seminars in **CELL & DEVELOPMENTAL BIOLOGY,** Vol 8, 1997: pp 113–120

The rat adrenal gland in the study of the control of catecholamine secretion

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Catecholamine secretion in the rat can be studied in freely moving and anaesthetized animals, in isolated-perfused adrenals, medullae slices and isolated cultured cells. In addition the rat offers the advantage over the more widely used bovine adrenal model that researchers can have access to animals of the same age, sex and feeding conditions. Catecholamine release is similar to other species although it gives robust secretion in response to stimuli such as muscarinic agonists, bradykinin or VIP. It also allows the study of neurotransmission at the splanchnic–adrenal synapse. The use of single-cell preparations (patch-clamp, microfluorimetry, amperometry or capacitance) has overcome the limitations of the number of cells obtained from a gland. It is possible to study secretion in animal models of hypertension, chronic stress or diabetes and rats can be genetically modified.

Key words: adrenal / chromaffin cell / catecholamines / excitation–secretion coupling / exocytosis

ADRENAL CHROMAFFIN tissues have been utilized for the study of secretory phenomena for over 40 years.[1-3] Perfused adrenal glands have been used to establish the concept of excitation–secretion coupling.[4] The introduction of tissue culture techniques in 1966 made chromaffin cells one of the most widely used models to study secretion.[5-7]

The requirement for a large number of cells, to ensure sufficient tissue to analyse catecholamines released or to carry out biochemical studies, has centered many studies on the use of isolated chromaffin cells of bovine origin. However, the arrival of single-cell techniques has overcome this problem. In addition, some limitations of bovine chromaffin cells have caused an increased interest in the use of other animal species. Bovine adrenals are currently obtained from local abattoirs. Researchers frequently ignore important facts like sex, age, feeding or hormone treatment received by the animals.[5,6] Recent diseases like bovine spongiform encephalophathy may limit the availability of bovine tissues.

Rodents have been the usual species for the development of an increasing number of experimental disease models for hypertension, diabetes or obesity. There is a considerable interest in the study of secretory responses of adrenal medulla from these animal models as well as from animals under chronic stress.[8-13] To study several phenomena like neurotransmission at the splanchnic–adrenal junction, the use of intact gland preparations offers a good alternative to cultured cells.[14,15] In spite of their popularity, cultured cells do not fully resemble the physiological properties of intact adrenal medullary tissues since they are denervated and cell to cell communication is lacking. Also, they are altered by enzyme digestion and, once in culture, they are away from the cyclic influence of cortical hormones. Although many neuropeptides are present within the adrenal gland, I have centered this review on catecholamines. I will discuss in this article some of the advantages and limitations of rat adrenomedullary tissues compared with other species.

Technical notes

Although methodological descriptions are not the major aim of the present review, I describe briefly some of the preparations used in the study of secretion from rat adrenal chromaffin cells.

In situ adrenals

Blood samples collected from aorta or from the adrenal vein have been used to estimate catecholamine secretion in response to several stimuli like insulin administration, controlled hypotension, or caused by pain or by stimulation of discrete CNS areas.[16-18] This technique can be used to study the release caused by direct stimulation of splanchnic nerves.[19]

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1084-9521/97/020113 + 08 $25.000/0/sr960130

Perfused glands

Adrenals removed from the animal can be perfused retrogradely through the adrenal vein. Buffer solution can be applied at high perfusion rate. Perfusate collected can then either be analysed by fluorimetric techniques or by HPLC although the more recent method of on-line electrochemical analysis gives excellent time-course records.[15, 19-22] On-line recording of catecholamine secretion can be assessed by passing the fluid emanating from the gland to an HPLC electrochemical detector. Oxidation currents are proportional to the concentration of catecholamines passing through.[20-23]

Slices

Iijima *et al* used 200–300 μm-thick adrenal medullary slices to examine the time course of intercellular electrical coupling.[24] This simple preparation can be useful to combine electrophysiology with intracellular calcium measurement and secretion studies.

