PHARMACOLOGICAL REVIEWS
Vol. 18, No. 1, Part I

Printed in U.S.A.

B. THE MECHANISM OF RELEASE OF CATECHOLAMINES FROM THE ADRENAL MEDULLA

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At the two previous meetings devoted to catecholamines and adrenergic mechanisms (41, 43), the adrenal medulla, which is the main repository of catecholamines in the body, was treated principally from the standpoint of synthesis and storage of the amines. Relatively little discussion was devoted to the question of the intimate mechanism of secretion of the catecholamines, that is to say, secretion in the sense of "extrusion" or "release." This emphasis reflects the uncertaintly then existing about the events occurring upon physiological stimulation of the chromaffin cell and leading to the discharge of the catecholamines. Although, as a result of the classical experiments of Feldberg *et al.* (27) and innumerable experiments of a pharmacological nature which followed, it was accepted that the immediate physiological stimulus to the chromaffin cell was acetylcholine, the neurohumor liberated by the secretomotor splanchnic nerves, there were few clues to suggest how acetylcholine might initiate secretory activity. Furthermore, the nature of the secretory process was itself quite uncertain. While an impressive body of evidence has been built up by cell fractionation studies after the pioneer work of Blaschko and Welch (3) and of Hillarp *et al.* (33) and by electron microscopy (see 28, 43 for references) to show that much of the catecholamine present in the chromaffin cells was lodged in membrane-limited chromaffin granules, some catecholamine also appeared to be present "free" in the cell sap. And which of these pools was immediately drawn on when the chromaffin cells were stimulated could only be speculated upon.

In the present paper my main purpose is to present evidence, much of it obtained since the previous meetings, which offers a clearer insight into the mechanism of the stimulant action of acetylcholine on the adrenal chromaffin cell, and which also provides some fresh clues to the nature of the catecholamine-extrusion process. I shall not attempt here an exhaustive review, for much of the ground has been covered in previous symposia (41–43), and a principal facet of the problem is to be covered in depth in a forthcoming review in the same journal. I shall presently concentrate, therefore, only in putting together the main pieces of evidence which have led me and my colleagues, Drs. R. P. Rubin and A. M. Poisner, to the view that acetylcholine excites the chromaffin cell by promoting an inward movement of calcium ions across the plasma membrane; and that the chromaffin cell thus excited draws immediately on the pool of catecholamines sequestered in the heavy "nucleotide-rich" chromaffin granules, and not, to any significant extent, on the other intracellular pools of catecholamine that have been described.

THE SITE AND MECHANISM OF ACETYLCHOLINE'S ACTION

The experiments that suggested such a mechanism of action of acetylcholine, were prompted by consideration of the existing evidence concerning the mode of

