*J. Physiol.* (1981), **313**, *pp.* 463–480
*With 7 text figures*
*Printed in Great Britain*



# STUDIES ON SECRETION OF CATECHOLAMINES EVOKED BY ACETYLCHOLINE OR TRANSMURAL STIMULATION OF THE RAT ADRENAL GLAND

BY ARUN R. WAKADE

*From the Department of Pharmacology, State University of New York, Downstate Medical Center, Brooklyn, New York 11203, U.S.A.*

(*Received 30 January 1980*)

SUMMARY

1. A method of studying the secretion of catecholamines (CA) in the isolated perfused rat adrenal gland by transmural stimulation or by application of acetylcholine (ACh) has been described.

2. Secretion of CA was practically linear in response to ACh administration, starting from 4·42 $\mu$M to 1·32 mM. Transmural stimulation enhanced secretion from a stimulation frequency of 0·5–3 Hz; the effect levelled at 10 Hz, and declined as frequency was raised to 30 Hz. The secretory response to transmural stimulation was maximal over 1 msec duration and 60 V.

3. Secretion evoked by transmural stimulation was blocked (70–95%) by 0·31 $\mu$M-tetrodotoxin (TTX) irrespective of stimulus duration, voltage and frequency of stimulation. Secretion evoked by ACh was depressed 43% by TTX. After mecamylamine (0·59 mM) treatment, secretory response evoked by either procedure was blocked by about 80%.

4. Adenosine (0·18 mM), adenosine monophosphate (0·28 mM), or adenosine triphosphate (0·19 mM) lowered CA secretion evoked by transmural stimulation by about 40%, but had no effect on secretion induced by ACh.

5. Isoprenaline (4·52 $\mu$M), propranolol (11·58 $\mu$M), clonidine (13·00 $\mu$M), phenoxybenzamine (3·30 $\mu$M), and 4-aminopyridine (3 mM) did not modify CA secretion evoked by transmural stimulation or by ACh.

6. Perfusion of the adrenal gland with 0·25 mM-Ca-Krebs solution completely abolished CA secretion evoked by transmural stimulation, but ACh-induced secretion was still 30–50% of the control value. 20 mM-Mg blocked electrically induced secretion by 60%, but that evoked by ACh was unaffected.

7. Perfusion with Ca-free Krebs solution for 2 hr did not completely abolish the response. However, treatment with EGTA (5 mM) for 30 min totally blocked ACh-induced secretion.

8. La or Mn were more effective in blocking transmurally evoked secretion than ACh-evoked secretion of CA. Verapamil (0·1 mM) had no significant effect on secretion evoked by either procedure. A 5-fold increase in its concentration caused about 75% blockade of secretion.

9. Differential effects of various ions and agents on CA secretion are explained on the basis that these compounds affect neurosecretory properties of the presynaptic splanchnic nerve terminals and of chromaffin cells differently.

0022-3751/81/3970-0762 $07.50 

## INTRODUCTION

Two mechanisms are involved in the secretion of adrenal medullary hormones. Upon excitation of splanchnic nerves, acetylcholine (ACh) is released from the nerve terminals, and then activates nicotinic (and muscarinic) receptors of the chromaffin cells, causing exocytotic secretion of catecholamines (CA). In the majority of experiments various aspects of the medullary secretion are investigated by studying the secretion of CA from chromaffin cells, using exogenous ACh, analogues of ACh, or other secretogogues. As a consequence, very little attention is given to the presynaptic component of the over-all secretory process of the medullary hormones. Therefore, the primary goal of the present investigation was to introduce a simple method in which secretion of ACh from splanchnic nerve endings and of CA from chromaffin cells can be studied and the effects of various compounds on both secretory processes can be investigated.

Among various compounds to be tested, the rationale for studying the effects of adenosine and its related analogues on CA secretion was that adenosine triphosphate and its metabolic products appear in significant amounts along with the CA during adrenal medullary secretion (Douglas & Poisner, 1966*a*, *b*). Furthermore, adenosine is known to interfere with stimulation-induced release of noradrenaline in a variety of peripheral sympathetic neuroeffector organs of the rat (Enero & Saidman, 1977; Clanachan, Johns & Paton, 1977; Wakade & Wakade, 1977, 1978), and of other species, such as dog (Verhaeghe, Vanhoutte & Shepherd, 1977), rabbit (Hedqvist & Fredholm, 1976; Su, 1978), and guinea-pig (Wakade & Wakade, 1978). Release of noradrenaline from the central noradrenergic neurones of the cortex and hypothalamus of the rat is also depressed by adenosine (Wakade, 1979). Michaelis, Michaelis & Myers (1979) have shown that release of dopamine from synaptosomes is reduced by adenosine. In the aforementioned studies it is implied that adenosine plays an important role in modulating neurotransmission of the sympathetic nerve terminals. However, to date there is no direct evidence for the idea that adenosine, released from either sympathetic nerves or from the effector organ, is capable of affecting noradrenaline release.

In a previous study it was shown that the postganglionic sympathetic nerves of the cat heart were insensitive to the inhibitory action of adenosine (Wakade & Wakade, 1978). Therefore, a possibility existed that the cat might be a species insensitive to adenosine, and that the perfused adrenal gland of the cat might not be a suitable preparation for investigating the effects of adenosine on CA secretion. In three preliminary trials we found that 0·4 mM-adenosine did not modify CA secretion evoked by exogenous ACh, or by electrical stimulation of splanchnic nerves (T. Khan & A. R. Wakade, unpublished results). Therefore, the present experiments were performed on the isolated perfused adrenal gland of the rat. It will be demonstrated that neurosecretory properties of the presynaptic nerve terminals and of the chromaffin cells are influenced differentially by adenosine and a number of other agents and ions. A preliminary report of this work was recently presented at a meeting of the Federation of American Societies for Experimental Biology (Wakade, Iyengar & Wakade, 1980).

## METHODS

Male rats weighing 300–400 g were anaesthetized with ether. The abdomen was opened by a mid-line incision, and the left adrenal gland and surrounding area were exposed by placing three hook retractors. The stomach, intestines and portions of the liver were not removed, but pushed over to the right side and covered by saline-soaked gauze pads to obtain enough working space for tying blood vessels and for cannulation.

As shown in Fig. 1, a cannula, used for perfusion of the adrenal gland (*A*), was inserted into the

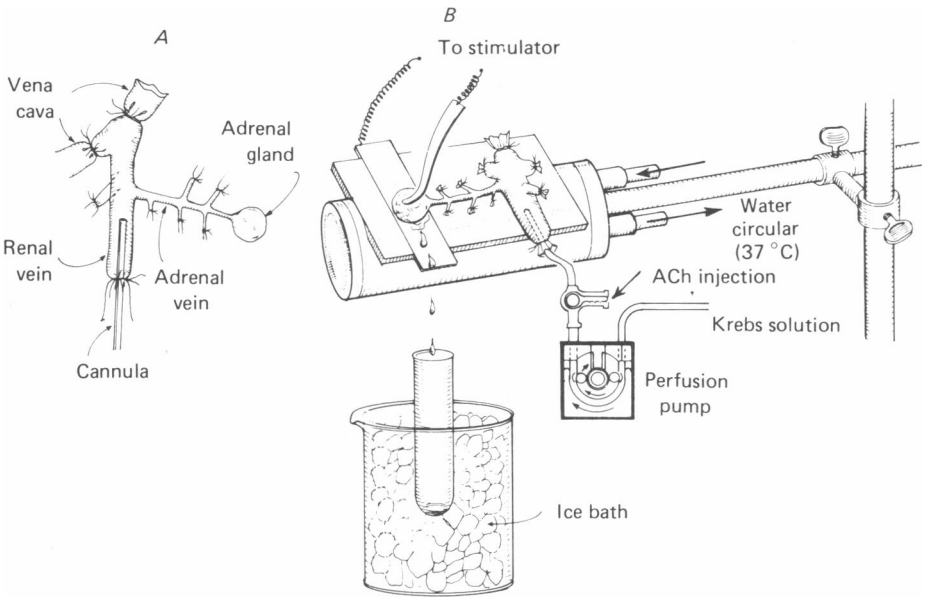


Fig. 1. Schematic drawing of the preparation used to study CA secretion in the isolated perfused gland of the rat. For further details, see the text.

