Changes in Tonicity of Perfusion Medium Cause Prolonged Opening of Calcium Channels of the Rat Chromaffin Cells to Evoke Explosive Secretion of Catecholamines

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Secretion of catecholamines (CA) from the isolated rat adrenal gland during and after perfusion with hypertonic Krebs bicarbonate solution was studied. Perfusion with hypertonic solution made by adding either 156 mM sodium chloride, choline chloride, arginine hydrochloride, sodium thiocyanate, or 312 mM sucrose had no effect on the spontaneous secretion of CA. The secretion evoked by splanchnic nerve stimulation (10 Hz for 30 sec) and nicotine (2 μ g) remained unaffected during perfusion with hypertonic Krebs solution (156 mM excess NaCl). After perfusion of the adrenal gland with hypertonic Krebs solution for 1 hr, if the medium was switched to normal Krebs solution the secretion of CA increased from about 15 to 450 ng; it remained elevated for over 1 hr and eventually returned to the control level after 150 min. Secretion of CA obtained in normal Krebs solution after perfusion of the adrenal gland with hypertonic medium was not affected by atropine plus hexamethonium, splanchnectomy, or tetrodotoxin.

After perfusion of the adrenal gland with Ca-free hypertonic (275 mM NaCl) Krebs solution for 1 hr, if the medium was changed to normal Krebs solution the secretion of CA increased from 10 to about 4000 ng in the first 10 min. The enhanced secretion was associated with an almost 70% reduction in the CA content of the adrenal medulla. A massive secretion of CA obtained after switchover from hypertonic to normotonic solution was not associated with an increase in lactate dehydrogenase content in the perfusate, nor was there any reduction in the lactate dehydrogenase content of the adrenal medulla. Immediately after the switchover from hypertonic to normotonic medium, the accumulation of Ca^{45} increased 6-fold over the control accumulation. The increase in Ca^{45} accumulation was detected as late as 120 min after the switchover, and was accompanied by an increase in the secretion of CA. The most likely explanation for these findings is that Ca channels, once opened after the switchover, inactivate very slowly and allow Ca ions to flow inside the chromaffin cells. The accumulation of tetraphenylphosphonium (C^{14} -TPP), a marker for the membrane potential, was 86 pg/mg in the adrenal medulla perfused with normal Krebs solution, and decreased to 32 pg/mg in the presence of 55 mM K-Krebs solution. After perfusion with hypertonic medium, the accumulation of C^{14} -TPP increased to about 190 pg/mg. Reperfusion with normal Krebs solution decreased the accumulation to about 105 pg/mg. The increase in C^{14} -TPP accumulation observed in hypertonic medium was prevented by inclusion of 30 mM K in the medium. High-K treatment also

prevented the explosive secretion of CA obtained after switchover from hypertonic to normotonic medium. It is suggested that perfusion of the adrenal medulla with hypertonic solution causes hyperpolarization of the chromaffin cell membrane, and switchover to normotonic medium moves the membrane potential toward the original resting level. This relative, rather than absolute, change in the membrane potential is of sufficient magnitude to open Ca channels and to raise intracellular Ca concentration high enough to evoke massive secretion of CA.

A number of investigators have used perfused adrenal glands of different species to evoke secretion of catecholamines (CA) by a variety of maneuvers in order to study the role of Ca ions in the secretory process (Baker and Rink, 1975; Douglas and Rubin, 1963; Kidokoro et al., 1979; Kirpekar and Cervoni, 1963; Marley and Paton, 1961; Robinson, 1967; Wakade, 1981a; Wilson and Kirshner, 1977). In the isolated perfused adrenal gland of the rat, it has been possible to evoke secretion by (1) activation of nicotinic and muscarinic receptors by nicotine, muscarine, or by ACh liberated from presynaptic splanchnic nerve terminals (Wakade and Wakade, 1983); (2) increasing the extracellular concentration of K, which is believed to cause depolarization of the chromaffin cell membrane (Kidokoro et al., 1979; Wakade and Wakade, 1983); (3) direct electrical stimulation of chromaffin cells using pulses of high strength or prolonged duration, which probably lead to nonpropagated local depolarization of the chromaffin cell membrane (Wakade and Wakade, 1982a); and (4) changing the ionic environment of the chromaffin cells by blocking the activity of the Na-K pump by means of cardiac glycosides or K-deprivation (Wakade, 1981b). In all of the above procedures, which have led to the secretion of CA, the indispensable role of calcium has been established.

The present study describes an entirely different approach to evoking secretion of CA in a Ca-dependent manner. We show that a switchover from hypertonic to normotonic perfusion medium results in a massive amount of CA secretion. A series of experiments has been designed to elucidate the mechanism of this secretion of CA. Preliminary reports of some of these results have already been presented (Sharma and Wakade, 1984; Wakade et al., 1985).

Materials and Methods

Retrograde perfusion of the adrenal gland

The left adrenal gland of the male rat (300–400 gm) was perfused retrogradely, as described earlier (Wakade, 1981a). Briefly, rats were anesthetized with ether, the left renal vein was cannulated, and the tip of the cannula remained near the junction of the renal and adrenal veins. All other blood vessels were ligated. The adrenal gland, along with tied blood vessels and the cannula, was removed from the rat and placed on a metal plate mounted in a Lucite chamber. The metal plate was made up of Ag/AgCl and served as one of the electrodes for stimulation

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This article is dedicated to the memory of the late Professor S. M. Kirpekar.

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of the gland; another plate electrode was placed on top of the gland (see below). The chamber was maintained at 37°C by circulating heated water. The gland was perfused at 0.35 ml/min by means of a Sigma motor pump. Perfusate escaped from a slit made in the adrenal cortex and was collected in chilled tubes.