Isolated and cultured cells

Cells from rat adrenal medulla can be successfully prepared by enzyme digestion. Although this procedure does not produce a high yield of cells, it can, however, provide enough cells to carry out single-cell experiments.[25-32]

Catecholamine synthesis

Rat adrenal glands constitute an excellent model to study catecholamine synthesis. They have two major advantages over bovine systems: (i) animals can be pre-treated with catecholamine-depleting drugs like reserpine or enzyme inhibitors; and (ii) glands are able to secrete for hours with stimuli like high potassium or muscarine.[33] Moreover, rat chromaffin cells do not take up catecholamines,[34] and they therefore require new synthesis to restore the initial catecholamine content. Stimulation of secretion causes the concomitant phosphorylation of tyrosine hydroxylase. New synthesis can be dramatically enhanced by addition of tyrosine or dopamine to the perfusion buffer.[33]

Physiological stimuli in the adrenal medulla

Chromaffin cells receive their physiological stimulation both from splanchnic nerves and blood-carried chemical secretagogues. Splanchnic branches release acetylcholine and peptides as a response to stress situations. Blood-carried stimuli include histamine, angiotensin II and bradykinin which are elevated in plasma as a part of the homeostatic responses to allergens or hypotension.[10,12,13,15,22]

Rat adrenals contain about 1 mg/g tissue of catecholamines with 90% of the total composed of adrenaline.[33] Some stimuli like histamine,[22,35] muscarinic agonists,[20] diencephalic stimulation [17] or hypoglycaemia[11,12] can selectively increase the release of adrenaline. Cold exposure[12] and some diencephalic stimulations[17] induce selective release of noradrenaline.

Excitation–secretion coupling

The rat adrenal gland is a valuable preparation for the study of the regulation of exocytosis. Catecholamine and peptide secretion can be triggered either by exogenous or by endogenous acetylcholine. Acetylcholine elicits secretion by activating nicotinic and/or muscarinic receptors.[36] The effect of acetylcholine can be attenuated in the perfused glands by tetrodotoxin (TTX)[14,37] but not in isolated chromaffin cells (R. Borges, unpublished results). In addition to this, TTX does not affect the secretion caused by high K^+ solutions. Acetylcholine induces Na^+-dependent membrane potential fluctuations which can be roughly reproduced by nicotine but not by muscarine. These observations suggest a role of nicotinic and sodium channels in the propagation of a depolarizing stimulus[38] and in the tissue synchronization of catecholamine secretion in response to acetylcholine.[37,39]

The role of both cholinergic receptors in the control of secretion has been extensively studied in rat chromaffin cells.[20,26,27,28,36,40] The time-course of the secretory responses varies depending on the duration of the stimulus applied. Brief pulses (5–15 s) of cholinergic agonists, applied intermittently at 5–10 min intervals, usually give very reproducible responses (Figure 1), whereas continuous application of secretagogues promotes the desensitization of the catecholamine output. Non-selective agonists like acetylcholine or carbachol produce a biphasic secretory response with a rapid elevation followed by a steady

state; this second phase can be suppressed by the muscarinic receptor antagonist atropine. Nicotinic agonists such as nicotine or dimethylphenylpiperazinium (DMPP) exhibit only the first component. These results show that muscarinic agonist-evoked release is less affected by desensitization than the nicotinic response.[20]

Recent observations using amperometric techniques with carbon microelectrodes on cultured cells have revealed important differences in the onset of secretory responses to cholinergic agonists. Nicotinic agonists cause a massive catecholamine release observed only within the first seconds after a brief drug application. In contrast, muscarinic drugs show a delay of the onset of secretion but secretory events last for over a minute.[28,29]

The nicotinic receptor of the rat chromaffin cell belongs to the neural nicotinic type.[41] Secretion elicited by nicotinic agonists occurs in a way similar to that described for bovine chromaffin cells. Nicotinic receptor activation results in depolarization and opening of voltage-dependent channels.[26] Nicotinic- but not muscarinic-evoked responses can be blocked by 1,4-dihydropyridine derivates (nifedipine-related drugs), a class of L-type calcium channel blockers[20] and by inorganic cations like Co^{2+} or Cd^{2+} (ref 39, R. Borges, unpublished observations).

Electrical stimulation

Wakade showed in 1981 that the secretion of catecholamines induced by transmural electrical stimulation of perfused adrenals can be almost abolished by a combination of muscarinic and nicotinic blockers.[14] This observation, widely supported by others in rats, cats[42] and guinea pigs, demonstrated that electrical field stimulation causes splanchnic acetylcholine release which causes catecholamine secretion through cholinoceptor activation. This observation opened a new methodological approach to study neurotransmission at the splanchnic–adrenal junction.

