Secretion of catecholamines from adrenal gland by a single electrical shock: Electrotonic depolarization of medullary cell membrane

(neurosecretory mechanism/calcium/tetrodotoxin/acetylcholine/cholinergic antagonist)

ARUN R. WAKADE* AND TARUNA D. WAKADE*

Abteilung Neurochemie, Max-Planck Institut für Psychiatrie, 8033 Martinsried b. München, Federal Republic of Germany

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ABSTRACT Transmural stimulation of the isolated adrenal gland of the rat and guinea pig results in secretion of catecholamines. The secretion is due to activation of cholinergic receptors of the adrenal medulla by acetylcholine released from splanchnic nerve terminals after transmural stimulation. Our aim was to see whether the same experimental technique could be used to directly excite the adrenal medullary cell membrane by electrical stimulation and whether such stimulation would result in secretion of catecholamines. We demonstrate here that a single electrical shock to the perfused adrenal gland of the rat results in massive secretion of epinephrine and norepinephrine. The secretion is directly related to the strength and duration of the applied stimulus over a wide range. Catecholamine secretion is unaffected by tetrodotoxin or hexamethonium/atropine but is abolished by Ca^{2+} lack or 3 mM Mn^{2+} . We suggest that the adrenal medullary membrane undergoes nonpropagated electrotonic depolarization on electrical stimulation and thereby voltage-dependent Ca^{2+} channels are opened to initiate secretion.

Acetylcholine (AcCho) released from splanchnic nerves causes activation of the cholinergic receptors of the adrenal medulla followed by inward movement of Ca^{2+} , and subsequent secretion of medullary hormones. However, the precise electrophysiological changes that occur as a result of the action of AcCho on the medullary cell membrane are not completely understood. Since the early work of Cannon and Rosenblueth (1), medullary cells have been regarded as electrically insensitive. However, recent studies have shown that depolarization (2), or even action potentials (3, 4), can be recorded from chromaffin cells under various conditions. Cultured adrenal chromaffin cells exhibit spontaneous action potentials and the frequency of such activity can be enhanced by AcCho (4) or excess K^+ (5). Therefore, it is proposed that alteration of medullary spike activity by AcCho plays a physiological role in secretion of catecholamines (CA).

Recently, it has been shown that, when the perfused adrenal gland is placed between plate electrodes and stimulated repetitively, CA are secreted (6, 7). The secretion was predominantly a result of activation of medullary nicotinic receptors by AcCho released from splanchnic nerves during stimulation. Thus, it was of particular interest to know whether medullary cell membrane could be excited by electrical stimulation and, if electrical stimulation did lead to a secretory response, the type of electrophysiological event that is responsible for the secretion of CA.

Pharmacological analysis of our data suggests that electrical stimulation of the adrenal gland may cause nonpropagated de-

polarization of the medullary cells and thereby increase Ca^{2+} influx and CA secretion.

MATERIALS AND METHODS

Perfusion of the left adrenal gland of the rat was carried out as described (6). Briefly, rats (250–350 g; either sex) were anesthetized with ether, and the left renal vein was cannulated after ligating other blood vessels. The adrenal gland, along with tied blood vessels and the cannula, was removed from the rat and placed on a platform in a Lucite chamber. The chamber was maintained at 37°C by circulating heated water. The adrenal gland rested on a flat Ag/AgCl plate mounted on the platform. The metal strip served as one of the electrodes for stimulation of the gland, and another plate electrode was gently placed on top of the gland (see figure 1 of ref. 6). The adrenal gland was perfused (0.2 ml/min) by means of a roller pump (LKB, model 2120). The perfusion medium was Krebs buffer/bicarbonate bubbled with 95% O_2 /5% CO_2 and the final pH was 7.4 to 7.5. The solution contained Na_2EDTA (10 $\mu\text{g/ml}$) to prevent oxidation of CA. Perfusate escaped from a slit made in the adrenal gland and was collected in chilled tubes containing perchloric acid (final concentration, 0.05 M).

Stimulation of the adrenal gland was achieved by connecting the plate electrodes to a Grass stimulator, model S 88. The resistance between plate electrodes, touching the adrenal gland, was fairly constant from one preparation to another ($\approx 350 \Omega$). The Grass stimulator was specifically wired to deliver supra-maximal current against a low resistance between electrodes. Stimulation parameters were either 600 shocks at 10 Hz (1.0-ms duration and 0.18-mA strength) or single shocks of various durations and strengths. In some cases, 130 μM AcCho was injected into the perfusion stream to evoke CA secretion.