distal end of the renal vein after all the branches of the adrenal vein, the renal vein (if any) and vena cava were ligated. A small slit was made into the adrenal cortex just opposite the entrance of the adrenal vein. Perfusion of the gland was started, to ensure that no leak was present, and the perfusion fluid escaped only from the slit of the adrenal gland. Then the adrenal gland, along with the tied blood vessels and the cannula, was carefully removed from the animal and placed on a platform of a Leucite chamber. The chamber was continuously circulated with water heated at 37 ± 1 °C (*B*). The adrenal gland rested on the flat Ag-AgCl strip mounted onto the platform. The metal strip served as one of the electrodes used for transmural stimulation of the gland, and the strip was also useful in maintaining the temperature of the adrenal gland and the perfusion fluid at near 37 °C.

*Perfusion of the adrenal gland*

The adrenal gland was perfused by means of a Sigmamotor pump at a rate of about 0·2 ml./min. The perfusion was carried out with Krebs-bicarbonate solution of the following composition (mM): NaCl, 118·4; KCl, 4·7; $CaCl_2$, 2·5; $MgCl_2$, 1·18; $NaHCO_3$, 25; $KH_2PO_4$, 1·2; glucose, 11·7. The solution was bubbled with 95 % $O_2$ + 5 % $CO_2$, and the final pH was 7·4–7·5. The solution contained disodium EDTA (10 μg/ml.) and ascorbic acid (100 μg/ml.), to prevent oxidation of CA. HEPES-Krebs solution was prepared by adding 11 mM of the buffer to a litre of Krebs solution from which $KH_2PO_4$ and $NaHCO_3$ were omitted. A final pH of 7·2 ± 0·2 was obtained by adjustment with 5 N-NaOH, and the solution was bubbled with 100 % $O_2$.

*Transmural stimulation*

The adrenal gland was transmurally stimulated by placing it between a Ag-AgCl strip (see above) and another plate electrode touching the gland, as shown in Fig. 1. Stimulation was carried out with a Grass stimulator, Model S6.

In four experiments, optimal stimulation parameters were determined, and the results of such experiments are shown in Fig. 2. It can be seen that maximum quantities of CA were secreted, with

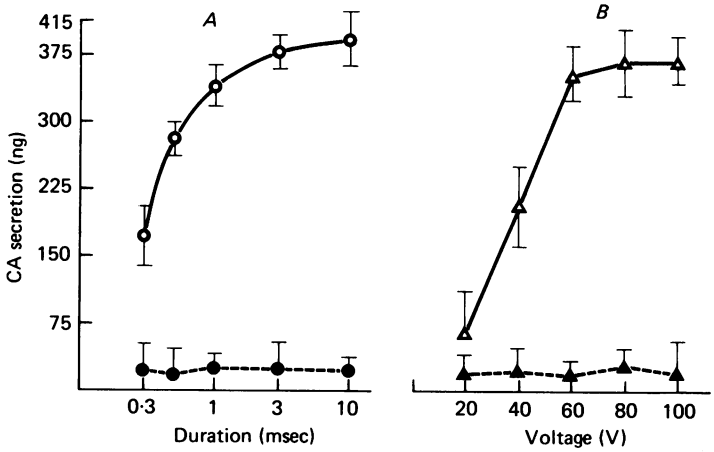


Fig. 2. The effect of increase in the duration or the intensity of the stimulus on secretion of CA. In one series of experiments (*A*), adrenal glands are perfused with Krebs solution (open circles) for 30 min, and then CA secretion was evoked by delivering 600 shocks (10 Hz and 80 V) at different durations of stimulus as shown. In each gland five different durations were tested in ascending order at 10 min intervals. The same gland was then perfused with 0·31 $\mu$M-TTX for 15 min, and in its presence 5 tests were again performed (solid circles). In another series (*B*), identical experimental protocal was followed, except that stimulus duration was kept constant at 1·5 msec and voltage was varied, as shown, in ascending order. Five tests at 10 min intervals were carried out on each gland before (open triangles) and after (filled triangles) TTX. Each sample was collected for 4 min. Four adrenal glands were used in each series. Vertical lines show S.E. of mean.

a stimulus duration and strength of over 1 msec and 60 V, respectively. Therefore, in subsequent experiments duration and voltage dials were set at 1·5 msec and 80 V. The secretory response was reproducible when duration and voltage were set at 1·5 msec and 60 or 80 V, but there was a rapid decline in secretion with the use of higher stimulus durations. In most experiments, 600 shocks were delivered at various frequencies.

*ACh or $K^+$ injection*

Different concentrations of ACh or excess $K^+$ were injected in a volume of 0·03 ml. into the perfusion stream via a three-way stopcock (Fig. 1). In preliminary experiments it was found that upon injection of various doses of ACh or $K^+$ the secretory response returned to preinjection level in about 4 min. Therefore, each sample was collected for 4 min after administration of ACh or $K^+$. Generally, the adrenal gland was perfused with Krebs solution for 30 min before stimulation. The adrenal perfusate was collected in chilled tubes. Details of the collection of samples are given in the Results section.

*Analysis of CA*

CA content of the perfusate was measured, in most cases, directly by the fluorometric method of Anton & Sayre (1962), without the intermediate purification on alumina. A volume of 0·2 ml. of the perfusate was used for the reaction. The CA content in the perfusate of stimulated glands

was high enough to obtain readings several-fold greater than the readings of control samples (unstimulated). The sample blanks were also lowest for perfusates of stimulated and non-stimulated samples. However, in some experiments, perfusates containing isoprenaline or high Mg interfered with the specific noradrenaline fluorescence and also gave high blanks. In those instances it was necessary to pass the perfusate over alumina (Shellenberger & Gordon, 1971) to remove the agent in the perfusate which interfered with the fluorometric determinations of CA. Standard solutions of noradrenaline were also passed over alumina, with recoveries ranging from 70 to 80%. Appropriate corrections for dilutions, recovery in alumina eluate where necessary, have been made. The content of CA in the perfusate was expressed in terms of noradrenaline (base) equivalents. All data are presented as means with standard errors, and differences were compared using Student's paired $t$ test.