Perfusion media

Krebs bicarbonate solution was used for the perfusion of the adrenal glands. The composition of the solution was as follows (mM): NaCl, 119; KCl, 4.7; CaCl₂, 2.5; MgSO₄, 1.2; KH₂PO₄, 1.2; glucose, 11; NaHCO₃, 25. Hypertonic solution was made by adding 156 mM of either sodium chloride, choline chloride, sodium thiocyanate, or arginine hydrochloride to Krebs bicarbonate solution. Hypertonic solution was also made by adding 156 and 312 mM sucrose. Ca-free medium was prepared by omitting CaCl₂ (2.5 mM) and adding 1 mM EGTA to either normal or hypertonic Krebs solution. K concentration of the medium was increased to 30 or 55 mM by adding excess amounts of KCl and omitting equivalent amounts of NaCl, unless stated otherwise. All solutions were bubbled with 95% O₂ + 5% CO₂, and the final pH was 7.4 ± 0.1 (*n* = 13). In all cases the adrenal gland was perfused for 30 min with Krebs solution before the beginning of the experiment.

Stimulation of the adrenal gland

Stimulation of the adrenal gland was achieved by connecting the plate electrodes to a Grass Model S88 stimulator. Stimulation parameters were 300 shocks at different frequencies (1.0 msec duration and 180 mA strength). In other cases, 2 µg nicotine was injected into the perfusion stream to evoke CA secretion. Perfusates were collected for varying periods of time, as described below.

C¹⁴-TPP accumulation

Thirty minutes after perfusion with Krebs solution, the medium was switched over to Krebs solution containing 2.2 µg/ml tetraphenylphosphonium (C¹⁴-TPP) (specific activity, 19.2 mCi/mmol; New England Nuclear) for 45 min, and then the gland was perfused with Ca-free Krebs solution at 4°C for 10 min. The adrenal medulla was separated, weighed, and homogenized in 0.1 N perchloric acid (1 ml), and after centrifugation (2000 rpm), the supernatant (0.5 ml) was counted in a Beckman liquid scintillation counter (Beckman Model LS7000). Total counts, after correction for dilution and quenching, were converted to picograms and the accumulation of C¹⁴-TPP was expressed in picograms per milligram. An identical protocol was carried out in other series of experiments, except that the adrenal gland was always pretreated with a modified Krebs solution (i.e., hypertonic or 55 mM K, etc.) for 10 min prior to switchover to modified Krebs solution containing C¹⁴-TPP.

Ca⁴⁵ accumulation

The adrenal gland was perfused with Krebs solution for 60 min and then the medium was changed for 10 min to Krebs solution containing 0.5 µg/ml Ca⁴⁵ (as Ca⁴⁵Cl₂, specific activity 23.69 mCi/mg; New England Nuclear). The gland was perfused for 30 min with radiolabel-free Krebs solution to wash out the extracellular Ca⁴⁵. In 4 experiments, the radioactivity in wash fluid was determined from the point of termination of perfusion with Ca⁴⁵-Krebs solution for up to 30 min. The first 5 min sample contained 96 ± 0.04 × 10⁴ cpm, whereas 20 and 25 min samples contained 0.023 ± 0.005 × 10⁴ and 0.021 ± 0.003 × 10⁴ cpm, respectively.

Basically the same protocol was followed in various types of experiments, except that Ca⁴⁵ was introduced at different times after initial perfusion of the adrenal gland with modified Krebs solution, as described in the text. Then the gland was quickly removed and the medulla was separated and rinsed several times with ice-cold Krebs solution. The medulla was gently blotted, weighed, and then homogenized in 1 ml of 0.1 N perchloric acid in a glass homogenizer. The homogenate was centrifuged (2000 rpm) for 5 min, and radioactivity in 0.5 ml supernatant was measured in a liquid scintillation counter (Beckman Model LS7000). Appropriate corrections for volume changes and quenching have been made. The amounts of Ca⁴⁵ accumulated in the control gland in each group of experiments were subtracted from those in the experimental gland to obtain the net accumulation of Ca⁴⁵; they are expressed as picograms per milligram of the wet weight of the adrenal medulla.

Measurement of CA

CA content in the perfusate was assayed directly by the fluorometric method of Anton and Sayre (1962). Because of low sample blanks, it was possible to detect up to 5 ng in a 2 ml perfusate without further concentration by alumina treatment.

CA content of the adrenal medulla was measured in some experiments. After removal of the adrenal gland the medulla was separated, weighed, and homogenized in 0.5 ml of 0.1 N perchloric acid. The homogenate was centrifuged and an aliquot (0.2 ml) was diluted 100× with ice-cold distilled water; 0.2 ml of this fluid was used for CA determination, using the method of Anton and Sayre (1962). Total tissue content was expressed as micrograms per milligram of adrenal medulla. The content of CA in the perfusate and the adrenal medulla was calculated in terms of the epinephrine base.

Lactate dehydrogenase

Lactate dehydrogenase in the perfusate and the adrenal medulla was analyzed by the method of Wroblewski and LaDue (1955), using Sigma Kit No. 340UV. One milliliter of the perfusate was used for the assay. In the case of the adrenal medulla, the tissue was homogenized in 1 ml of ice-cold 0.1 M phosphate buffer, centrifuged, and the supernatant was divided into 2 portions. One part was diluted 10× with phosphate buffer and used for determination of lactate dehydrogenase. The other portion was first diluted 10× with 0.5 N perchloric acid, then diluted 10× with water and used for CA estimation.

All data were presented as means with standard errors, and differences were compared using Student's *t* test.