CA content was analyzed by HPLC after treatment of the perfusate with alumina (8). Samples (0.05 ml) of the alumina eluate were injected onto the column for electrochemical detection of norepinephrine (NE), epinephrine (EPI), and dopamine (9, 10). Appropriate standard solutions were also treated with alumina, with recoveries ranging from 65% to 80%. Corrections for dilutions and recovery in alumina eluate have been made. The CA values are expressed in terms of base. Data are presented as mean $\pm$ SEM, and differences were compared by using Student's paired *t* test. Drugs used were AcChoCl, hexamethonium bromide, and atropine sulfate (Sigma), and tetrodotoxin (Calbiochem).

Abbreviations: AcCho, acetylcholine; CA, catecholamine(s); EPI, epinephrine; NE, norepinephrine; TTX, tetrodotoxin.

* Present address: Dept. of Pharmacology, State University of New York, Downstate Medical Center, 450 Clarkson Ave., Brooklyn, NY 11203.

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RESULTS

Secretion of CA evoked by various experimental procedures is shown in Fig. 1. Stimulation of the adrenal gland by a single shock resulted in marked increases in NE and EPI secretion over the spontaneous secretion. The secretory response increased with increase in the duration of the single shock up to 300 ms (A). The same adrenal gland was also stimulated by a train of 600 shocks at 10 Hz. Fig. 1B shows that secretion of NE and EPI was increased several-fold by the train of shocks at 10 Hz from the gland that had been stimulated earlier with single shocks of increasing duration. In another series of experiments, secretory response was evoked by single shocks of various stimulus strengths (C) or by injecting 130 μ M AcCho into the perfusion stream (D). Increase in stimulus strength from 0.08 to 0.28 mA produced a >10-fold increase in CA secretion. Adrenal glands stimulated at higher stimulus strength when perfused with Krebs buffer/AcCho secreted large quantities of CA (Fig. 1D). Perfusates analyzed 4 or 8 min after termination of the stimulus did not contain excess amounts of CA, and the amounts were comparable with those found prior to

FIG. 1. CA secretion in response to a single shock, a train of shocks, and AcCho. (A) Fifteen minutes after the beginning of perfusion with Krebs solution, the adrenal gland was stimulated by single shocks (0.18 mA) of various durations. A 10-min rest was given between stimulation periods. The sample-collection time was 4 min for the 30-ms shock and 8 min for the others. Spontaneous secretion in the absence of stimulation was determined for 4- and 8-min periods. (B) Fifteen minutes after the last stimulation, the adrenal gland was stimulated at 10 Hz (60 s). In another series, the adrenal gland was stimulated by a single shock (30 ms) at various strengths (C) and, 15 min later, it was stimulated with 130 μ M AcCho (D). The sample-collection time was 4 min for the 0.08-mA and AcCho stimulations and 8 min for the other stimulations. $\circ$ and $\square$, NE; $\bullet$ and $\blacksquare$, EPI; — and $\square$, spontaneous secretion. Six adrenal glands were used in each series of experiments.

stimulation. In all instances, EPI secretion was ≈ 3 times greater than that of NE. This was expected, because the NE/EPI ratios in control and experimental (perfused and stimulated) adrenal glands were similar [control, NE = 4.28 ± 0.22 and EPI = 11.69 ± 0.9 μ g per medulla ($n = 7$)]. The corresponding dopamine values were 0.16 ± 0.02 and 0.22 ± 0.03 μ g per medulla ($n = 7$).

Comparison of the amounts of CA secreted on one shock and a train of 10-Hz shocks is shown in Table 1. The amounts of NE and EPI secreted on 1 shock and 10 Hz when calculated as nanograms per shock showed marked differences between two modes of stimulation. The amount of CA secreted upon one shock (100 ms) was >300 times that secreted after a train of 10-Hz shocks. Increase in duration to 300 ms led to an almost 800-fold increase in secretion. If the secretory response is expressed in terms of ng/ms duration, the amounts of CA secreted after 1 shock or a train of 10-Hz shocks were also significantly different (2- to 3-fold).

