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THE ROLE OF ENERGY METABOLISM IN CALCIUM-EVOKED SECRETION FROM THE ADRENAL MEDULLA

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SUMMARY

1. Experiments were carried out on cat adrenal glands perfused with Locke solution to study the effects of inhibition of metabolism on calcium-evoked catecholamine release.

2. In the presence of sodium cyanide (CN, 0·2 mM), a low concentration of glucose (1 mM) prevented the gradual decline in the secretory response to sequential exposures to calcium. Furthermore, when the secretory response was almost completely blocked by perfusing with a glucose-deprived solution containing CN, restoration of secretion was correlated with the glucose concentration in the perfusion medium.

3. In the presence of CN, 2-deoxyglucose blocked both the protective effect and the restorative effect of glucose. The deoxyglucose inhibition of the glucose-dependent restoration of secretion was antagonized by a higher concentration of glucose.

4. Restoration of calcium-evoked secretion was also observed after the washout of CN. The extent of this restoration was not at all related to the glucose concentration and was not affected by various inhibitors of carbohydrate metabolism, including deoxyglucose.

5. Analysis of adrenal glands which had been perfused first with a glucose-free solution containing CN and subsequently with the normal medium indicated that no discernible synthesis of catecholamines had taken place during the experimental procedures.

6. The data provide further evidence that the action of calcium to trigger medullary secretion requires the presence of metabolic energy and support the hypothesis that an interaction between calcium and high-energy nucleotides is a step in the sequence of events leading to the extrusion of catecholamines from the chromaffin cell.

INTRODUCTION

Although studies on a variety of secretory systems, including the adrenal medulla, have verified the key role played by calcium in the secretory process, the mechanism by which calcium exerts its effects is still undefined (see Douglas, 1968). However, a recent investigation has shown that the release of catecholamines from the adrenal medulla in response to challenges with calcium eventually fails when oxidative and glycolytic pathways are simultaneously blocked by cyanide (CN) plus glucose deprivation (Rubin, 1969). This finding indicated that the action of calcium requires metabolic energy and suggested that calcium might interact with high energy nucleotides to elicit secretion.

The present experiments have been carried out to examine further the role of energy metabolism in the activity of calcium as a secretogogue in order to obtain additional information on the calcium receptive secretory process. These results confirm that metabolic energy is indispensable for the action of calcium and also show that the chromaffin cell can obtain this energy by utilizing either endogenous or exogenous substrates.

METHODS

The acutely denervated cat adrenal gland was perfused at room temperature with a modified Locke solution containing excess potassium (K^+), which had the following composition (mM); NaCl 100; KCl 56; $NaHCO_3$ 12; glucose 10. In certain experiments the glucose concentration was reduced or completely omitted (see Results). Calcium chloride was added when needed to give a final concentration of either 0·5 or 3·0 mM.

The perfusate was analysed for adrenaline and noradrenaline by a modification of the fluorometric method of Anton & Sayre (1962). Oxidation of a given sample of effluent by potassium ferricyanide was carried out both at pH 6 and pH 3 (see Rubin & Jaanus, 1966). The outputs are expressed as total catecholamine (μg adrenaline plus noradrenaline base/min).

Whole adrenal glands were homogenized in 4 ml. 0·2 N perchloric acid and the homogenate spun at 20,000 rev/min for 15 min. An aliquot of the supernatant was diluted tenfold with distilled water and assayed for adrenaline and noradrenaline.

Agents used. Sodium cyanide (Merck); 2-deoxyglucose, adenosine-5-triphosphate (Calbiochem); creatine phosphate (Boehringer); iodoacetic acid (Nutritional Biochemical); oxamic acid (Sigma). The latter agent was neutralized with 1 N-NaOH before use.

