphosphate; 1) which in turn mobilized intracellular Ca^{2+} to promote the secretory process. However, it was found in this study that the muscarinic secretory response

was inhibited by Cd^{2+} (Fig. 3Bc). This argues against the above notion that the intracellular Ca^{2+} store plays an essential role for the muscarine-evoked secretion if Cd^{2+} exerts its effect exclusively on Ca^{2+} channels to block Ca^{2+} -influx. At present, however, we can not rule out a possibility that a very small amount of Cd^{2+} may enter chromaffin cells and interfere with intracellular secretory process.

As another possibility, the activation of muscarinic receptors on chromaffin cells may induce a Ca^{2+} -influx which is not inhibited by nifedipine but by Cd^{2+} . There are now various, novel proposals (4, 7) in which second messengers or GTP-binding proteins in various cell types could activate non-traditional Ca^{2+} channels to induce Ca^{2+} -influx to trigger cellular responses following agonist-receptor interactions.

We studied previously the characteristics of the CA secretion induced by bradykinin (20). Bradykinin-stimulated rat adrenal medulla cells selectively secreted adrenaline and this secretion was not suppressed by methoxy-verapamil (D600) but inhibited by Co^{2+} . Moreover, the time course of bradykinin-induced secretion quite resembled that of muscarine-induced secretion, exhibiting a brief, initial output followed by a maintained secretion. These characteristics coincide all with those of muscarine-evoked secretion presented in this study. We therefore presume the occurrence of common intracellular processes in muscarine- and bradykinin-evoked secretions.

ACh Receptors Activated by Splanchnic Nerve Stimulation

The CA secretion caused by stimulation of splanchnic nerve contained a very little muscarinic contribution (Fig. 4). Wakade and Wakade (19) have reported a similar result when the nerve in the adrenal gland was transmurally stimulated. It seems that nicotinic receptors are concentrated in the synaptic region on rat adrenal medullary cells while most of muscarinic receptors occur in the extrajunctional region.

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