FIG. 2. Effects of TTX and cholinergic blocking agents on CA secretion evoked by electrical stimulation. Secretion of CA was evoked in Krebs buffer or in 0.3 μ M TTX/Krebs buffer in the same adrenal gland by a train of impulses (600 shocks for 1 min) or by a single shock (100 ms, 0.18 mA). (A) Fifteen minutes after perfusion with Krebs buffer, the adrenal gland was stimulated by a 10-Hz train of shocks or by single shocks at 10-min intervals. Then, 0.3 μ M TTX/Krebs buffer ($\downarrow$) was introduced for 15 min and secretion was evoked as before. In another set of experiments (B), the identical protocol was followed, except that 0.14 mM hexamethonium/1.44 μ M atropine was used instead of TTX. A 10-min rest was given between stimulation periods. The sample-collection time was 4 min for the multiple-shock stimulation and 8 min for the one-shock stimulation. Spontaneous secretion of NE and EPI in the absence of stimulation $\square$ was determined for 4- or 8-min periods just prior to stimulation. $\square$ and $\blacksquare$, NE; $\square$ and $\blacksquare$, EPI. Five adrenal glands were used in each series of experiments. Results represent mean $\pm$ SEM.

Experiments were carried out with tetrodotoxin (TTX) to determine the involvement of Na^+ action potentials in CA secretion induced by the two types of stimulation procedures. The results of such experiments are shown in Fig. 2A. Secretion of NE and EPI was induced first by a train of 10-Hz shocks and then, 15 min later, by a single shock (100 ms). Both types of stimulation caused a marked increase in CA secretion over the spontaneous secretion. Introduction of $0.3 \mu\text{M}$ TTX for 15 min reduced the CA secretion induced by the train of shocks. In contrast, secretion of NE and EPI induced by 1 shock was unaffected by TTX in the same adrenal gland. The secretory response induced by the train of shocks was restored to the control level 15 min after washout of TTX (data not shown).

Attempts were made to study CA secretion induced by one shock and by a train of 10-Hz shocks in low- Na^+ Krebs buffer. Unfortunately, decreasing the Na^+ content from a control value of 144 mM to 25 or 50 mM (while replacing it with equimolar LiCl) resulted in a marked increase in spontaneous secretion of CA (up to 100 ng) and, therefore, it was not possible to accurately determine the effects of low- Na^+ medium on CA secretion evoked by stimulation.

Whether cholinergic receptors play any role in one-shock-induced secretion of CA was examined by using hexamethonium ion/atropine. These results are shown in Fig. 2B. A combination of nicotinic and muscarinic antagonists produced a marked blockade of CA secretion induced by a train of 10-Hz shocks. However, one-shock-induced secretion of NE and EPI in the presence of hexamethonium ion/atropine was almost identical to that found in the absence of these antagonists.

It was of critical interest to know whether Ca^{2+} was needed for CA secretion evoked by a single shock. The results of such experiments are shown in Fig. 3. CA secretion was evoked by a train of impulses (600 shocks at 10 Hz) and by one shock of 30- or 300-ms duration in 2.5 mM Ca^{2+} medium. Earlier, it was reported that omission of Ca^{2+} from the perfusion medium is not sufficient to abolish CA secretion and inclusion of EGTA

is necessary to completely block AcCho-induced secretion in the rat adrenal gland (6, 7). Therefore, we perfused the adrenal gland with Ca^{2+} -free Krebs buffer 1 mM EGTA for 30 min and then evoked secretion by either a train of impulses or one shock. As shown in Fig. 3, secretion of NE and EPI evoked by either a train of 10-Hz shocks or a single shock (30- or 300-ms duration) was undetectable over the spontaneous secretion. Reperfusion of the adrenal gland with 2.5 mM Ca^{2+} -containing Krebs buffer completely restored the secretory response evoked by both types of stimulation procedures.

The effects of 3 mM Mn^{2+} , known to block stimulation-evoked secretion of AcCho and CA in the rat adrenal gland (6), were tested. The results of such experiments are summarized in Table 1. The amounts of NE and EPI induced by one shock (100-ms duration) and those induced by a train of 10-Hz shocks were suppressed after perfusion of the adrenal gland with 3 mM Mn^{2+} . Secretion (evoked by stimulation) was restored to normal level after perfusion with Krebs buffer (data not shown).