RESULTS

Anoxia plus glucose deprivation gradually depresses the secretory response of the adrenal gland to acetylcholine and calcium (Rubin, 1969). In the present study, cyanide (CN) rather than nitrogen was employed to produce anaerobiosis since in a glucose-free medium, CN brings about a more consistent and predictable decline in the response to calcium. The

stimulant effect of calcium was restored within 12 min either by adding glucose in the continued presence of CN or by removing the CN (Fig. 1). The outputs obtained when cyanide was removed were somewhat higher than those obtained when glucose was added in the presence of CN. Thus, subsequent to the block of calcium-evoked secretion, the addition of glucose (10 mM) increased the catecholamine output in response to calcium

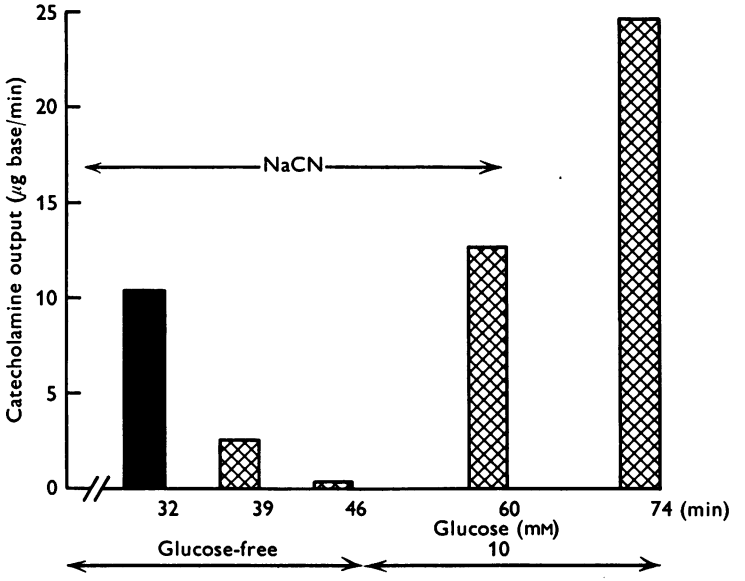

Fig. 1. The effect of cyanide and glucose deprivation on the secretory response to calcium. Adrenal glands were perfused for 46 min with glucose-free, calcium-free high K^+ Locke solution, which also contained sodium cyanide (NaCN, 0·2 mM). Glucose (10 mM) was then added to the perfusion medium for the next 14 min and the NaCN was removed for the final 14 min of perfusion. Calcium (0·5 or 3·0 mM) was added for 2 min periods every 7 or 14 min.

The vertical columns depict the total catecholamine outputs obtained in response to calcium (■ 0·5 mM and ▨ 3·0 mM).

(3·0 mM) to 8·40 ± 1·50 μg/min (mean of four experiments ± S.E.); whereas the restoration of aerobiosis by the removal of CN from the perfusion medium increased the calcium-evoked output to 14·05 ± 1·74 μg/min (eight experiments). Secretion was negligible under all conditions in the absence of calcium.

Anoxia, produced either by nitrogen or CN, causes a striking change in the differential secretion of catecholamines, so that most of the output during anoxia is adrenaline (Rubin, 1969). When calcium-evoked secretion was restored, either by the addition of glucose or by the removal of CN,

output was still predominantly adrenaline. The mean percent adrenaline secreted in response to calcium after the restoration of glucose was $84{\cdot}8 \pm 3{\cdot}7$ %, that after the washout of CN was $87{\cdot}2 \pm 3{\cdot}3$ %.

Catecholamine secretion in the presence of cyanide

The role of glucose. Cyanide potentiates the secretory response to calcium in the presence of the normal concentration of glucose (10 mM), but when glucose is omitted from the perfusion medium, CN causes a gradual

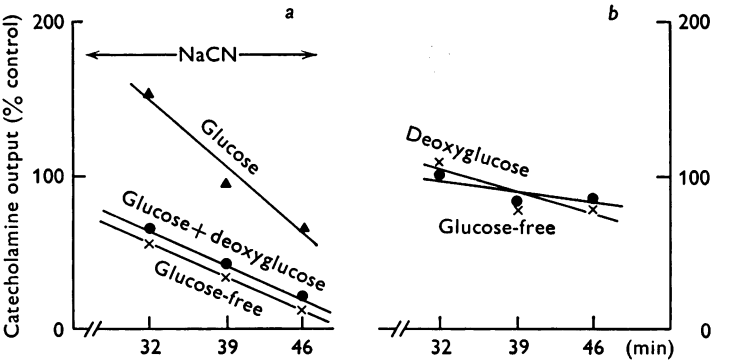

Fig. 2. The effect of glucose on the secretory response to calcium in the presence and absence of cyanide. Glands were perfused for 46 min with calcium-free, high K^+ Locke solution in the presence and absence of NaCN (0·2 mM). In certain experiments where indicated, glucose (1·0 mM) and/or deoxyglucose was also present. Calcium (0·5 mM) was added during the 30–32nd min and 3·0 mM calcium was added during the 37–39th min and 44–46th min of perfusion.