Drugs and chemicals used

The following drugs and chemicals were used in these experiments: nicotine (Aldrich Chemical Corp., Milwaukee, WI); TTX (Calbiochem-Behring Corp., La Jolla, CA); atropine sulfate, hexamethonium bromide (Sigma Chemical Corp., St. Louis, MO); choline chloride (Eastman Kodak Company, Rochester, NY); sodium chloride and sucrose (Fisher Scientific Corp., Fairlawn, NJ); arginine hydrochloride (Nutritional Biochem, Cleveland, OH); and sodium thiocyanate (Merck and Company, Rahway, NJ).

Results

Effect of hypertonic Krebs solution on spontaneous and stimulation-evoked secretion of CA

The spontaneous secretion of CA from the rat adrenal gland perfused with Krebs bicarbonate solution remained steady and at a very low level over prolonged periods of time (15 ± 2 ng at 60 min and 10 ± 3 ng at 240 min; *n* = 6). If the adrenal gland was perfused with hypertonic Krebs solution (made by adding 156 mM NaCl) for several hours, there was no change in the normal pattern of spontaneous secretion of CA (15 ± 10 ng vs 10 ± 2 ng; *n* = 13).

In subsequent experiments the effect of hypertonic medium on stimulation-evoked secretion of CA was examined. Secretion of CA evoked by nerve stimulation (5 Hz for 1 min) or 2 µg nicotine in hypertonic medium was almost identical to that obtained in normal medium in the same adrenal gland (142 ± 11 vs 155 ± 18 ng for 5 Hz and 139 ± 11 vs 134 ± 16 ng for nicotine; *n* = 3). Each sample was collected for 5 min.

Secretion of CA after exposure of the adrenal gland to hypertonic Krebs solution

If the perfusion medium was switched to normal Krebs solution after 1 hr perfusion with hypertonic medium, there was a massive secretory response within the first 5 min period (Fig. 1). The secretion remained at that level (about 400 ng) for the next 10 min and gradually declined to about 100 ng after 30 min. The secretion remained near the 50 ng level even 90 min after switchover to the normal medium. As is shown in Figure 1, after perfusion with Krebs solution for about 2 hr, the secretion of CA approached the basal control levels.

Figure 1. Secretion of CA in normal Krebs solution following exposure to hypertonic medium. After 30 min perfusion with Krebs solution, the medium was changed to hypertonic Krebs solution (156 mM NaCl added to Krebs solution) for 60 min. Four 5 min samples within the last 20 min of perfusion with hypertonic solution were collected for CA assay (open columns). After 60 min, the perfusion medium was changed to normal Krebs solution and 5 min serial samples were collected immediately after the switchover of the solution, up to 30 min (shaded columns). Additional samples were collected, as shown, at various time intervals. Each column represents a mean of 4–7 experiments. Vertical lines, SEM.

Secretion of CA after perfusion with hypertonic Krebs solution made by the addition of different agents

Results of these experiments are summarized in Table 1. As stated above, perfusion of the adrenal gland with Krebs solution throughout the experiment did not modify the secretion. However, 1 hr exposure to hypertonic Krebs solution made by the addition of either 156 mM sodium chloride, choline chloride, arginine hydrochloride, or sodium thiocyanate, followed by perfusion with normal Krebs solution, resulted in an enhanced secretion of CA. Hypertonic solution made with 312 mM sucrose was also effective in inducing the secretion. It should be pointed out that perfusion of the adrenal gland with 156 mM of different agents in Krebs solution did not affect the basal secretion of CA.

Figure 2. Secretion of CA after second exposure to hypertonic medium. The experimental protocol was identical to that described in Figure 1, except that 156 mM sodium thiocyanate was used to make hypertonic Krebs solution. Secretion of CA was determined first in normal Krebs solution ($\blacktriangle$) and then in hypertonic medium after 60 min perfusion ($\times$). A series of samples were collected as shown ($\bullet$) when the medium was switched to normal Krebs (at the arrow). After 150 min, the adrenal gland was reperfused with hypertonic Krebs solution for 60 min, a sample was removed ($\times$), and the medium was switched over to normal Krebs (at the arrow), and another series of samples were collected at various points ($\bullet$) for CA analysis. Each sample was collected for 5 min. Each point is a mean of 3 observations. Vertical lines, SEM.

Reproducibility of CA secretion after repeated exposure to hypertonic Krebs solution

Because of the unexpected and unusual nature of the secretion of CA obtained following, but not during, exposure to the hypertonic medium, it was essential to establish that such a secretion was not a result of tissue damage. Two types of experiments were designed to examine this point. In one, the ability of the adrenal gland to secrete CA in response to electrical stimulation (10 Hz) was compared in normal Krebs solution

Table 1. Catecholamine secretion after perfusion with hypertonic solution made by addition of various agents to the Krebs solution

Type of hypertonic Krebs solution	Secretion of catecholamines (ng)		
	Krebs	Hypertonic Krebs	Krebs
Regular Krebs (119 mM NaCl)	15 $\pm$ 10	25 $\pm$ 15	20 $\pm$ 10
+ 156 mM sodium chloride	25 $\pm$ 5	15 $\pm$ 5	460 $\pm$ 40
+ 156 mM choline chloride	20 $\pm$ 10	35 $\pm$ 15	370 $\pm$ 45
+ 156 mM arginine hydrochloride	10 $\pm$ 5	10 $\pm$ 10	150 $\pm$ 40
+ 156 mM sodium thiocyanate	20 $\pm$ 5	20 $\pm$ 5	270 $\pm$ 85
+ 156 mM sucrose	25 $\pm$ 10	15 $\pm$ 10	10 $\pm$ 10
+ 312 mM sucrose	5 $\pm$ 5	20 $\pm$ 5	515 $\pm$ 94

After 30 min perfusion with normal Krebs solution, one 5 min sample was collected, and then the adrenal gland was perfused for 1 hr with Krebs solution made hypertonic by addition of 156 and 312 mM of the agents shown above. A 5 min sample during the last 60 min perfusion period with hypertonic Krebs solution was analyzed, and then the medium was changed to normal Krebs solution. Immediately after the switchover, a 5 min sample was removed. CA analyzed in each 5 min sample were expressed as total secretion in nanograms. Each value is the mean of 3–12 observations.