action of acetylcholine as a chemical transmitter at other sites of cholinergic transmission in the body, such as the neuromuscular junction, the parasympathetic neuroeffector synapses in the heart, and the synapses of autonomic ganglia. At each of these sites the action of acetylcholine appeared to be localized to the *membrane* of the postjunctional elements—indeed, there was evidence that its action was on the outer surface of the membrane (8); and in each instance, the critical effect of acetylcholine seemed to be the production of an increase of the permeability of the postsynaptic membrane to common species of inorganic ions although different ions were involved at the different sites. Such evidence prompted Douglas and Rubin (22) to test whether a similar sort of mechanism might underlie the actions of acetylcholine as a medullary secretogogue, and they tried the simple experiment of examining how the secretory response to acetylcholine was influenced by the ionic composition of the extracellular environment. For this purpose they used cats' adrenal glands, acutely denervated and perfused, *in vivo* or *in vitro*, with Locke's solution or appropriate variations of that solution. The experiments soon showed that acetylcholine still caused secretion when all sodium and potassium was omitted from the perfusion medium (tonicity being maintained with sucrose). Indeed, the response to acetylcholine was potentiated by reduction in the concentration of these two cations (22, 24). By contrast, the response to acetylcholine was profoundly depressed when calcium was omitted from the perfusion medium; and over a wide range of calcium concentrations the response to acetylcholine increased with increasing calcium concentration so that at 17.6 mM (the highest concentration of calcium studied) the output of catecholamines to the standard concentration of acetylcholine was about doubled. In addition, as will be described in fuller detail below, evidence was obtained that calcium itself could evoke catecholamine secretion. These findings, coupled with the known ability of acetylcholine to increase the permeability of membranes at other sites in the body, led to the hypothesis, first put forward at meetings four summers ago (20, 21), that acetylcholine probably stimulated the chromaffin cell to secrete by promoting an increased uptake or influx of calcium ions. Experimental support for this hypothesis has continued to accumulate: it has been demonstrated that acetylcholine does indeed increase calcium-45 uptake in the adrenal medulla (14); and evidence has gradually built up to show that the catecholamine extrusion process may indeed be set in motion by calcium. A valuable piece of evidence of the latter sort was obtained in experiments which catecholamine secretion was induced by raising the potassium concentration of the perfusion medium. Excess potassium was known, from the work of Vogt (44) to evoke catecholamine secretion by a direct action on the chromaffin cell. This required calcium in the extracellular environment (22); moreover, the intensity of the secretory response to excess potassium varied with the extracellular calcium concentration over a wide range (24); and excess potassium greatly increased the uptake of calcium-45 in the medulla (13). The principal interest attaching to these experiments is, of course, that a secretogogue of a chemical nature distinct from that of acetylcholine again requires calcium for its action and promotes calcium uptake. Furthermore, just as with acetylcholine, there are grounds for supposing that potassium must owe its action to

an effect on the membrane. Thus, we must suppose that the concentration of potassium inside the chromaffin cell (which is developmentally homologous with the sympathetic postganglionic neurones) is already high and unlikely to be significantly changed by the elevations in extracellular potassium sufficient to evoke secretion: the first requirement of any stimulus is, after all, that it should alter the environment of the object to be stimulated. Presumably, the effect of excess potassium is due to a reduction in the ratio of potassium inside the cell to that outside of it with a consequent depolarization of the chromaffin cell membrane. Certainly this is accepted as the mechanism of action of potassium in provoking inward movement of calcium in muscles and nerves (40). That calcium entry, the common factor in stimulation by the two secretogogues, acetylcholine and potassium, is indeed responsible for secretion, is made further likely by evidence that a variety of secretogogues of diverse chemical constitution are all ineffective when calcium is omitted from the extracellular environment. These secretogogues include: nicotine and related drugs (23); muscarine, pilocarpine and methacholine; the amines, histamine and 5-hydroxytryptamine; and the polypeptides, angiotensin and bradykinin (37). And it is relevant that the bulk of evidence suggests that these diverse drugs owe most of their pharmacological effects at other sites in the body to actions on the cell membrane, and that, in smooth muscle at least an important aspect of their action appears to be concerned with increasing the inward movement of calcium (7).

A different and perhaps more telling piece of evidence to support the view that calcium entry is a critical link in "stimulus-secretion coupling" has come from experiments in which calcium itself in the absence of any of the familiar secretogogues evokes catecholamine secretion. Although calcium has little direct stimulant effect on the adrenal medulla in normal circumstances, it becomes a powerful secretogogue if it is introduced after a period of perfusion with a calcium-free medium (22). In such conditions, restoration of the conventional amount of calcium (2 mM) to the perfusion medium causes a brief, but violent, secretory response comparable in intensity with that provoked by large doses of acetylcholine under normal conditions. A simple explanation of the phenomenon (see 11, 12, 22) is that it is attributable to calcium penetrating the plasma membranes of the chromaffin cells as a result of their having been rendered unduly leaky by calcium deprivation. This explanation, which is consistent with what is, perhaps, the most familiar consequence of omitting calcium from the extracellular environment of various cells, can, of course, be regarded as support for the view that acetylcholine acts on the membrane of the chromaffin cell to increase its permeability to calcium, and that during exposure to acetylcholine calcium enters the chromaffin cell by running down its electrochemical gradient from the extracellular to the intracellular compartments. That stimulation involves inward movement of calcium from the extracellular fluid, and not merely displacement of, for example, membrane calcium, is suggested by the promptness of the change in rate of catecholamine secretion which occurs in response to changes in the extracellular concentration in cells continuously exposed to acetycholine or excess potassium (24).