Each point represents the mean calcium-evoked output, as a % mean output obtained under control conditions. The control solutions contained NaCN plus glucose (10 mM) and glucose (10 mM). The mean outputs were calculated from at least three experiments.

The data of the control experiments were taken from a previous study (Rubin, 1969).

decline in calcium-evoked secretion (Rubin, 1969). This block of the action of calcium could be prevented by the addition of a low concentration of glucose (1 mM, Fig. 2*a*). When calcium-elicited secretion was profoundly depressed by CN plus glucose deprivation, restoration of the response to calcium could be correlated with the concentration of glucose in the perfusion medium in the continued presence of CN (Fig. 3*a*). Furthermore, with a low concentration of glucose (1 mM), the catecholamine output varied directly with the amount of calcium added (Fig. 4*a*); with 10 mM glucose, 0·5 mM and 3·0 mM calcium elicited approximately equal outputs (Fig. 4*a*).

The effect of deoxyglucose. Deoxyglucose (2 mM) antagonized the protective effect of glucose on calcium-evoked secretion, so that the response to calcium in the presence of glucose plus deoxyglucose paralleled the response observed in the absence of glucose (Fig. 2*a*). The restorative action of glucose on calcium-evoked secretion was also antagonized by deoxyglucose, and this inhibitory action of deoxyglucose was overcome by increasing the glucose concentration (Fig. 4*b*).

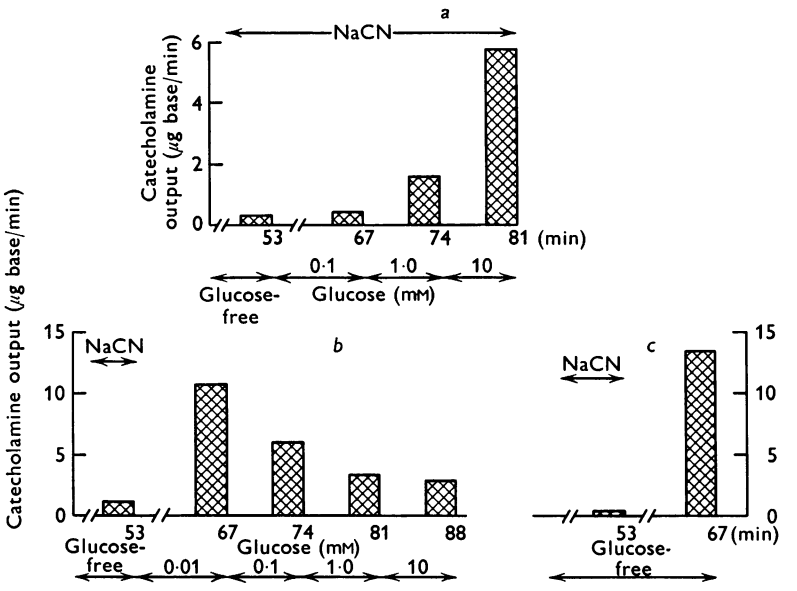

Fig. 3. The effect of glucose on the restoration of the secretory response following the exposure to cyanide plus glucose deprivation. Glands were perfused for 53 min with glucose-free, calcium-free high K^+ Locke solution which also contained NaCN. In certain experiments NaCN was present throughout the entire experiment (*a*) and in other experiments NaCN was removed after the first 53 min of perfusion (*b*, *c*). Glucose was then added in increasing concentrations (*a*, *b*) or was withheld from the perfusion medium (*c*). Calcium was added for 2 min every 7 or 14 min beginning with the 30–32nd min of perfusion; however, only the outputs beginning with the exposure to calcium at 51–53rd min of perfusion are shown.