*Drugs*

Drugs used were: acetylcholine chloride, 4-aminopyridine, propranolol, mecamylamine, adenosine, adenosine monophosphate and adenosine triphosphate (Sigma Chemical Corp., St Louis, MO); isoprenaline bitartrate (Sterling-Winthrop Research Institute, Rensselear, NY); clonidine hydrochloride (Boehringer Ingelheim Ltd, Elmsford, NY); tetrodotoxin (Calbiochem-Behring Corp., La Jolla, CA); lanthanum chloride and manganous chloride (Fisher Scientific Corp., Fairlawn, NJ). Verapamil was a generous gift from Knoll Pharmaceutical Co., Whipanny, NJ.

## RESULTS

### *CA secretion in response to ACh or transmural stimulation of the adrenal gland*

As shown in Fig. 3*A*, injection of 4·42 μM-ACh into the perfusion stream caused significant secretion of CA over the background secretion. A gradual increase in ACh administration resulted in greater amounts of CA to appear in the perfusate. Almost 640 ng CA were secreted in 4 min after injection of 1·32 mM-ACh. Fig. 3*B* shows the relationship between frquency of transmural stimulation and secretion of CA. Detectable amounts appeared in the perfusate upon stimulation at 0·5 Hz. CA secretion increased linearly with increase in frequency of stimulation to 1 and 3 Hz. A further increase in stimulation frequency to 10 Hz caused no additional secretion over that obtained at 3 Hz, and at 30 Hz CA secretion was reduced from a maximum value of about 224 to about 96 ng.

### *Secretion of CA during successive periods of either transmural stimulation, ACh injection, or excess K administration*

CA secretion evoked by giving 600 shocks at different frequencies of stimulation (Fig. 3*B*) was reproducible during successive periods of stimulation at 30 min apart (Table 1). Secretory response to a low frequency of stimulation (1 Hz) was depressed by about 40% during the 4th stimulation period. CA secretion evoked by repeated exposure to ACh was also maintained at near 90% of that in the $S_1$ period. In one experiment (marked with an asterisk in Table 1), after evoking secretion with ACh the adrenal gland was stored at 4 °C for 20 hr and then reperfused at 37 °C. When challenged with ACh, almost 52% of the CA was secreted from such gland in comparison to the amounts secreted during $S_1$ period about 20 hr earlier. The 23·6 and 35·4 mM-K-induced secretion of CA were also reproducible during two stimulation periods.

*The effect of TTX and mecamylamine on CA secretion evoked by transmural stimulation or by ACh*

In each adrenal gland the effects of TTX and mecamylamine were tested on CA secretion evoked by either ACh or transmural stimulation, and the results of such experiments are shown in Fig. 4. After evoking secretion of CA at 3, 10 and 30 Hz or by 0·13 mM-ACh under control conditions, the adrenal gland was perfused with 0·31 μM-TTX for 20 min, and, in the presence of TTX, secretion was evoked by

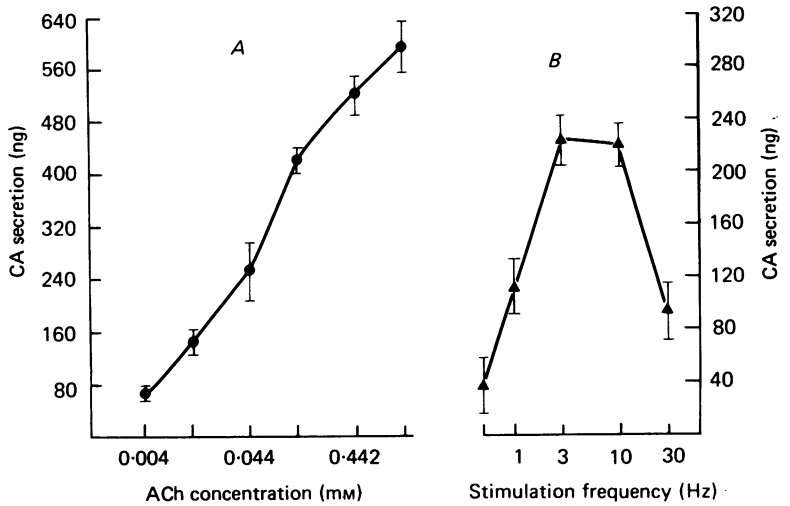


Fig. 3. CA secretion in response to ACh or transmural stimulation of the adrenal gland. *A*, 30 min after the beginning of perfusion with Krebs solution, various concentrations of ACh were injected at 10 min intervals into the perfusion stream, as described in Methods. Each gland was challenged, first with increasing order of ACh concentrations, and then with decreasing order, and the averaged values from six to eight experiments are represented by each dot. After each injection, the perfusate was collected for 4 min. Vertical lines represent S.E. of mean. *B*, in another series of experiments, the adrenal gland was transmurally stimulated (600 shocks), using different frequencies shown. A 10 min rest was given between stimulation periods. The adrenal perfusates were collected for varying periods of time. In the case of 0·5, 1 and 3 Hz, the collection time was 22, 12 and 5 min, respectively. At 10 and 30 Hz the perfusate was collected for 4 min. In each experiment the gland was stimulated in ascending order, then in descending, and the averaged values from twelve to sixteen experiments are shown by each triangle. Vertical lines show S.E. of mean.

transmural stimulation or by ACh. As shown in Fig. 4*B*, CA secretion evoked at all the three frequencies of stimulation was reduced, a maximum effect (70%) being obtained at 3 and 10 Hz. ACh-induced secretion was depressed by 43% in the presence of TTX. The reduction was statistically significant ($P > 0·05$). Thirty min after washout of TTX, the secretory response to transmural stimulation and ACh was fully restored (Fig. 4*C*). Perfusion of the adrenal gland with 0·59 mM-mecamylamine for 30 min produced over 80% blockade of CA secretion evoked by transmural stimulation

or by ACh (Fig. 4*D*). As shown in Fig. 4, spontaneous secretion of CA was not greatly affected by various drug treatments.

The effect of TTX on CA secretion evoked by various durations and strengths of stimulus is shown in Fig. 2. With increase in duration or voltage, there was an increase in CA secretion up to 1 msec and 60 V, respectively. Further increases in either of these two parameters had no significant effect on secretion. Fig. 2 also shows that TTX reduced CA secretion evoked by low or high stimulus duration and strength to the same level (about 20% of the control).

TABLE 1. Secretion of CA from the isolated perfused rat adrenal gland

| Type of stimulus to evoke secretion‡ | *n*† | Secretion of CA as a percentage of that in the first stimulation period ($S_1$) | | | | |
|---|---|---|---|---|---|---|
| | | $S_1$ (ng) | $S_2$ | $S_3$ | $S_4$ | $S_5$ |
| Transmural stim. (600 shocks) | | | | | | |
| 1 Hz | 6 | 119±15 | 82±10 | 74±8 | 61±6 | — |
| 3 Hz | 7 | 234±19 | 96±6 | 92±4 | 80±11 | 72±9 |
| 10 Hz | 10 | 229±18 | 93±7 | 89±10 | 84±7 | 79±8 |
| ACh | | | | | | |
| 0·013 mM | 6 | 149±18 | 92±8 | — | — | — |
| 0·13 mM | 12 | 438±19 | 101±4 | 98±10 | 91±8 | 52* |
| $K^+$ | | | | | | |
| 20·6 mM | 3 | 41±15 | — | — | — | — |
| 23·6 mM | 4 | 92±11 | 105±14 | — | — | — |
| 35·4 mM | 4 | 328±19 | 97±13 | — | — | — |

* See text.