Further evidence of a calcium-activated mechanism for extrusion of amines

has come from studies of the closely related alkaline earth metal, barium. Barium has been supposed to stimulate medullary secretion through the splanchnic nerves (29), but pharmacological analysis shows that it also has a powerful direct stimulant effect on the chromaffin cells (26), and will, for example, evoke secretion when introduced in small amounts to glands perfused with Locke's solution. In this respect it is quite distinct from calcium, which evokes vigorous secretion only when the cell is exposed to one or other of the various secretogogues mentioned above, or is altered in some way by calcium deprivation. It is significant that barium is known to be able to penetrate muscle membranes much more readily than calcium (35). Barium seems to act in its own right and not merely by freeing calcium from sites of binding: it is fully effective in media free of calcium and containing EDTA (26). It has been argued (26) that barium owes its stimulant effect on medullary secretion to its penetrating the chromaffin cell readily and there activating the catecholamine extrusion mechanism that is normally set in motion by calcium ions entering during exposure to acetylcholine. This mechanism can also be activated by strontium but not by a number of other divalent ions (25). Magnesium is quite without stimulant effect on cats' adrenal glands. Its action, on the contrary, is strongly inhibitory. It opposes not only the stimulant effects of calcium and barium, but also those of acetylcholine and potassium. This strengthens the view that calcium is critically involved in the action of these last two secretogogues (24).

Taken together, all these studies provide evidence that the role of acetylcholine at the adrenal medullary synapse is similar to its role at, for example, the neuromuscular junction or ganglionic synapse, and is confined to the production of an increase in permeability of the postjunctional cell membrane to allow increased diffusion of common species of inorganic ions—in this instance the critical event apparently being inward movement of calcium. On this view, calcium propagates the signal for catecholamine release that is initiated at the plasma membrane by the action of the synaptic transmitter, acetylcholine, and thus acts as a link in the chain of events which may conveniently be described as "stimulus-secretion coupling" (22). This role is comparable with the proposed role of calcium in "excitation-contraction coupling" (40), and the many striking parallels between these two seemingly disparate processes have been commented on previously (11, 12, 22). The interesting possibility arises that nature employs a single stratagem, influx of calcium (or its translocation), to initiate both contraction and certain secretory responses.

THE NATURE OF THE CATECHOLAMINE EXTRUSION PROCESS

The question that obviously arises at this juncture concerns the nature of the catecholamine extrusion process which, it seems, is set in motion by calcium. An answer to this can be arrived at only by taking into account what is now known of the intracellular disposition of catecholamines. According to present understanding, the catecholamines are, for the most part, sequestered in membrane-limited "chromaffin granules," with an ill-defined proportion of catecholamines "free" in the cell sap (see 31). One of the most interesting discoveries has

been that chromaffin granules contain, in addition to catecholamines, enormous amounts of ATP: the molar ratio of catecholamines to ATP (about 4:1) being such as to suggest that ATP serves as an anion paired with the basic catecholamines, and that the complex so formed is an integral part of the intragranular mechanism for catecholamine storage. But although this may be true of most chromaffin granules, Hillarp (31) has presented evidence that another type of granule exists in which catecholamines are stored without ATP. He has calculated that this pool, together with a third pool of "free" cytoplasmic amines, accounts for about 20% of all the catecholamines in the chromaffin cell. This is a very large amount, theoretically enough to sustain secretory activity for a prolonged period. The question that confronts us then, is this: Which of these three pools of catecholamines is immediately drawn on when the chromaffin cell is stimulated to secrete by physiological means?