Figure 3. Degree of hypertonicity and time of exposure to hypertonic solution essential to initiate CA secretion. *a*, Adrenal glands were perfused with Krebs solution to which increasing amounts of NaCl were added, to a final concentration of 275 mM. The adrenal gland was perfused with each hypertonic solution for 1 hr. The increasing strengths of the hypertonic solutions were tested in the same gland after the basal secretion had reached a low control value (about 5–10 ng/5 min sample). *b*, Hypertonic solution (156 mM sodium chloride added to Krebs solution) was used to perfuse the adrenal gland for varying periods of time, as shown, and then the medium was switched over to normal Krebs solution. The same adrenal gland was used to test various exposure periods after basal secretion was restored toward control level (see above). In both series of experiments, CA secretion within the first 10 min of switchover was measured, and these amounts are expressed as ng/min. Each point is a mean of 4–5 experiments. Vertical lines, SEM.

before and after exposing the adrenal gland to the hypertonic solution. In 3 experiments it was found that the secretory response of the adrenal gland after hypertonic challenge (60 min of hypertonic medium followed by 150 min perfusion with normal medium) was still $81 \pm 16\%$ of the control value obtained in Krebs solution at the start of the experiment. Similarly, nicotine-evoked secretion was unaffected by treatment of the adrenal gland with hypertonic Krebs solution followed by 150 min perfusion with normal Krebs solution (267 ± 17 ng vs 252 ± 29 ng).

In another series of experiments, viability was tested by perfusing the same adrenal gland for the second time with hypertonic solution and then examining the secretion during perfusion with normal Krebs solution. The results of such experiments are shown in Figure 2. When the secretory response approached

essentially control value after the first exposure to hypertonic and then to normotonic media, the adrenal gland was reperfed with 156 mM sodium thiocyanate-Krebs solution for 1 hr, and then with normal Krebs solution. As is shown in Figure 2, the secretion of CA increased soon after the return to normal medium and remained elevated over a 10 min period. The adrenal gland was not only capable of responding to a second challenge of hypertonic medium but, in fact, the secretion of CA was even further enhanced after the second exposure to the hypertonic medium.

Degree of hypertonicity and time of exposure to hypertonic solution required to evoke secretion of CA

Sodium chloride concentration of the Krebs solution was increased in separate experiments from a control value of 119 mM to 275 mM to study the effect of gradual increases in the degree of tonicity on the secretion of CA. As is shown in Figure 3a, a significant increase ($p < 0.05$) in CA secretion occurred after perfusion of the adrenal gland with $1.75 \times$ the normal concentration of sodium chloride in the Krebs solution. The secretory response increased sharply (over 13-fold) when the sodium chloride concentration was raised to more than 2-fold the normal concentration (119 + 156 mM).

In another series of experiments, 275 mM sodium chloride-Krebs solution was used to perfuse the adrenal gland for varying periods of time, and then secretion was examined in normal Krebs solution to establish the time course (Fig. 3b). A 15 min exposure of the adrenal gland to hypertonic solution was sufficient to evoke significantly enhanced secretion during perfusion with normal Krebs solution. A further increase in the exposure period to 30 and 45 min led to a still enhanced secretion of CA. Secretion was not further increased if the exposure period was increased to 60 min.

Effect of various procedures on the secretion of CA evoked after exposure to a hypertonic medium

The secretion of CA obtained after treatment of the adrenal gland with hypertonic medium could be a result of activation of nicotinic and muscarinic receptors of the chromaffin cells by ACh released from the splanchnic nerve terminals during switchover from hypertonic to normotonic media. To determine the role of the presynaptic component in the present phenomenon, several types of experiments were performed. In one, identical experiments—as described in Figure 1—were carried out in chronically splanchnectomized adrenal glands. As is shown in Table 2, the secretory response normally observed after perfusion with hypertonic Krebs solution, followed by normal me-

Table 2. Effects of splanchnectomy, TTX, and cholinergic antagonists on catecholamine secretion evoked after exposure to hypertonic Krebs solution

Treatment	Secretion of catecholamines (ng) ^a		
	Krebs	Hypertonic Krebs	Krebs
None ^b	15 ± 5	15 ± 5	460 ± 10
Splanchnectomy ^c	5 ± 1	20 ± 5	505 ± 15 ^f
Tetrodotoxin ^d (1 μM)	5 ± 1	5 ± 2	470 ± 120 ^f
Atropine (1.5 μM) + hexamethonium (138 μM) ^e	12 ± 5	7 ± 1	370 ± 55 ^f

^a Each value represents a mean of 3–7 experiments.

^b Experimental protocol identical to that in Table 1. The first row of values represents a “control” for the remaining types of experiments carried out after different treatments.

^c Left adrenal gland was surgically denervated and used for perfusion 2–3 weeks after the operation.

^d TTX was added during the last 30 min perfusion with hypertonic Krebs solution and also was present when the medium was switched over to the Krebs solution.

^e These agents were added to hypertonic and normal Krebs solutions in a manner identical to that described in *d*.

^f Statistically insignificant when compared with the control value in *a*.