† Number of experiments

‡ Thirty minutes after perfusion with Krebs solution, the adrenal gland was stimulated in each experiment with either transmural stimulation at different frequencies shown, with two concentrations of ACh, or with increasing concentrations of $K^+$ as described in Methods. Interval between stimulation periods was 30 min. The perfusate was collected for 4 min, when the adrenal gland was stimulated by ACh or K. At 1 Hz the collection period was 12 min, and at 3 or 10 Hz it was 5 min.

*The effects of adenosine on CA secretion evoked by transmural stimulation or by ACh*

Since the results obtained in Fig. 4 indicated that CA secretion evoked by transmural stimulation results primarily by the action of ACh released from excitation of splanchnic nerve terminals on nicotinic (and muscarinic) receptors of the chromaffin cells, it was therefore of particular interest to study the effects of adenosine on CA secretion evoked by transmural stimulation and that evoked by exogenous ACh. The results of these experiments are shown in Fig. 5. Perfusion of the adrenal gland with 0·18 mM-adenosine for 30 min resulted in 30–40% reduction in CA secretion evoked at a stimulation frequency of either 1 or 3 Hz. A 2-fold increase in adenosine concentration did not produce a significant additional decrease in CA secretion at either of the frequencies of stimulation.

Fig. 5 also shows that the same adrenal glands used for transmural stimulation

when challenged with exogenous ACh before and after adenosine (0·36 mM) showed no change in CA secretion.

*The effects of miscellaneous agents on CA secretion evoked by transmural stimulation or by ACh*

As shown with adenosine (Fig. 5), its related compounds, adenosine monophosphate and adenosine triphosphate, produced a significant reduction in CA secretion evoked by transmural stimulation, but not that evoked by ACh (Table 2). Alpha- and

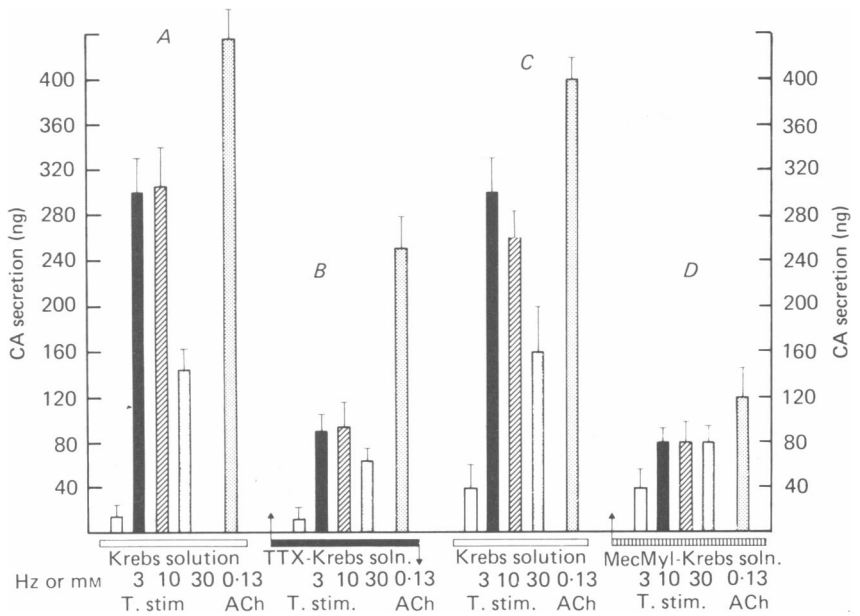


Fig. 4. The effects of TTX and mecamylamine on CA secretion evoked by transmural stimulation or by ACh. Secretion of CA was evoked 30 min after perfusion of the adrenal gland with either Krebs solution (*A*, *C*), 0·31 μM-TTX (TTX-Krebs solution) (*B*), or 0·59 mM-mecamylamine (MecMyl)-Krebs solution (*D*). Shaded columns show CA secretion in response to transmural stimulation (T. stim., 600 shocks at frequencies shown), or to ACh injection (0·13 mM). Each adrenal gland was subjected to the above treatments in the order described, and CA secretion was evoked in each medium by electrical stimulation and by ACh. Open columns represent spontaneous CA secretion in the absence of stimulation. Each column represents a mean of six to eight experiments. Vertical lines on top of columns show S.E. of mean.

beta-adrenoceptor agonists or antagonists, or 4-aminopyridine, had no significant effect on CA secretion evoked by either procedure. None of the above agents modified spontaneous secretion of CA.

*Relationship between external Ca and CA secretion evoked by transmural stimulation or by ACh*

Evoked secretion of CA in the presence of different Ca concentrations is shown in Fig. 6. As the Ca concentration was reduced from a control value of 2·5–1·25 mM, the secretory response evoked by transmural stimulation was depressed by about 50%,

whereas that evoked by ACh was near control value. A further reduction in Ca concentration to 0·5 mM reduced transmurally evoked CA secretion over 80%. ACh-evoked CA secretion in 0·5 mM-Ca was still 72% of the control. A 10-times reduction in Ca concentration caused complete blockade of CA secretion evoked by transmural stimulation. However, ACh-evoked secretion was still over 50% of that obtained in 2·5 mM-Ca-Krebs solution. Perfusion of the adrenal gland with Ca-free

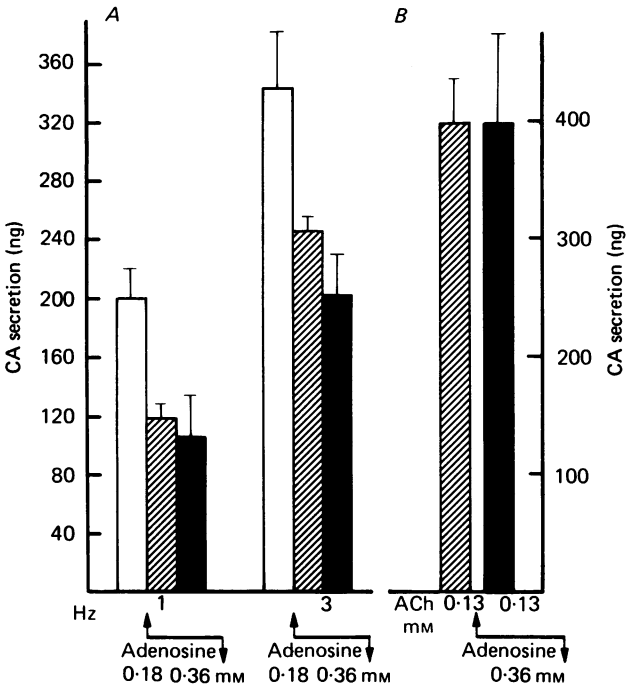


Fig. 5. The effect of adenosine on CA secretion evoked by transmural stimulation or by ACh. In each adrenal gland CA secretion was evoked first in Krebs solution by transmural stimulation (600 shocks at 1 or 3 Hz) (*A*), and then by injection of 0·13 mM-ACh (*B*). The same gland was perfused for 30 min with 0·18 or 0·36 mM-adenosine, and in the presence of adenosine CA secretion was evoked as mentioned above. Sample collection time was 12 and 5 min in the case of electrical stimulation, and 4 min after ACh stimulation. Each column represents a mean of six experiments. Vertical lines on top of each column show S.E. of mean.