One possibility, which has been suggested repeatedly, is that it is the "free" pool of cytoplasmic amines. Thus, Blaschko and Welch (3) speculated that liberation of acetylcholine from the splanchnic nerve might result in an increased permeability of the membrane of the chromaffin cell which would lead to a loss of amine present in the cytoplasmic sap, and that the role of the chromaffin granules might be to act as stores which would replenish the cytoplasmic amines depleted during secretory activity. And similar conjectures have been advanced by many others (*e.g.*, 36, 38). Alternatively, it has been suggested, on various grounds, that the chromaffin granules are immediately involved (9, 32). But no persuasive evidence has been offered for either view. Electron microscopic studies have not resolved the problem; they clearly cannot examine the possibility of migration of "free" cytoplasmic amine; and they have offered conflicting testimony on the involvement of chromaffin granules (9, 46). A most interesting observation bearing on the problem was made some years ago by Carlsson and Hillarp (4), who examined adrenal glands taken from rats after several hours of medullary stimulation and found that the granule fraction was poor in catecholamines and ATP. Similar observations were later made in sheep (5). Such a result indicates clearly that the classical ATP-rich chromaffin granules are somehow involved. But it leaves unanswered the critical question whether this involvement is immediate and central to the release process, or whether granule depletion is secondary to loss of cytoplasmic amines—the shift of granule amine to cytoplasm restoring the balance between the two pools as suggested by Blaschko and Welch (3). And, of course, such observations do not tell of the possible involvement of the other sizeable catecholamine pools (ATP-free) that were later described by Hillarp (31).

Recently Douglas *et al.* (12, 19) devised an alternative approach which overcomes some of these difficulties and offers more direct evidence on the pool of amines immediately involved in the acute secretory response. In experiments on cats' adrenal glands perfused with Locke's solution, they evoked catecholamine secretion with acetylcholine and other medullary secretogogues, and examined the venous effluent for ATP and metabolites. Whenever catecholamines appeared in the venous effluent in response to such stimuli, large amounts of

AMP were also present along with traces of ADP and ATP. This finding at once offered evidence on the hitherto mysterious (45) fate of granule ATP, and indicated that the ATP-rich, "heavy," chromaffin granules were immediately involved in the acute secretory response. That the "heavy" chromaffin granules were the source of the nucleotide found in the venous effluent was probable because no other source in the gland seemed rich enough in nucleotide to supply such a large efflux; moreover, the amount of AMP escaping was highly correlated with the catecholamine output; and the molar ratio of catecholamines to AMP (about 6:1) was, by calculation, about what would be expected if the granules were the source of both the catecholamines and the nucleotide.

This conclusion has been strengthened with the demonstration (16) that stimulation of the medulla by the splanchnic nerves yields a similar result; and, further, that the total amount of ATP and metabolites (ADP, AMP and adenosine) escaping in the adrenal vein with a given amount of catecholamine yields a ratio (4.22 ± 0.7) close to the corresponding ratio of catecholamines to adenine nucleotides found by Hillarp and Thieme (34) in the chromaffin granules of the cat. Moreover, in successive drops of adrenal venous effluent as they emerged after beginning splanchnic stimulation, the nucleotide appeared as soon as the catecholamines, within a second or two of the onset of stimulation, and the effluxes of nucleotide and catecholamines paralleled one another closely thereafter (16). This argues against any "secondary" involvement of granules subsequent to hypothetical escape of "free" amine from the cytoplasm, a possibility which is rendered still more unlikely by considerations to be developed later. Thus, the evidence squarely indicates that the nucleotide-rich, "heavy," chromaffin granule is the immediate source of amines that escape when the chromaffin cell is stimulated to secretion by physiological means. Any possible contribution from nucleotide-free pools, such as the "light" chromaffin granules or "free" cytoplasmic amine, can be, at most, a minor one.