Figure 4. Secretion of CA after switchover from Ca-free and hypertonic Krebs solution to normal medium. *a*, After 30 min perfusion with Krebs solution, one 5 min sample (0–5 min) was collected and the medium was changed to Ca-free, 275 mM NaCl-Krebs solution for 60 min, and one 5 min sample collected. Then the medium was changed to normal Krebs solution, and 5 min serial samples were collected immediately after the switchover of the solution, up to 40 min. All samples were assayed for CA. *b*, Some of the samples derived from the experiment (*a*) were also assayed for lactate dehydrogenase activity, as described in Materials and Methods. Each column represents a mean of 4–13 experiments. Vertical lines, SEM.

dium, remained unaffected by degeneration of splanchnic nerve terminals. The success of splanchnectomy was attested to in each adrenal gland at the beginning of the experiment by applying transmural stimulation, which is known to evoke release mainly by activation of the splanchnic nerves (Wakade, 1981b). In none of the splanchnectomized adrenal glands used in the present study did 10 Hz stimulation cause a significant secretion of CA in comparison to that in the innervated glands (data not shown).

In other experiments, adrenal glands were perfused with hypertonic medium containing a combination of atropine (1.5 μ M) and hexamethonium (138 μ M) in order to inactivate muscarinic and nicotinic receptors, respectively (Wakade and Wakade, 1983). When this medium was changed over to normal Krebs solution containing atropine plus hexamethonium, the typical secretory response was still present (Table 2).

The possibility was considered that during switchover from hypertonic to normotonic solution there may be repetitive firing along the splanchnic nerves (and chromaffin cells) that is mainly responsible for the prolonged secretion of CA seen after such a maneuver. However, treatment of the adrenal gland with 1 μ M TTX failed to modify the secretory response seen after switchover from hypertonic to normal Krebs solution (Table 2).

Figure 5. Effect of Mn on the explosive secretion of CA. After 30 min perfusion with Krebs solution, the medium was changed to Ca-free and 275 mM NaCl-Krebs solution for 40 min, and 3 mM Mn was added to the same medium for an additional 20 min. Then the medium was changed to Krebs solution containing 3 mM Mn, and 5 min serial samples were collected immediately after the switchover of solutions. After 20 min, the medium was changed to regular Krebs solution and 5 min samples were collected immediately and for up to 30 min. For this series of experiments, Ca-free Krebs solution was prepared by adding 11 mM HEPES to the solution, in the place of NaHCO_3 , and KH_2PO_4 , pH was adjusted to 7.4, and the solution was bubbled with 100% O_2 . To this solution, 2.5 mM CaCl_2 was added to obtain normal Krebs solution, and 156 mM NaCl was added as required. Each bar represents a mean of 4 experiments. Vertical lines, SEM.

The obligatory role of calcium in the secretion of CA evoked after exposure to hypertonic Krebs solution

Although a number of the above-described experiments revealed that the secretion of CA obtained from the adrenal medulla after exposure to hypertonic solution was not a result of tissue damage, it was essential to know whether Ca was needed in this secretory process. Therefore, several types of experiments were carried out to establish the role of Ca in the secretion evoked after hypertonic treatment.

As is shown in Figure 4*a*, if Ca was omitted during 1 hr perfusion with hypertonic medium (275 mM NaCl-Krebs solution) and introduced in a normotonic solution, the secretion of CA increased from 10 to about 4000 ng within the first 10 min, and gradually declined to 400 ng 40 min after the switchover. The secretory response persisted for another 40 min (data not shown). Because of the explosive nature of the secretion observed in these experiments, it was essential to rule out the possibility of medullary cell lysis. Therefore, lactate dehydrogenase content of the adrenal perfusates was estimated during perfusion with different types of solution. Figure 4*b* shows that the enzyme content remained unchanged during the entire course of the experiment.

Two types of control experiments were carried out for the above series of experiments. In one series, the adrenal gland was perfused with Ca-free Krebs solution for 1 hr and then switched over to Krebs solution. There was no massive increase in the secretion of CA (13 ± 6 ng vs 21 ± 8 ng; $n = 4$). In the other, the adrenal gland was perfused with 275 mM NaCl and Ca-free Krebs solution for 1 hr and then changed over to 275

Figure 6. Accumulation of Ca^{45} and secretion of CA at various times after switchover from Ca-free and hypertonic Krebs to normal Krebs solution. *a*, Adrenal gland was perfused with Ca-free and hypertonic Krebs solution for 1 hr and then with regular Krebs, as described in Figure 5. In another series of experiments, after perfusion of the adrenal gland with Ca-free and 275 mM NaCl-Krebs solution, the medium was switched to Ca-free Krebs solution (normotonic) for 30 (*b*), 60 (*c*) and 120 (*d*) min prior to perfusion with Krebs solution. Six 5 min samples were collected in each group to determine the secretion of CA. Each bar is a mean of 3–16 experiments. In another group of adrenal glands, the entire protocol (*a*–*d*) was repeated as described above, except that normal Krebs solution (after perfusion with hypertonic medium) contained $0.5 \mu\text{g/ml}$ $\text{Ca}^{45}\text{Cl}_2$ for 10 min, which was washed for 30 min prior to removal of adrenal medulla for estimation of radioactivity due to Ca^{45} (solid bar), as described in Materials and Methods. In still another group, the above protocol was repeated, except that the adrenal glands were perfused with Ca-free Krebs solution (normotonic) for 60 min prior to switchover to Ca-free normotonic solution for various periods of time prior to 10 min exposure to Ca^{45} . These control experiments were performed to determine the accumulation of Ca^{45} under “nonstimulation condition.” The amounts of Ca^{45} accumulated in these control experiments (229 ± 42 pg/mg; $n = 8$) have been subtracted from the above group to obtain net uptake of Ca^{45} for each group. In still 7 other glands, the uptake of Ca^{45} was examined 30 min after perfusion with regular Krebs solution (207 ± 40 pg/mg; $n = 7$). There was no significant difference between these two values. Each solid bar is a mean of 3–5 experiments. Vertical lines, SEM.

mM NaCl-Krebs solution. This change also did not result in a marked increase in the secretion of CA (7 ± 4 ng vs 17 ± 9 ng; $n = 3$).