The effect of phosphorylated compounds. An attempt was made to restore the secretory response during anaerobiosis and glucose deprivation by perfusing a gland with a very high concentration of adenosine-5-triphosphate (ATP, 10 mM). During the infusion of the nucleotide, ethylenediaminetetraacetic acid (EDTA, 0·3 mM) was also present to prevent the breakdown of the ATP by endothelial phosphatases (Douglas & Poisner, 1966). However, after a 10 min exposure to ATP and with CN in the perfusion

medium, the catecholamine output in response to calcium (3·0 mM) was only 0·47 μg/min. In addition, in three different preparations which had been exposed to CN plus glucose deprivation, the secretory response to 3·0 mM calcium in the presence of either creatine phosphate (5 mM), phosphoenolpyruvate (1 mM), or adenosine cyclic 3′,5′-monophosphate (3′,5′-AMP) (1 mM) was only 1·13, 0·62 and 0·35 μg/min, respectively. By comparison, in the presence of 1 and 10 mM glucose, mean outputs in response to calcium were 2·13 and 8·49 μg/min respectively.

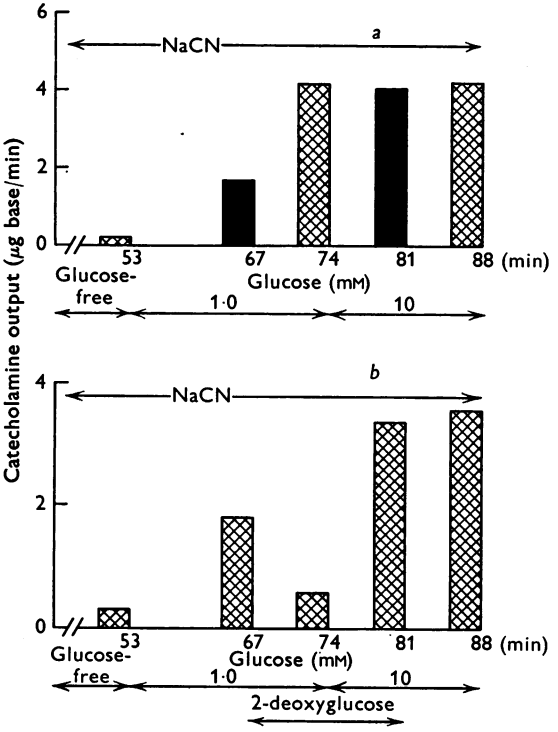

Fig. 4. The effect of (*a*) the calcium concentration and (*b*) deoxyglucose on the restoration of secretion with a low and a high glucose concentration. Secretion was blocked by perfusion with a glucose-free modified Locke solution containing NaCN, as described in the previous Figures. Glucose concentrations of 1 and 10 mM were sequentially added to the perfusion medium.

In *a*, responses to 0·5 (■) and 3·0 mM (▨) calcium were tested with each glucose concentration.

In *b*, responses to 3·0 mM calcium were tested after the addition of each glucose concentration both in the presence and absence of 2-deoxyglucose (2 mM).

A different preparation was used for each set of responses.

Catecholamine secretion in the absence of cyanide

The role of glucose. Under aerobic conditions glucose deprivation does not interfere with the secretory action of calcium. The striking restoration of calcium-evoked secretion upon the removal of CN from the perfusion medium was not at all related to the glucose concentration in the perfusion fluid (Fig. 3*b*). In fact, a high output of catecholamines could be elicited by calcium in the complete absence of glucose (Fig. 3*c*).

TABLE 1. The effect of various metabolic inhibitors on the secretory response to calcium following the removal of cyanide from the perfusion medium

Expt.	Inhibitor	Concn. (mM)	Catecholamine output (μg/min) Experimental*	Control
1	Deoxyglucose	10	35·0	16·9
2	Deoxyglucose	10	16·4	5·3
3	Iodoacetic acid	0·2	10·1	6·8
4	Oxamic acid	2·0	11·2	4·7

* Glands were perfused with cyanide plus glucose-free solutions for 53 min and exposed to calcium for 2 min every 7 min, beginning with the 30–32nd min of perfusion. Cyanide was then replaced by a metabolic inhibitor for 20 min before calcium was reintroduced. Finally, the inhibitor was removed and the control response to calcium obtained.