It is appropriate to indicate here that the concept of catecholamine release from "free" cytoplasmic amine, for all its superficial plausibility, has rested on evidence that is either insecure or indirect. Since the earliest cell fractionation studies it has been recognized that some, at least, of the "free" cytoplasmic amine is probably an artifact of the fractionation procedure. And although Hillarp (31) offered a variety of arguments to support the view that a pool of "free" amines does normally exist within the chromaffin cell, its true size remains conjectural and may be extremely small. Moreover, the theoretical view that an increase in the permeability of the chromaffin cell, such as might be brought about by acetycholine, should allow outward leakage of free amines, is not corroborated by experiment. Thus calcium deprivation (either alone or with the addition of EDTA) which is well known to increase the permeability of membranes (and promotes potassium efflux from the adrenal (18)) does not result in an increased rate of release of catecholamines. On the contrary, it reduces spontaneous output far below the normal level. Nor can secretion be evoked in such circumstances by further permeability-increasing maneuvers, such as exposure to acetylcholine or to depolarizing concentrations of potassium (16, 22, 24). This

evidence is not only counter to the hypothesis of outward diffusion of "free" cytoplasmic amine, but offers further grounds for doubting that any sizeable amine pool of this sort exists.

RELATION OF ATP HYDROLYSIS TO CATECHOLAMINE RELEASE

The possibility that splitting of the ATP lodged within the heavy granule might be the critical link in catecholamine release, gained plausibility from Hillarp's (30) report that ATPase activity was present in the chromaffin granule fraction. Although this finding was subsequently challenged (10) it has recently been confirmed (2). Hillarp (30) suggested that when the chromaffin cell is stimulated, the ATPase associated with the chromaffin granules, perhaps in their membranes, may be activated in some way enabling it to attack the ATP of the catecholamine storage complex and thus freeing amines to diffuse or be transported out of the granule, and ultimately out of the cell. For the writer and his colleagues, this scheme seemed most attractive. Their experiments had suggested that acetylcholine evoked secretion by promoting an inward movement of calcium, and calcium was known to be an activator of certain ATPases. Indeed, the critical role of calcium in excitation-contraction coupling in muscles is clearly associated with increased splitting of ATP. And to these theoretical considerations there was soon joined the experimental evidence that massive amounts of ATP metabolites appeared in the adrenal venous effluent from glands secreting catecholamines (19). However, subsequent events have dispelled the charm of this neat hypothesis. It was early appreciated (19) that the presence of ATP metabolites in the venous effluent from the adrenal might be attributable to endothelial enzymes, for control perfusions with ATP in appropriate concentrations showed that little of it survived passage through the adrenal vessels, and that most was broken down to AMP and adenosine, the main metabolites recovered in the effluent from adrenals secreting catecholamines. To reduce profoundly the rate of hydrolysis of ATP in the adrenal vasculature, it was found sufficient to remove calcium and magnesium from the perfusion medium and to add 1 to 2 mM EDTA: in such conditions, more than 80% of the ATP passed through the gland in control perfusions survived. Since acetylcholine is ineffective in calcium-free media (22), barium was used to evoke catecholamine secretion. In seven tests (17), the mean molar ratio of catecholamines to ATP in the venous effluent during brief exposure to barium (2 to 5 mM) was 11.5 ± 2.9, and in two of these experiments the ratio was less than 6. This result is in vivid contrast to the results obtained during perfusion with solutions allowing intravascular destruction of ATP, where, in response to acetylcholine (or barium), the ratio of catecholamines to ATP in the venous effluent was many hundreds to one, and where the principal nucleotide was AMP (17).