DISCUSSION

We have previously shown that passage of a train of impulses across the isolated adrenal gland causes excitation of splanchnic nerve terminals and that AcCho released from these nerves activates medullary cholinergic receptors to evoke CA secretion (6, 7). Thus, the effect of stimulation by a train of 10-Hz shocks was predominantly on presynaptic components of the adrenal gland. In this study, we have shown that application of a single electrical shock leads to massive secretion of CA from a saline-perfused adrenal gland of a rat *in vitro*. The questions are which of the two sites in the adrenal gland (splanchnic nerve terminals or medullary cells) are affected by a single shock and what kind of electrophysiological changes are brought about by such stimulation to trigger secretion. By using various physiological and pharmacological criteria, we have concluded that the adrenal medullary cell membrane can be excited directly by electrical stimulation. The membrane undergoes nonpropagated electro-

FIG. 3. Importance of Ca^{2+} in secretion of CA evoked by 1 shock. Fifteen minutes after perfusion with 2.5 mM Ca^{2+} /Krebs buffer, CA secretion was evoked by 600 shocks for 1 min. Ten minutes later it was evoked by a single shock (30 ms; 0.18 mA) and, 10 min later, by still another shock (300 ms; 0.18 mA). The gland was then perfused with Ca^{2+} -free Krebs buffer/ 1 mM EGTA for 20 min ($\downarrow$), and secretion was evoked as before. Finally, the perfusion medium was switched to 2.5 mM Ca^{2+} Krebs buffer and secretion was again evoked. The sample-collection time was 4 min for the multiple-shock and 30-ms-duration single-shock stimulations, and 8 min for 300-ms-duration single-shock stimulations. Spontaneous secretion of NE and EPI in the absence of stimulation ($\square$) was determined for 4- and 8-min periods just prior to stimulation. $\square$ and $\square$, NE; $\blacksquare$ and $\blacksquare$, EPI. Results represent mean $\pm$ SEM of seven experiments.

Table 1. Secretion of CA after stimulation of adrenal gland with a single shock or a train of shocks and blockade by Mn^{2+}

Treatment and stimulation	CA secretion, ng			
	Per shock		Per ms	
	NE	EPI	NE	EPI
Krebs buffer*				
One shock (100 ms)	26.5 $\pm$ 1.8	77.5 $\pm$ 5.5	0.26 $\pm$ 0.01	0.77 $\pm$ 0.05
One shock (300 ms)	75.3 $\pm$ 4.7	189 $\pm$ 16.3	0.25 $\pm$ 0.03	0.63 $\pm$ 0.075
Train of shocks (10 Hz, 1 min)	0.08 $\pm$ 0.001	0.25 $\pm$ 0.02	0.08 $\pm$ 0.001	0.25 $\pm$ 0.02
3 mM Mn/Krebs buffer†				
One shock (100 ms)	4.0 $\pm$ 0.06	5.7 $\pm$ 2.1		
Train of shocks (10 Hz, 1 min)	0.01 $\pm$ 0.001	0.04 $\pm$ 0.003		

CA secretion is calculated as nanograms per shock by dividing total amounts of NE and EPI secreted by number of shocks given in the stimulation period and as ng/ms by dividing total amounts of NE and EPI by total stimulus duration in each stimulation period. Net secretion was calculated by subtracting spontaneous secretion during an equivalent time period from total secretion.

*The experimental protocol was identical to that for Fig. 1 A and B. Six adrenal glands were used.

†The experimental protocol was identical to that for Fig. 2 A and B. Three adrenal glands were used for each series of experiments.

tonic depolarization on electrical stimulation, causing influx of Ca^{2+} and subsequent secretion of CA.

The lack of effect of cholinergic blocking agents on one-shock-induced secretion of CA indicates that the main target of stimulus is the medullary cells and not the presynaptic cholinergic nerve endings. Another important piece of evidence is that splanchnic nerves exhibit optimal stimulation parameters for secretory response (6) but, in this study, the secretion increased with increase in stimulus strength and duration without reaching a plateau. This observation is unique, because stimulation of sympathetic nerves of the guinea pig heart with one pulse of extended duration or strength does not lead to release of NE (unpublished observations). Finally, comparison of the amounts of CA induced by a train of 10-Hz shocks or one-shock stimulation shows a marked difference. Taken together, these findings suggest that electrical stimulation causes excitation of the medullary cell membrane. During the course of this study, it was reported that bovine adrenal medullary slices secreted CA on electrical stimulation (11).