The effect of metabolic inhibitors. Deoxyglucose (10 mM), in the presence or absence of glucose, had no inhibitory effect on the secretory response to calcium in the absence of CN (Fig. 2*b*), although 2 mM deoxyglucose markedly depressed secretion in the presence of CN with glucose present (Fig. 2*a*). In addition, deoxyglucose did not inhibit the calcium-evoked secretion that occurred after removal of CN from a glucose-deprived perfusion medium that contained CN (Table 1). The large secretory response to calcium after removal of CN was also tested in the presence of either iodoacetic acid or oxamic acid, two other inhibitors of carbohydrate metabolism. The concentration of iodoacetic acid employed (0·2 mM) depresses the initial response to calcium by 50% under aerobic conditions and by 90% under anaerobic conditions (Rubin, 1969). However, in the concentrations tested these inhibitors were unable to depress significantly the powerful rate of calcium-evoked secretion which follows the washout of CN (Table 1).

The effect of cyanide and glucose-deprivation on the catecholamine content of the adrenal gland

In order to ascertain whether the restoration of the secretory response that follows the resumption of either glycolysis or oxidative metabolism might be the result of a resynthesis of medullary catecholamine, perfused glands were analysed for their catecholamine content after exposure to CN plus glucose-deprivation and the subsequent addition of glucose. Since the total catecholamine content of the cat left adrenal gland does not differ from that of the right gland (Butterworth & Mann, 1957), any significant resynthesis of catecholamine in the perfused left gland could be detected by comparing the sum of the amine content of the perfused gland and the total output with the amine content of the non-perfused right gland. Perfused glands were found to contain much less catecholamine than their respective controls (Table 2). Furthermore, the amount of amine released during each experiment plus the amount of amine remaining in the depleted gland very closely approximated the amine content of the control gland (Table 2).

TABLE 2. The effect of cyanide and glucose deprivation on the catecholamine content of perfused adrenal glands

Expt.	(*a*) Content of perfused gland*	(*b*) Total output†	(*a*) + (*b*)	(*c*) Content of unperfused gland
1	81·6	72·1	153·7	151·6
2	68·9	125·5	194·4	199·1
3	70·8	42·5	113·3	123·2

* Glands were perfused with a glucose-free medium containing CN and exposed to calcium in manner described in Table 1. Glucose was then added to the perfusion medium and response to calcium repeated.

† Outputs obtained during 6–8 2 min exposures to calcium (0·5 and 3·0 mM).

All values are expressed as μg catecholamine.

DISCUSSION

The present data provide further evidence to support the hypothesis that calcium requires the presence of metabolic energy to trigger the release of catecholamines from the adrenal medulla. Under anaerobic conditions the block of the secretory response to calcium produced in the absence of exogenous substrate was rapidly reversed by the addition of glucose to the perfusion medium or by the restoration of aerobiosis by the washout of the CN. The block and restoration of the secretory response could not have been due to a depletion and subsequent resynthesis of medullary catecholamines for the following reasons: the return of the

response to calcium was obtained within 8–12 min; there was no loss of catecholamine in the absence of calcium despite the inhibition of metabolism; and most significantly, analysis of the perfused glands showed that no discernible synthesis had taken place during the period of perfusion.

Since it is well established that the chromaffin granules contain a large concentration of ATP (Blaschko, Born, D'Iorio & Eade, 1956; Hillarp, Högberg & Nilson, 1955) which is utilized for the storage of catecholamine and is independent of metabolic processes (see Schümann, 1966), it would appear that the metabolic energy required for the secretory action of calcium must involve a small, more labile, extragranular pool of ATP. Indeed, preliminary experiments show that perfused adrenal glands, in which both glycolytic and oxidative pathways are blocked and the response to calcium is abolished, contain concentrations of ATP which approximate those found in control perfused glands (M. B. Feinstein & R. P. Rubin, unpublished).

The medulla employs a variety of biochemical mechanisms to produce the metabolic energy for sustaining its specialized function of secretion; and the present, as well as the previous investigation (Rubin, 1969), have shown that block of both glycolytic and oxidative pathways is necessary to prevent the chromaffin cell from producing the metabolic energy required for the maintenance of the secretory process. It appears that exogenous glucose is not a critical substrate under aerobic conditions. When the response to calcium is almost completely abolished by perfusion with a glucose-deprived solution containing CN, glucose is also not utilized when the response is restored after CN has been removed from the perfusion fluid. This conclusion is based upon the findings that the return of the response following the removal of the CN is not at all correlated with the glucose concentration in the perfusion medium and that various inhibitors of carbohydrate metabolism have no discernible effect on the response to calcium following the removal of CN. Furthermore, it is not likely that endogenous glycogen is still being utilized since it has been shown that during perfusion with a medium lacking glucose and containing CN the glycogen stores of the medulla are profoundly depleted (Rubin, 1969). Although these results do not rule out the possibility that an endogenous store of carbohydrate is utilized to restore secretory activity following anaerobiosis, they do suggest that a non-carbohydrate substrate, such as lipid, might be oxidized under these circumstances.