From such experiments it must be concluded that splitting of intragranular ATP is not a critical step in the catecholamine release process, and that most such ATP is extruded intact to the cell exterior. This result compels us to abandon what had seemed, on various grounds, an attractive scheme for catecholamine release. In addition, it appears to place an obstacle in the way of accept-

ance of *any* hypothesis involving *intracellular* release of catecholamines from storage granules. Thus, it seems unlikely that if catecholamines and ATP were to be liberated from the granules to the cell sap, the ATP should, on the one hand, escape hydrolysis by intracellular enzymes, and, on the other hand, should penetrate the chromaffin cell membrane so readily that the ratio of catecholamines to ATP in the venous effluent is maintained at a value not far removed from that existing in the granules (ATP is known to penetrate cell membranes poorly).

The hypothesis of intracellular release from granules has been espoused by many following its advocacy by Hillarp *et al.* (32). Indeed it provides the rationale for much work on isolated chromaffin granules, directed toward attaining an understanding of the events involved in physiological secretion of catecholamines. Yet there is no compelling evidence in its favor. One of the few direct pieces of evidence was the observation of Schümann and Philippu (39) that the addition of calcium to suspensions of chromaffin granules *in vitro* accelerates the loss of catecholamines from the granules to the suspension medium. The relevance of this phenomenon to the physiological events in the intact cell has been questioned (24, 25) on the following grounds: first, that rather high concentrations of calcium were required and they produced only small effects; and second, that magnesium was also effective although it strongly inhibits catecholamine release from the intact chromaffin cell.

The large amounts of ATP in the effluent from adrenal glands secreting catecholamines, suggests that the release of granule catecholamine and ATP most probably occurs close to the cell surface by some means that prevents any access to intracellular ATPases. Such a mechanism might be the extrusion of whole granules (1, 6) or extrusion of granule contents by reverse micropinocytosis (9), or perhaps a transient fusion of granule and cell membranes allowing ATP and catecholamines unimpeded access to the cell exterior. A most interesting and relevant observation was made some years ago by Carlsson and Hillarp (4) when they found the protein content of the granule fraction isolated from adrenals that had been stimulated for some hours, was not lowered to the same extent as the catecholamine and ATP components of this fraction. One interpretation of this would be that the granules give up catecholamines and ATP while retaining most of the protein (4), but other explanations are possible: for example, that new granule protein had formed during the prolonged stimulation. Measurements of the protein content of the adrenal effluent during catecholamine secretion (15) have yielded values lower than would be expected were whole granules to be extruded. Further extension of these approaches may clarify the picture. But although the details are uncertain, the principal conclusion remains, namely, that "heavy," nucleotide-rich, chromaffin granules provide most—and possibly all—of the catecholamines extruded by the medullary chromaffin cell when it is acutely stimulated by physiological means.

In summary, the evidence available suggests that the main events in "stimulus-secretion coupling" in the adrenal medulla may be as follows: 1) reaction of the synaptic transmitter, acetycholine, with the plasma membrane of the

chromaffin cell, probably the outer surface of that membrane; 2) an increased permeability of the plasma membrane to calcium ions; 3) an inward movement of calcium ions down their electrochemical gradient to some strategic site (possibly no further than the inner surface of the plasma membrane); 4) the initiation of some process that causes the "heavy" chromaffin granules to be released or, more probably, to release their content of catecholamines and ATP (unhydrolyzed) to the cell surface; and, 5) termination of secretion upon the disappearance of acetylcholine by diffusion or hydrolysis and by binding or extrusion of the calcium that has penetrated the cell.

While it seems that the outlines of the cellular mechanism involved in the physiological release of catecholamines are beginning to take shape and, one hopes, what is learned of the chromaffin cell will be applicable to its developmental homologue, the adrenergic neurone (12, 22), it is pleasing to recognize that there remains, besides the necessary task of devising experiments to test the various hypotheses, the exciting challenge of explaining the intimate nature of the several component steps.

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