Whether the explosive secretion seen in Figure 4 required Ca when the perfusion medium was changed from hypertonic and Ca-free to Ca-containing Krebs solution was tested in the next series of experiments.

Antagonism by manganese

Earlier, it was shown that Mn blocked the Ca-dependent secretion of CA evoked by ACh and splanchnic nerve stimulation in the rat adrenal gland (Wakade, 1981a). Therefore, the effects of 3 mM Mn were investigated in the present study; the results are shown in Figure 5. During the final 15 min of 1 hr perfusion with hypertonic and Ca-free Krebs solution, Mn was introduced into the medium, and was also present when the solution was changed to normal Krebs solution. As is shown in Figure 5, Mn prevented the secretion of CA during switchover. Figure 5 also shows that, as Mn was being washed out, the secretory response began to appear at a significant level in the same adrenal gland.

Secretion of CA at various times after exposure to calcium

In another series of experiments, Ca was presented at various time intervals after the adrenal gland was exposed to Ca-free, hypertonic medium and then to Ca-free, normotonic medium. The results of these experiments are summarized in Figure 6. Immediate exposure to Ca after the switchover produced a massive secretory response (Fig. 6a), as has also been found in previous experiments (Fig. 4). However, if Ca was presented

after 30 min, there was still a large increase in the secretion of CA (from essentially undetectable levels to 800 ng in 5 min; Fig. 6b). If introduction of Ca was delayed as much as 60 min after the gland had been perfused with Ca-free and hypertonic solution for 1 hr, and then another 60 min with Ca-free Krebs (normotonic) solution, a marked increase in the secretion occurred (Fig. 6c). Finally, as shown in Figure 6d, significant quantities of CA were secreted even 120 min after presentation of Ca to the gland.

Ca^{45} accumulation

To determine if the secretion of CA obtained in the above series of experiments was a result of the influx of Ca, a study was made of the accumulation of Ca^{45} at various times, as has been described in previous experiments. The results are included in Figure 6. The accumulation of Ca^{45} increased 6-fold over the control when Ca^{45} was presented immediately after the switchover from hypertonic to normotonic solution (Fig. 6a). Although the accumulation of Ca^{45} gradually declined with an increase in the time interval between presentation of Ca^{45} and termination of perfusion with Ca-free and hypertonic solution, a significant accumulation of Ca^{45} was detected even 2 hr after the change (Fig. 6d).

Assessment of membrane potential by C^{14} -TPP accumulation

Changes in the membrane potential of the perfused adrenal medulla were monitored by measuring the accumulation of C^{14} -TPP. Several investigators have shown that the accumulation of C^{14} -TPP decreases when the cell membrane depolarizes, and

Figure 7. C^{14} -TPP accumulation of the adrenal medulla perfused with normal and excess K-Krebs solution. *a*, After 30 min perfusion with Krebs solution, the medium was changed over to Krebs solution containing C^{14} -TPP for 45 min, and then the adrenal gland was perfused with ice-cold Krebs solution for 10 min. The adrenal medulla was removed for analysis of radioactivity due to C^{14} -TPP (open bar), as described in Materials and Methods. In another group, the identical protocol was carried out except that 55 mM K was present 10 min before and during 45 min perfusion with C^{14} -TPP Krebs solution (hatched bar). *b*, The above 45 min samples from each group were analyzed for CA and expressed as $\mu\text{g}/\text{mg}$ of adrenal weight. *c*, C^{14} -TPP accumulation in the adrenal medulla perfused with hypertonic and then hypertonic followed by normotonic solutions. The experimental protocol was identical to that described in *a*. After initial perfusion with Krebs solution, the medium was changed over to 275 mM NaCl-Krebs solution for 30 min, then to the same solution containing C^{14} -TPP for 45 min, and washed with ice-cold Krebs solution, as above, prior to removal of the adrenal medulla to determine C^{14} -TPP content (hatched bar). In another group the adrenal gland was perfused with 275 mM NaCl-Krebs solution for 60 min; then the medium was changed over to Krebs solution containing C^{14} -TPP for 45 min, followed by a 10 min wash prior to removal of the adrenal medulla (cross-hatched bar). *d*, The above 45 min samples from each group were analyzed for CA. Each bar is a mean of 5 observations. Vertical lines, SEM.

increases when the membrane hyperpolarizes (Kiefer et al., 1980; Lichtshtein et al., 1979; Wakade and Wakade, 1982b; Wakade et al., 1983; Wastek et al., 1981).

First, the applicability of the technique in assessing the membrane potential by using C^{14} -TPP was determined by comparing the accumulation of C^{14} -TPP in the adrenal medulla perfused with normal medium with the accumulation of C^{14} -TPP in 55 mM K-Krebs solution. Figure 7*a* shows that the accumulation of C^{14} -TPP was markedly reduced (63%) in 55 mM K-Krebs solution, which was associated with a marked increase in the secretion of CA (0.02–0.45 $\mu\text{g}/\text{mg}$; Fig. 7*b*).