Krebs solution for 30 min reduced ACh-evoked secretion by about 70%. In none of the five experiments was secretion evoked by ACh completely blocked, but that evoked by nerve stimulation was undetectable. Fig. 6 also shows that a 3-fold increase in external Ca enhanced CA secretion evoked by ACh or transmural stimulation by about 180 and 280%, respectively. Spontaneous secretion of CA was not modified by changes in Ca concentrations.

TABLE 2. Effects of miscellaneous agents on CA secretion evoked by transmural stimulation or by ACh

| Treatment | No. of expts. | CA secretion evoked by‡ Transmural stimulation | ACh |
|---|---|---|---|
| None | 24 | 189±18 | 376±35 |
| Adenosine monophosphate (0·28 mM) | 3 | 133±22* | 419±54† |
| Adenosine triphosphate (0·19 mM) | 3 | 86±35* | 394±20† |
| Isoprenaline (4·52 μM) | 3 | 165±18† | 332±39† |
| Propranolol (11·58 μM) | 3 | 167±16† | 385±31† |
| Clonidine (13·0 μM) | 3 | 161±16† | 394±35† |
| Phenoxybenzamine (3·30 μM) | 3 | 148±31† | 398±16† |
| 4-aminopyridine (3 mM) | 3 | 161±24† | 383±22† |

* $P < 0.05$.

† Not significantly different from controls.

‡ In each experiment, secretory response was evoked by transmural stimulation (3 Hz, 600 shocks) or by ACh (0·13 mM), 15 min apart, first in Krebs solution, and then in the presence of one of the agents. Secretion of CA is expressed in terms of ng. The perfusate was collected for 4 min.

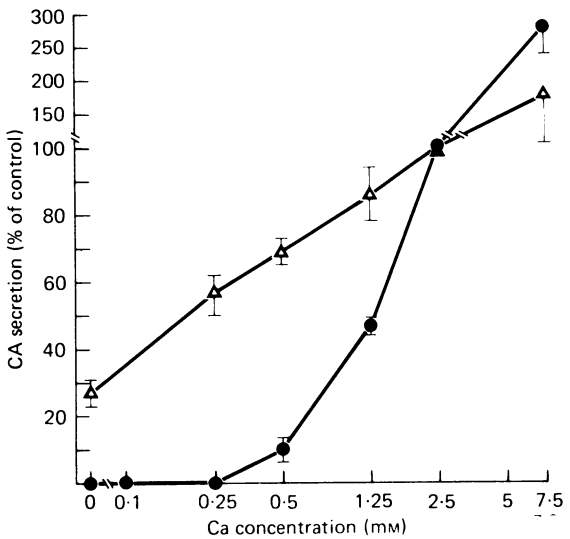


Fig. 6. Relationship between external Ca and CA secretion evoked by transmural stimulation or by ACh. Thirty minutes after perfusion with Krebs solution (2·5 mM-Ca), CA secretion was evoked by transmural stimulation (●) (600 shocks at 3 Hz), and 6 min later by ACh (△) (0·13 mM). The gland was then perfused with two different concentrations of Ca (randomly selected) for 30 min each, and secretion was evoked in respective media by electrical stimulation or by ACh. Finally, the perfusion medium was switched to 2·5 mM-Ca before evoking secretion. In each instance the perfusate was collected for 4 min. Secretion of CA is expressed as a percentage of the control obtained in Krebs solution after transmural stimulation (197±34 ng, $n = 18$) and after ACh (387±52 ng, $n = 14$). Each point, except the control, represents a mean of four to six experiments. Vertical lines show S.E. of mean.

*The influence of prolonged perfusion with Ca-free medium or EGTA on CA secretion evoked by ACh*

Since the secretory response evoked by ACh was not completely blocked by perfusion of the adrenal gland with Ca-free Krebs solution for 30 min, it was decided to wash the gland with Ca-free medium for longer periods of time. As shown in Fig. 7, there was a gradual decline in secretory response to ACh with increasing exposure to Ca-free medium. After perfusion with Ca-free Krebs solution for 2 hr, CA secretion was depressed by about 90% when evoked by either four or two challenging doses of ACh. Fig. 7 also shows the influence of EGTA on secretory response evoked by ACh in Ca-free medium. In four experiments, perfusion of the adrenal gland with

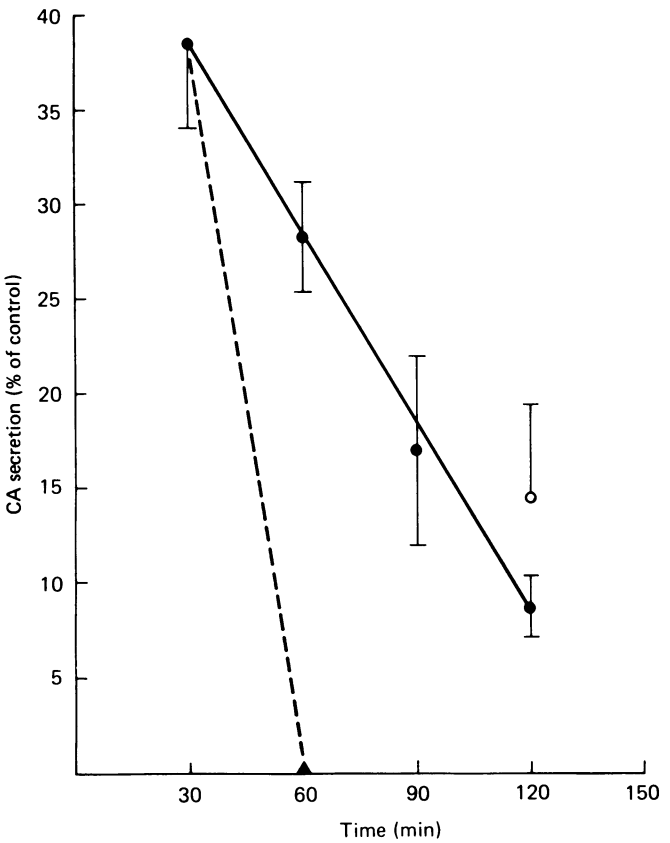


Fig. 7. The influence of prolonged perfusion with Ca-free medium or EGTA on CA secretion evoked by ACh. In all experiments, CA secretion was evoked first in Krebs solution, which averaged $406 \pm 35$ ng, $n = 13$, and represented the control value; then some glands ($n = 3$) were perfused with Ca-free Krebs solution for 2 hr and challenged once with ACh (open circle) and others ($n = 6$) were perfused in Ca-free Krebs for 2 hr and challenged with ACh (filled circles) at 30 min intervals. In still another series of experiments ($n = 4$), after obtaining control response the glands were perfused for 30 min with 5 mM-EGTA in Ca-free Krebs solution and then stimulated with ACh (interrupted line). 0·13 mM-ACh was used for evoking CA secretion. Each sample was collected for 4 min. Vertical lines over and below each point show S.E. of mean.