Secretion of CA increased with the duration and strength of the stimulus, and the response did not level off at 300-ms duration or 0.28-mA strength. The simplest explanation may be that medullary cell membrane undergoes electrotonic depolarization, and the degree of such depolarization depends largely on the extent of stimulus strength and duration. The amounts of Ca^{2+} entering the cell would be related to the stimulus strength and duration. On the other hand, if a conducted action potential(s) was involved in one-shock-induced secretion, we would not expect an increase in secretory response beyond a critical stimulation level (i.e., supramaximal parameters). Further support for the assumption that Na^+ action potentials are not involved in one-shock-induced secretion of CA comes from TTX experiments. Secretion evoked via splanchnic nerves by a train of 10-Hz shocks was markedly reduced by TTX but that evoked by one shock was unaffected. However, involvement of Ca^{2+} action potentials cannot be ruled out from these experiments.

The NE/EPI ratios in the perfusate after various types of stimulation and those in the control and stimulated adrenal glands were comparable. Furthermore, spontaneous secretion of CA was not elevated after stimulation with one shock, and AcCho was able to evoke secretion after one-shock treatment. These data suggest that secretion evoked by one shock had no deleterious effects on storage and release of CA. The most important piece of evidence for the fact that we are dealing with a physiological event comes from Ca^{2+} lack and Mn^{2+} experi-

ments. Almost complete and reversible blockade by these procedures means that Ca^{2+} is intimately involved in single-shock-induced secretion of CA. Since Ca^{2+} -dependent secretion increased with increase in the duration of stimulus, it is likely that the amount of Ca^{2+} entering the cell via voltage-dependent channels also increases with the duration of the shock.

Action potentials have been recorded from dissociated chromaffin cells maintained in tissue culture medium for several days (3, 4, 5) but not from intact adrenal glands perfused *in vitro* with physiological salt solution (12). Furthermore, there is a discrepancy between the effect of AcCho on the frequency of action potentials in dissociated chromaffin cells and that on CA secretion from the perfused adrenal gland. In the former case, the effect of AcCho was saturable at 3–10 μ M, but CA secretion induced by AcCho was nonsaturable even at 300 μ M (5, 6). Thus, the physiological significance of adrenal medullary action potentials in secretion of CA remains obscure. One possibility is that, after stimulation, the adrenal chromaffin cell membrane can undergo nonpropagated local depolarization to bring about the voltage-dependent increase in Ca^{2+} influx and subsequent secretion of CA.

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1. Cannon, W. B. & Rosenbluth, A. (1937) *C. R. Seances Soc. Biol. Paris* **124**, 1262–1264.
2. Douglas, W. W., Kanno, T. & Sampson, S. R. (1967) *J. Physiol. (London)* **188**, 107–120.
3. Biales, B., Dichter, M. & Tischler, A. (1976) *J. Physiol. (London)* **262**, 743–753.
4. Brandt, B. L., Hagiwara, S., Kidokoro, Y. & Miyazaki, S. (1976) *J. Physiol. (London)* **263**, 417–439.
5. Kidokoro, Y. & Ritchie, A. K. (1980) *J. Physiol. (London)* **307**, 199–216.
6. Wakade, A. R. (1981) *J. Physiol. (London)* **313**, 463–481.
7. Wakade, A. R. (1981) *J. Physiol. (London)* **313**, 481–498.
8. Shellenberger, M. K. & Gordon, J. H. (1971) *Anal. Biochem.* **39**, 356–372.
9. Felice, L. J., Felice, J. D. & Kissinger, P. G. (1978) *J. Neurochem.* **31**, 1461–1465.
10. Wakade, A. R. & Wakade, T. D. (1981) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **317**, 302–309.
11. Brooks, J. C., Burke, D. H. & Trembl, S. (1980) *Brain Res.* **194**, 269–272.
12. Ishikawa, K. & Kanno, T. (1978) *Jpn. J. Physiol. (Shinrigaku Kenkyu)* **28**, 275–289.