In contrast to the accumulation of C^{14} -TPP (about 80 pg/mg) in the adrenal medulla perfused with normal medium, the accumulation increased to about 190 pg/mg after perfusion with 275 mM NaCl-Krebs solution. After switching to hypertonic medium, if the adrenal gland was perfused with normotonic medium, the accumulation of C^{14} -TPP was reduced to 104 pg/mg (Fig. 7*c*). In these experiments the perfusates, before and during C^{14} -TPP exposure, were assayed for CA; the results are shown in Figure 7*d*. The amounts of CA secreted in various media were just as expected from the results obtained in previous experiments.

Figure 8. C^{14} -TPP accumulation in the adrenal medulla perfused with Ca-free and 275 mM NaCl-Krebs solution, followed by Krebs solution. *a*, The experimental protocol (for the first 3 bars, starting from the left) was identical to that described in Figure 7*c*, except that hypertonic solution was deprived of Ca, as described in Figure 4. The same figure also shows the inhibitory effect of 30 mM K on the accumulation of C^{14} -TPP and CA secretion obtained after switchover of Ca-free and hypertonic solution to normotonic solution. After initial perfusion with normal medium, the adrenal gland was perfused with Ca-free and 275 mM NaCl-Krebs solution for 50 min, followed by 30 mM K-, Ca-free, and 275 mM NaCl-Krebs solution for 10 min and then with 30 mM K-Krebs containing C^{14} -TPP for 45 min, washed for 10 min prior to removal of the adrenal medulla (solid bar in *a*). *b*, The above 45 min samples from each group were analyzed for CA. In this series of experiments, 30 mM K was added to Krebs solution without removing the equivalent amounts of NaCl. Each bar is the mean of 4 experiments. Vertical lines, SEM.

Identical results were obtained in another group of experiments, in which C^{14} -TPP accumulation was determined in the adrenal medulla perfused with 275 mM NaCl and Ca-free medium, and after switching from this medium to normal Krebs solution (Fig. 8).

If the explosive secretion of CA was a result of change in the membrane potential (i.e., "depolarization"), then the presence of excess K should interfere with the secretion during change-over from hypertonic to normotonic solution. The results of experimental studies are shown in Figure 9. As expected, perfusion with 30 mM K-Krebs solution caused a good secretion of CA compared to the spontaneous secretion. The secretion was essentially zero when Ca was omitted from 275 mM NaCl- and 30 mM K-Krebs solution. However, switchover from such medium to 30 mM K-Krebs solution (normotonic) almost completely prevented the explosive secretion of CA otherwise observed after such a switchover in 5.9 mM K-Krebs solution. This represented over a 90% inhibition of the explosive secretion by 30 mM K during switchover. To ensure that the inhibitory effect of excess K was a real one, we repeated the identical protocol in the same adrenal gland without increasing K concentration of the medium over 5.9 mM. Figure 9 shows that immediately after the changeover from hypertonic to normotonic solution, the secretion of CA increased over 1700 ng in a 10 min period.

The antagonism between excess K and changes in tonicity of the perfusion media in producing explosive secretion of CA was examined on the accumulation of C^{14} -TPP. As can be seen in Figure 8, the accumulation of C^{14} -TPP of about 190 pg/mg in

Figure 9. Inhibition by excess K of explosive secretion of CA evoked by changes in tonicity of the perfusion medium. After 30 min perfusion with Krebs solution, one 5 min sample (0–5 min) was collected to determine spontaneous secretion of CA, and then medium was changed to 30 mM K-Krebs solution for 15 min. The gland was then perfused with Ca-free and 275 mM NaCl-Krebs solution for 50 min, followed by 30 mM K-, Ca-free, and 275 mM NaCl-Krebs solution for 10 min. The medium was changed over to 30 mM K-Krebs solution and three 5 min samples were collected. Finally, the medium was changed to Ca-free and 275 mM NaCl-Krebs solution for 60 min, followed by regular Krebs solution. In this series of experiments, 30 mM K was added to the Krebs solution without removing equivalent amounts of NaCl. Each column represents a mean of 4 observations. Vertical lines, SEM.

hypertonic medium was reduced to about 50 pg/mg, if 30 mM K was present in the medium. The inhibition of C^{14} -TPP accumulation was associated with a reduction in secretion, just as described in the previous section.

CA content of the adrenal medulla

Since the secretion of CA was of such an immense magnitude in the present study, it was of interest to determine the CA content of the adrenal gland. The results are shown in Figure 10. Perfusion of the adrenal gland with Krebs solution for over 2 hr did not modify CA content, as compared with that of the unperfused contralateral control gland. However, after perfusion with hypertonic and Ca-free medium for 60 min, if the perfusion was changed over to regular medium for 60 min, there was about a 70% reduction in CA content. As shown above in several experiments, this treatment caused a large increase in the secretion of CA ($8.25 \pm 0.17 \mu\text{g}$; $n = 4$). Figure 10 shows that the reduction in CA content was not associated with any significant loss in the lactate dehydrogenase content of the same adrenal glands.

Discussion

It was surprising to observe that perfusion of the rat adrenal gland with hypertonic Krebs solution for prolonged periods of time had no adverse effect on the amounts of CA that were secreted spontaneously, or those evoked by excitation of chromaffin cells via splanchnic nerve terminals. Although the secretion of CA was not affected during perfusion with the hypertonic medium, it was increased over 10-fold immediately upon restoring the perfusion medium to a normal physiological salt solution. The secretory response lasted more than 1 hr after the switchover, and was reproducible in the same adrenal gland.