TABLE 3. Influence of 'Ca antagonists' on CA secretion evoked by transmural stimulation or by ACh

| Experimental solution | CA secretion (ng) In absence of stimulation | $n$† | Evoked by‡ Transmural stimulation Before | During | After | ACh Before | During | After |
|---|---|---|---|---|---|---|---|---|
| Krebs | 3·7±1·5 | 16 | 377±37 | 334±39 | 319±28 | 479±50 | 458±35 | 436±3 |
| 20 mM-Mg-Krebs | — | 6 | 404±52 | 157±48* | 413±56 | 609±45 | 648±41 | — |
| Verapamil | | | | | | | | |
| 0·1 mM | 2·8±0·9 | 6 | 317±52 | 236±15** | — | 312±50 | 246±24 | — |
| 0·5 mM | 2·8±0·9 | 5 | — | 84±9* | 157±39 | — | 114±15* | 189±15 |
| HEPES-Krebs | 3·7±1·8 | 3 | 355±31 | 309±37 | — | 372±37 | 394±28 | — |
| La | | | | | | | | |
| 1 mM | 54±22 | 5 | 524±31 | 37±13* | — | 347±58 | 295±48 | — |
| 3 mM | 126±37 | 5 | — | 0 | 503±56 | — | 71±11* | 208±33 |
| Mn | | | | | | | | |
| 1 mM | 3·7±1·8 | 3 | 430±33 | 223±24* | — | 601±41 | 308±31* | — |
| 3 mM | 5·6±3·7 | 4 | — | 52±5* | 432±35 | — | 107±13* | 595±39 |

† Number of experiments.

‡ In each experiment, secretory response was evoked by transmural stimulation (3 Hz, 600 shocks) or by ACh (0·13 mM) 15 to 30 min apart, first in Krebs solution, in the presence of one of the agents, and then after washout of the agent. The perfusate was collected for 4 min.

* $P < 0.001$, ** $P < 0.05$.

Ca-free Krebs solution containing 5 mM-EGTA for 30 min led to complete disappearance of the response.

After EGTA treatment, if the adrenal gland was perfused with Krebs solution for 10 min, followed by 30 min of perfusion with Ca-free solution, the secretory response was fully restored to the initial control (38%) level (not shown).

*The effect of 'Ca antagonists' on CA secretion evoked by transmural stimulation or by ACh*

The results obtained with different 'Ca antagonists' on CA secretion are summarized in Table 3. 20 mM-Mg lowered CA secretion evoked by transmural stimulation about 60%, but had no effect on secretion evoked by ACh. High concentrations of verapamil (0·1 mM) did not modify CA secretion evoked by either stimulus. However, 0·5 mM depressed the secretion by 65–75%. 1 mM-La caused over 90% blockade of CA secretion induced by transmural stimulation of the adrenal gland, but the same concentration was without significant effect on secretion caused by ACh. 3 mM-La caused about 80% inhibition of secretion by ACh. Finally, 1 mM-Mn lowered secretion evoked by both procedures up to 50% of control value, and higher concentrations inhibited secretion over 80%. Except La, which enhanced spontaneous secretion by 15- to 30-fold, none of the other agents had an effect on secretion in the absence of stimulation.

## DISCUSSION

In the present study, electrical stimulation of the isolated perfused adrenal gland could possibly evoke secretion of CA in two ways. One is, electrical excitation could generate action potentials in the presynaptic splanchnic nerve terminals, causing release of ACh, which could subsequently activate nicotinic (and muscarinic) receptors of the chromaffin cells to evoke CA secretion. Another way is that the chromaffin cell membrane could undergo sufficient depolarization upon transmural stimulation of the adrenal gland to lead to opening of voltage-dependent Ca channels and subsequent secretion of CA. Some evidence has been accumulated in support of the idea that fast Na channels exist in the chromaffin cell membrane which can be opened by electrical stimulation (Biales, Dichter & Tischler, 1976; Brandt, Hagiwara, Kidokaro & Miyazaki, 1976) or by veratridine (Ito, Nakazato & Ohga, 1979; Kirpekar & Prat, 1979). In the present study, data obtained with TTX offer no clues as to which of the above two ways is involved in CA secretion by transmural stimulation of the adrenal gland, because TTX could block fast Na channels of the chromaffin cell and splanchnic nerves, as well. However, a marked interference with secretion by mecamylamine strongly suggests that transmural stimulation causes release of ACh by depolarizing splanchnic nerve terminals, which in turn activates nicotinic (and probably muscarinic) receptors of the chromaffin cell to evoke CA secretion.

Confirmative evidence for such a suggestion was obtained by demonstrating that transmural stimulation of the chronically denervated adrenal gland caused practically no secretion of CA, but ACh was still effective in such a preparation (Wakade, 1981).

TTX lowered ACh-induced secretion of CA by about 45%. Previously Kirpekar & Prat (1979) also reported a similar degree of depression by TTX of CA secretion induced by ACh in the cat adrenal gland. These observations imply that a part of the secretory response by ACh may be due to the opening of fast Na channels. Biales

*et al.* (1976) and Brandt *et al.* (1976) have reported that action potentials can be generated in the chromaffin cells of gerbil or rat which are susceptible to TTX and removal of Na. Furthermore, the frequency of spontaneous action potentials can be increased upon application of ACh. On the other hand, Ritchie (1979) has found that secretion of dopamine from pheochromocytoma cell lines by carbamylcholine was insensitive to TTX or removal of external Na.

Adenosine or its analogues reduced CA secretion evoked by transmural stimulation of the adrenal gland, but not that evoked by exogenous ACh. These observations can be interpreted on the basis of differential electrical properties of pre- and postsynaptic membranes of the rat adrenal medulla. For example, splanchnic nerve terminals are capable of generating action potentials upon electrical excitation (see above), and if adenosine interferes with the secretory process by shortening the duration of nerve action potential and thereby reducing Ca influx (Wakade & Wakade, 1978), adenosine could reduce ACh release, and thereby CA secretion. On the other hand, ACh-induced secretion of CA is not always associated with the generation of action potentials in the chromaffin cell; therefore, it is not surprising to see the lack of effect of adenosine on ACh-induced CA secretion. Whether adenosine has a physiological role in the secretion of medullary hormones is still a matter of speculation.

Failure of adrenergic agents to affect CA secretion evoked by electrical stimulation or by ACh is indicative of a lack of adrenoceptors, comparable to those suggested for sympathetic nerve terminals (for references, see Langer, Starke & Dubocovich, 1979), in the regulation of hormone secretion in the rat adrenal gland. However, Gutman and coworkers (Gutman & Boonyaviroj, 1974, 1975; Boonyaviroj & Gutman, 1977) have proposed that $\alpha$- and $\beta$-adrenoceptors are involved in the regulation of CA secretion in the rat and human adrenal glands. Unfortunately, various experimental designs used by these workers to extend the hypothesis of the autoregulatory role of adrenoceptors in the adrenal gland are open to criticism. For example, in one case the effect of exogenous noradrenaline (1 mg/kg, s.c.) on CA secretion evoked by insulin shock was judged by measuring residual CA content of the adrenal gland; and in another case, the effect of phenylephrine was tested on the spontaneous leakage of CA from the human adrenal gland slices, without using a secretagogue. Starke, Görlitz, Montel & Schümann (1974) found that among various $\alpha$-receptor antagonists only phenoxybenzamine had a modest but significant effect in enhancing CA secretion evoked by excess K in bovine adrenal gland, and therefore, these authors concluded that $\alpha$-receptors are involved in the regulation of CA secretion. Earlier, Kirpekar & Cervoni (1963) reported that phenoxybenzamine, but not phentolamine, had an enhancing effect on CA secretion evoked by stimulation of splanchnic nerves of the dog adrenal gland.