By contrast, exogenous glucose appears to be a critical substrate under anaerobic conditions. Glucose prevented the decline and restored the response to calcium in the presence of CN. The observed effects of glucose appear to be related to its utilization as an energy-producing substrate. The restorative effect of glucose was directly related to its concentration

in the perfusion medium, and the effects of glucose were antagonized by deoxyglucose, a glucose analogue which interferes with the uptake and utilization of glucose (see Webb, 1966). These data suggest that under anaerobic conditions glucose enters the chromaffin cells and is metabolized through glycolytic pathways to produce the energy necessary for secretion. The inability of infused ATP to restore the response to calcium indicates that either ATP was not able to traverse the chromaffin cell membrane or was not able to gain access to the critical site within the chromaffin cell.

If the amount of energy produced is related to the concentration of glucose added, then it is of interest that the rate of secretion was correlated with the calcium concentration in a low, but not in a high concentration of glucose. It suggests that either calcium influx into the chromaffin cell is dependent on energy metabolism or the amount of amine secreted depends upon some stoicheiometric relation between the calcium concentraction and the high-energy intermediate. The former possibility is not deemed likely, since evidence suggests that calcium entry into the chromaffin cell involves passive diffusion down a concentration gradient (see Rubin, 1969).

Both during and after anaerobiosis, calcium-evoked secretion was overwhelmingly in the form of adrenaline. This finding indicates that the adrenaline-containing cells of the medulla are adapted to perform their specialized function of secretion both under aerobic and anaerobic conditions and suggests that the adrenaline cells manifest greater metabolic flexibility than the noradrenaline cells. The increase in the amount of adrenaline secreted during anoxia is apparently the result of an increase in the uptake and utilization of glucose in the adrenaline-secreting cells. In the mammalian sympathetic neurone, which is a homologue of the medullary chromaffin cell, Larrabee & Bronk (1952) showed that anoxia produced by either CN or nitrogen causes an increase in glucose uptake.

The results from the experiments carried out on medullary tissue *in vivo* and *in vitro* are strikingly similar to those obtained in studies of the anaphylactic release of histamine from tissue *in vitro*. Calcium is required for histamine release from platelets and lung tissue in response to an antigenic stimulus (Humphrey & Jaques, 1955; Mongar & Schild, 1958); glucose can prevent the CN-induced inhibition of the anaphylactic release of histamine from mast cells (Chakravarty, 1968); and, finally, ATP stimulates the release of histamine from isolated rat mast cell preparations (Diamant & Krüger, 1967), just as it stimulates the release of catecholamines from isolated chromaffin granules (Oka, Ohuchi, Yoshida & Imaizumi, 1967; Poisner & Trifaro, 1967). It will require further study to determine whether a similar mechanism underlies the release of other biogenic amines, as well as other secretory substances which are not

chemically related to catecholamines and histamine, but which also require calcium for secretion.

Further parallels between secretion and contraction. Attention has been previously drawn to the many similarities between the basic physiological processes of secretion and contraction (Douglas & Rubin, 1961, 1963, 1964). The similarities discussed were in regard to the effects of various ionic species on these two processes. Now further analogies can be made in regard to the metabolic events involved. For example, in isolated mammalian cardiac muscle, glucose is poorly utilized under aerobic conditions (Williamson, 1962), whereas anaerobiosis greatly augments glucose uptake and utilization (Morgan, Henderson, Regen & Park, 1961), and causes a profound depletion of tissue glycogen (Fisher & Williamson, 1961). Contraction, like secretion, requires both ATP and calcium ion (see Sandow, 1965). ATP splitting is the immediate energy source for contraction, since contraction is inhibited when ATP splitting is depressed by inhibitors of ATPase (Weber, 1958). Likewise, ATPase inhibitors prevent the ATP-induced release of catecholamines from isolated granules (Poisner & Trifaro, 1967), so that splitting of ATP may be required for secretion. How much further the analogy between 'excitation–contraction coupling' and 'stimulus–secretion coupling' can be extended, remains to be determined.

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