Certain basic characteristics of this unusual secretory phenomenon were established, such as exposure time and degree of hypertonicity, and the types of agent used to make up the

Figure 10. CA and lactate dehydrogenase contents of the adrenal gland after evoking explosive secretion of CA. The left adrenal gland was perfused with Krebs solution for 150 min, and the medulla removed to determine CA content (solid bar in a). In another group, the protocol was identical to that described in Figure 4 (solid bar in b). For both groups, the contralateral right adrenal gland was removed and assayed for CA (open bars). Each medulla was homogenized in ice-cold 0.1 M phosphate buffer (pH 7.5) and used for estimation of CA (a) and lactate dehydrogenase (b), as described in Materials and Methods. Each bar is mean of 4 experiments. Vertical lines, SEM.

hypertonic Krebs solution. It was essential that the adrenal gland be exposed to hypertonic solution for at least 15 min, and that a sodium chloride concentration be $1.75 \times$ the normal value to initiate the secretion in the postperfusion period with normal medium. Hypertonicity resulting from excess sodium or chloride ions was not the major reason for this unusual secretory phenomenon, because the increase in tonicity achieved by addition of choline chloride, arginine hydrochloride, sodium thiocyanate, and sucrose gave essentially similar results as those achieved with excess sodium chloride.

The primary site from which the secretion begins after change-over from hypertonic to normotonic solution has been determined. Experiments with the antagonists of nicotinic and muscarinic receptors and chronically denervated adrenal glands ruled out the possibility of activation of the cholinergic receptors of chromaffin cells by ACh liberated from splanchnic nerves during switchover from hypertonic to normal Krebs solution. Thus, the effect of the hypertonic medium appears to be directly on the chromaffin cell and not on presynaptic splanchnic nerve terminals.

The importance of Ca ions in the secretion of CA by a variety of secretagogues has been amply documented (Baker and Knight, 1984; Douglas, 1968; Douglas and Rubin, 1961; Knight and Kesteven, 1983; Viveros, 1975). In the present study we found that Ca was essential for the secretion of CA evoked by changes in tonicity of the perfusion medium, since the secretory response was virtually absent if Ca ions were omitted from the normal medium, or if Mn was added to the medium. Most important, the accumulation of Ca^{45} increased almost 6-fold during switchover from hypertonic to normotonic medium. The need for Ca suggests that exocytosis must have been a major mechanism of

secretion of CA in the present study as well. Although secretion of other vesicular markers was not monitored, lack of increase in the secretion of the cytoplasmic enzyme, lactate dehydrogenase, eliminated the possibility of leakage of CA via cytoplasm.

The major question is how extracellular Ca ions are made available to the secretory process when the environment of chromaffin cells is changed from hypertonic to normal. Currently, it is believed that Ca channels are opened either by activation of nicotine receptors of the chromaffin cell membrane (Kilpatrick et al., 1982; Knight and Kesteven, 1983; Viveros, 1975; Wakade et al., 1986) or by depolarization of the cellular membrane (Hagiwara and Byerly, 1981). The possibility that cholinergic receptors and Na action potentials are the primary stimulus for opening Ca channels of chromaffin cells has been eliminated in the present study (Table 2). However, it is likely that electrochemical properties of chromaffin cell membrane may change during perfusion with hypertonic medium. One hour exposure to hypertonic solution may cause a loss of cellular fluid, causing shrinkage of chromaffin cells. As a result, intracellular concentration of K may increase and lead to hyperpolarization of chromaffin cell membrane, as one would expect from the electrophysiological properties of cell membrane. If indeed the uptake of C^{14} -TPP is inversely related to the membrane potential, as many reports show (see Results), then the more than a 2-fold increase in C^{14} -TPP accumulation seen in the adrenal medulla perfused with hypertonic solution must reflect a considerable degree of hyperpolarization. And when the perfusion medium was changed from hypertonic to normotonic solution, the accumulation of C^{14} -TPP declined toward the control value. This observation suggests that the hyperpolarized membrane returned toward a more positive membrane potential. Since, during this maneuver, the secretion of CA commences, it is our opinion that the relative change in membrane potential was responsible for opening voltage-sensitive Ca channels and for an increase in cytosolic levels of Ca ions (see below). In support of such a proposal, studies demonstrated that if the cell membrane was depolarized by prior exposure to 30 mM K, then the changes induced by hypertonic medium were prevented. The inhibition by excess K was obtained on the C^{14} -TPP accumulation (Fig. 8), as well as on CA secretion (Fig. 9).

A number of observations rule out the question of tissue damage and lysis in the present phenomenon. It is remarkable that an explosive secretion of CA (from 4 to 4000 ng) obtained after changes in tonicity of the medium did not result in the release of the cytoplasmic enzyme, lactate dehydrogenase. Furthermore, several interventions reversibly prevented the explosive secretion in a specific manner. For example, when 3 mM Mn or 35 mM K were added to the medium prior to switchover from Ca-free hypertonic to normotonic solution, the secretory response was virtually absent, and removal of these agents caused the expected secretion in the same adrenal glands. Most important, the massive secretion—which was associated with about 70% reduction in CA content of the adrenal medulla—did not produce any alteration in the lactate dehydrogenase content of the tissue.

The massive and prolonged secretory response that begins immediately after switchover from hypertonic to normotonic medium cannot be a result of transient entry of Ca ions within the first few seconds after voltage-sensitive Ca channels open, or a gradual disposition of free cytosolic Ca ions over the next 40 min. It is likely that Ca channels may remain in an open state for a very long period of time once they are opened by changes in tonicity of the medium. It is clear from the results of Figure 6 that even if Ca was presented to the adrenal medulla as late as 30–120 min after the switchover from hypertonic and Ca-free to normotonic solution, Ca was still transported inside

the chromaffin cells and was effective in evoking the secretion of CA.

In summary, an entirely new procedure is introduced to evoke CA secretion in a Ca-dependent manner that should help to explore the properties of Ca channels, storage, and turnover of CA as well as other constituents of chromaffin granules.

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