4-Aminopyridine has been reported to block delayed K current and thereby increase the duration of nerve action potential in a number of test preparations (cockroach nerves, Pelhate & Pichon, 1974; squid giant axon, Meves & Pichon, 1977; frog skeletal muscle, Gillespie & Hutter, 1975). Enhanced secretion of ACh (Lundh & Thesleff, 1977; Horn, Lambert & Marshall, 1979) and of noradrenaline (Kirpekar, Kirpekar & Prat, 1977) evoked by nerve impulses has been ascribed to increased influx of Ca resulting from blockade of K current and lengthening of nerve action potential by

4-aminopyridine. In the present work, 4-aminopyridine had no effect on CA secretion evoked by nerve stimulation or by ACh. It should be pointed out that 4-aminopyridine facilitates [$^3$H]noradrenaline release upon stimulation of sympathetic nerves of the rat heart by 5- to 10-fold (A. R. Wakade, unpublished results). The present findings are consistent with the earlier reports (Lund, Leander & Thesleff, 1977; Kirpekar *et al.* 1977) that 4-aminopyridine is devoid of any curare-like effects.

The indispensable role of Ca in the neurosecretory process has been thoroughly established, and in the present work removal of external Ca markedly depressed CA secretion evoked by either transmural stimulation or by ACh. However, although the secretory response evoked by transmural stimulation was totally extinguished in 0·25 mM-Ca-Krebs solutions, that evoked by ACh was sustained at the level of 50–30 % in low or zero Ca medium. In the case of the cat adrenal gland, Douglas & Rubin (1963) obtained over 99 % blockade of CA outflow in response to ACh in Ca-free Locke solution. In perfused bovine adrenal gland (Philippu & Schümann, 1962) and bovine adrenal slices (Oka, Ohuchi, Yoshida & Imaizumi, 1965) a complete blockade of CA secretion by ACh in Ca-free medium was seen. Ishikawa & Kanno (1978) obtained over 95 % inhibition of adrenaline secretion from perfused rat adrenal gland in response to ACh.

In the present work, the reasons for the considerable response to ACh in Ca-free medium are not clear. It may be that chromaffin cells of the rat adrenal gland contain an intracellular store of Ca which participates in the secretion of CA. Such a store may not be easily depleted by removal of external Ca. Intracellular stores of Ca have been shown to play some role in contraction of smooth muscle produced by noradrenaline or ACh in Ca-free medium (Bozler, 1959; Ohashi, Takewaki & Okada, 1974; Casteels & Raeymaekers, 1979).

Different cations known to interfere with the Ca channels also exhibited differential effects on the secretory processes of the adrenal gland. Excess Mg and lower concentrations of La and Mn blocked release of ACh evoked by transmural stimulation. However, 20 mM-Mg, which is known to block CA secretion in the cat adrenal gland (Douglas & Rubin, 1963), had no effect on such secretion in the present work. Since Mg is believed to inhibit medullary secretion by depressing Ca flux between the gland and the perfusate (Hazard & Wurmser, 1939; Douglas & Rubin, 1963), one possibility is that mobilization of extracellular Ca is not utilized by the rat medullary cells upon stimulation with ACh. As suggested earlier, the internal pool of Ca may be involved in ACh-induced secretion, and Mg may not have any effect on internal mobilization of Ca, whereas La and Mn can block such mobilization. La causes a marked increase in CA secretion in the absence of stimulation. Borowitz (1972) also found that La enhanced spontaneous CA secretion in the bovine adrenal gland. Such secretion could result from either increased release of ACh from presynaptic nerves, or by increased release of CA from medullary cells by the action of La on internal stores of Ca (Kajimoto & Kirpekar, 1972).

Since the secretory response of the rat adrenal gland was depressed only when the concentration of verapamil was raised to 0·5 mM, it may be that Ca channels of pre- and post-synaptic structures of the rat adrenal gland are not as sensitive to the drug as the Ca channels of the heart and smooth muscle are (Kass & Tsien, 1975; Reuter,

1973; Fleckenstein, 1977). Recently, Nachshen & Blaustein (1979) also showed that verapamil or D-600 have very little effect on Ca channels of the rat brain synaptosomes.

In summary, the present study describes a simple method to evoke secretion of CA in the isolated rat adrenal gland by stimulation of splanchnic nerve endings or by ACh. Furthermore, the secretory mechanisms of nerve endings and chromaffin cells are differentially affected by various ions and agents.

This material is based upon work supported in part by the National Institutes of Health grant no. HL-18601, and in part by the National Science Foundation grant no. BNS79-23019.

## REFERENCES

ANTON, A. H. & SAYRE, D. F. (1962). A study of the factors affecting the aluminum oxide–trihydroxyindole procedure for the analysis of catecholamines. *J. Pharmac. exp. Ther.* **138**, 360–375.

BIALES, B., DICHTER, M. & TISCHLER, A. (1976). Electrical excitability of cultured adrenal chromaffin cells. *J. Physiol.* **262**, 743–753.

BOONYAVIROJ, P. & GUTMAN, Y. (1977). Inhibition of $PGE_2$ and by phenylephrine of catecholamine release from human adrenal in vitro. *Eur. J. Pharmac.* **41**, 73–75.

BOROWITZ, J. L. (1972). Effect of lanthanum on catecholamine release from adrenal medulla. *Life Sci. Oxford* **11**, 959–964.

BOZLER, E. (1969). Role of calcium in initiation of activity of smooth muscle. *Am. J. Physiol.* **216**, 671–674.

BRANDT, B. L., HAGIWARA, S., KIDAKORO, Y. & MIYAZAKI, S. (1976). Action potentials in the rat chromaffin cell and effects of acetylcholine. *J. Physiol.* **263**, 417–439.

CASTEELS, R. & RAEYMAEKERS, L. (1979). The action of acetylcholine and catecholamines on an intracellular calcium store in the smooth muscle cells of the guinea-pig taenia coli. *J. Physiol.* **294**, 51–68.

CLANACHAN, A. S., JOHNS, A. & PATON, D. M. (1977). Presynaptic inhibitory actions of adenine nucleotides and adenosine on neurotransmission in rat vas deferens. *Neuroscience* **2**, 597–602.

DOUGLAS, W. W. & POISNER, A. M. (1966*a*). Evidence that the secreting adrenal chromaffin cell releases catecholamines directly from ATP-rich granules. *J. Physiol.* **183**, 236–248.

DOUGLAS, W. W. & POISNER, A. M. (1966*b*). On the relation between ATP-splitting and secretion in the adrenal chromaffin cell: Extrusion of ATP (unhydrolysed) during release of catecholamines. *J. Physiol.* **183**, 249–256.

DOUGLAS, W. W. & RUBIN, R. P. (1963). The mechanism of catecholamine release from the adrenal medulla and the role of calcium stimulus-secretion coupling. *J. Physiol.* **167**, 288–310.

ENERO, M. A. & SAIDMAN, B. Q. (1977). Possible feed-back inhibition of noradrenaline release by purine compounds. *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **297**, 39–46.

FLECKENSTEIN, A. (1977). Specific pharmacology of calcium in myocardium, cardiac pacemakers, and vascular smooth muscle. *A. Rev. Pharmacol. Toxicol.* **17**, 149–166.

GILLESPIE, J. I. & HUTTER, O. F. (1975). The action of 4-aminopyridine on the delayed potassium current in skeletal muscle fibers. *J. Physiol.* **252**, 70–71*P*.

GUTMAN, Y. & BOONYAVIROJ, P. (1974). Suppression by noradrenaline of catecholamine secretion from adrenal medulla. *Eur. J. Pharmac.* **28**, 384–386.

GUTMAN, Y. & BOONYAVIROJ, P. (1975). Regulation of catecholamine release from adrenal medulla in vitro by $\alpha$- and $\beta$-receptors and by prostaglandins. *Sixth Int. Congress of Pharmacology, Helsinki*, p. 423.

HAZARD, R. & WURMSER, L. (1939). Magnesium et acetylcholine. Differenciation par le magnesium des effets de l'ion potassium de ceux de l'acetylcholine. *C.r. séances soc. Biol.* **130**, 1424–2426.

HEDQVIST, P. & FREDHOLM, B. B. (1976). Effects of adenosine on adrenergic transmission, prejunctional inhibition and postjunctional enhancement. *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **293**, 217–223.

HORN, A. S., LAMBERT, J. J. & MARSHALL, I. G. (1979). A comparison the the facilitatory actions of 4-aminopyridine methiodide and 4-aminopyritine on neuromuscular transmission. *Br. J. Pharmac.* **65**, 53–62.

ISHIKAWA, K. & KANNO, T. (1978). Influence of extracellular Ca and K concentration on adrenaline release and membrane potential in the perfused adrenal medulla of the rat. *Jap. J. Physiol.* **28**, 275–289.

ITO, S., NAKAZATO, Y. & OHGA, A. (1979). The effect of veratridine on the release of catecholamines from the perfused adrenal gland. *Br. J. Pharmac.* **65**, 319–330.

KAJIMOTO, N. & KIRPEKAR, S. M. (1979). Effect of manganese and lanthanum on spontaneous release of acetylcholine at frog motor nerve terminals. *Nature, New Biol.* **235**, 29–30.

KASS, R. S. & TSIEN, R. W. (1975). Multiple effects of calcium antagonists on plateau currents in cardiac Purkinje fibres. *J. gen. Physiol.* **66**, 169–192.

KIRPEKAR, S. M. & CERVONI, P. (1963). Effect of cocaine, phenoxybenzamine and phentolamine on catecholamine output from spleen and adrenal medulla. *J. Pharmac. exp. Ther.* **142**, 59–70.

KIRPEKAR, M. KIRPEKAR, S. M. & PRAT, J. C. (1977). Effect of 4-aminopyridine on release of noradrenaline from the perfused cat spleen by nerve stimulation. *J. Physiol.* **272**, 517–528.

KIRPEKAR, S. M. & PRAT, J. C. (1978). Release of catecholamine from perfused cat adrenal gland by veratridine. *Proc. natn. Acad. Sci. U.S.A.* **76**, 2081–2083.

LANGER, S. Z., STARKE, K. & DUBOCOVICH, M. L. (1979). Presenaptic receptors. In *Advances in the Biosciences*, vol. 18, pp. 1–389. Oxford: Pergamon.

LUNDH, H., LEANDER, S. & THESLEFF, S. (1977). Antagonism of the paralysis produced by botulinum toxin in the rat. *J. neurol. Sci.* **32**, 29–43.

LUNDH, H. & THESLEFF, S. (1977). The mode of action of 4-aminopyridine and quanidine on transmitter release from motor nerve terminals. *Eur. J. Pharmac.* **42**, 411–412.

MEVES, M. & PICHON, Y. (1975). Effects of 4-aminopyridine on the potassium current in internally perfused giant axons of the squid. J. Physiol. **251**, 60–62*P*.

MICHAELIS, M. L., MICHAELIS, E. K. & MYERS, S. L. (1979). Adenosine modulation of synaptosomal dopamine release. *Life Sci. Oxford* **24**, 2083–2092.

NACHSHEN, D. A. & BLAUSTEIN, M. P. (1979). The effects of some organic 'calcium antagonists' on calcium influx in presynaptic nerve terminals. *Molec. Pharmacol.* **16**, 579–586.

OHASHI, H., TAKEWAKI, T. & OKADA, T. (1974). Calcium and the contractile effect of carbachol in the depolarized guinea-pig taenia caecum. *Jap. J. Pharmac.* **24**, 601–611.

OKA, M., OHUCHI, T., YOSHIDA, H. & IMAIZUMI, R. (1965). The importance of calcium in the release of catecholamine from the adrenal medulla. *Jap. J. Pharmac.* **15**, 348–356.

PELHATE, M. & PICHON, Y. (1974). Selective inhibition of potassium current in the giant axon of the cockroach. *J. Physiol.* **243**, 90–91*P*.

PHILLIPU, A. & SCHÜMANN, H. J. (1962). Der Einfluss von Calcium auf die Brenzcatechinamin freisetzung. *Experientia* **18**, 133–140.

REUTER, H. (1973). Divalent ions as charge carriers in excitable membranes. *Prog. Biophys. molec. Biol.* **26**, 1–43.

RITCHIE, A. K. (1979). Catecholamine secretion in a rat pheochromacytoma cell line: Two pathways for calcium entry. *J. Physiol.* **286**, 541–561.

SHELLENBERGER, M. K. & GORDON, J. H. (1971). A rapid, simplified procedure for simultaneous assay of norepinephrine, dopamine and 5-hydroxytryptamine from discrete brain areas. *Analyt. Biochem.* **39**, 365–372.

STARKE, K., GÖRLITZ, B.-D., MONTEL, H. & SCHÜMANN, M. J. (1974). Local $\alpha$-adrenoceptor mediated feed-back inhibition of catecholamine release from the adrenal medulla? *Experientia* **30**, 1170–1171.

SU, C. (1978). Purinergic inhibition of adrenergic transmission in rabbit blood vessels. *J. Pharmac. exp. Ther.* **204**, 351–361.

VERHAEGHE, R. H., VANHOUTTE, P. M. & SHEPHERD, J. T. (1977). Inhibition of sympathetic neurotransmission in canine blood vessels by adenosine and adenine nucleotides. *Circulation Res.* **40**, 208–215.

WAKADE, A. R. (1979). Modulation of $^3$H-norepinephrine release in the rat hypothalamus and cortex by adenosine. In *Advances in the Biosciences*, vol. 18, pp. 377–383, ed. LANGER, S. Z., STARKE, K. & DUBOCOVICH, M. L. Oxford: Pergamon.

WAKADE, A. R. (1981). Facilitation of secretion of catecholamines from rat and guinea-pig adrenal glands in potassium-free medium or after ouabain. *J. Physiol.* **313**, 481–498.

WAKADE, A. R. & WAKADE, T. D. (1977). Another endogenous modulator of sympathetic neurotransmission at the synaptic level – adenosine. *Pharmacologist* **19**, 129.

WAKADE, A. R. & WAKADE, T. D. (1978). Inhibition of noradrenaline release by adenosine. *J. Physiol.* **282**, 45–49.

WAKADE, A. R., IYENGAR, P. & WAKADE, T. D. (1980). Facilitation by K-deprivation of catecholamine secretion evoked by transmural stimulation of rat adrenal gland. *Fedn Proc.* **39**, 1005.