Wakade's group also demonstrated the presence of a non-cholinergic component in the secretory products of splanchnic nerves which was partially sensitive to high concentrations of naloxone.[15,43] The same authors identified this substance as vasoactive intestinal peptide (VIP). VIP can be responsible for the catecholamine release in response to low frequency splanchnic nerve activity.[44,45]

In spite of the large catecholamine content of the adrenal tissues, the functional presence of sympathetic nerve terminals in the adrenal can be studied separately. Sympathetic nerves but not chromaffin cells can take up [^{3}H] noradrenaline when perfused through the adrenal gland. Tyramine and ephedrine release tritium but not catecholamines whereas acetylcholine, nicotine and muscarine release catecholamines but not tritium; on the other hand, electrical stimulation promotes the release of both tritium and catecholamines.[46] This elegant preparation can be used to study the mechanisms undergoing the release of catecholamines simultaneously from the sympathetic nerve terminals and the chromaffin cells of the same adrenal gland.

Some experimental data support the existence of substance P in the splanchnic nerves. Perfusion of adrenal glands with submicromolar concentrations of substance P increased the acetylcholine released from splanchnic nerves and prevented the inactivation of nicotinic receptors.[47] The effect of substance P seems to be important only under high frequency of nerve stimulation (10 Hz).[47] These findings opened an exciting field of research on the role of substance P in situations of maintained stress.

Muscarinic receptors

Perhaps one of the most interesting advantages of rat medullary tissues is the study of muscarinic receptor transduction mechanisms. It largely differs from bovine chromaffin cells where usually muscarinic receptors do not trigger secretion.[48,49] The fact that removal of external calcium causes a significant reduction of muscarinic agonist-induced secretion together with the lack of effect of nifedipine, indicates that Ca^{2+} is entering into the cell through a different pathway. In the cat adrenal a calcium ionophore coupled to an M_2 muscarinoceptor has been proposed.[50]

Finnegan *et al*[29] studied the time course of secretion and intracellular free calcium in single bovine and rat adrenal cells in response to muscarinic stimulation. Methacholine, a pure muscarinic agonist, evokes a rise in the intracellular Ca^{2+} in all of the cells studied, whereas only the cells with elevated basal calcium levels respond to the drug. Methacholine can increase the intracellular free calcium even when the external cation has been removed although only a limited number of secretory spikes can be observed.[29] Recently Guo *et al*[49] have performed elegant experiments that confirm data from perfused adrenals

about the role of intracellular calcium stores in muscarinic agonist-mediated secretion.

Patch-clamp studies have been used to establish the relation of muscarinic receptors to other ionic channels. Muscarine induces a dose-dependent elevation of resting potential, due to the inhibition of a Ca^{2+}-dependent K^+ current.[27,51,52]

Other receptors

Histamine H_1 receptor activation promotes a rise in intracellular free calcium and secretion in rat chromaffin cells.[21,22,29,35,53,54] Although histamine has been detected within adrenal tissues, most of it is located in the cortex.[55] Blood concentrations of histamine during anaphylactic shock are in the range of the concentration that triggers catecholamine secretion from the adrenal medulla.[22] In the absence of adrenals the hypotensive responses to intravenous administration of histamine are enhanced. Histamine preferentially releases adrenaline.[22,35] Adrenaline can be considered as the physiological antagonist of histamine; it can reverse most of the effects of histamine including vasodilatation, bronchoconstriction and oedema.

Bradykinin promotes catecholamine release through B_2 receptor activation. This peptide is a potent secretagogue; concentrations as low as 1 nM cause an increase in the catecholamine output[56] as well as increases in intracellular free calcium concentration even in the absence of external calcium. As with histamine,[54] a combination of external free calcium solution and an intracellular Ca^{2+} antagonist (TMB-8) is necessary to suppress secretion and calcium signals caused by bradykinin.[57]

Angiotensin II-triggered secretion appeared to be mediated by AT_2 receptor subtype.[58] Contrary to the long-lasting effects on inositol metabolism observed in bovine chromaffin cells,[59] secretory responses elicited by angiotensin II exhibit a rapid desensitization in the rat adrenal (Figure 1).

Receptor transduction mechanisms

Due to the difficulties in carrying out biochemical studies in the adrenal medullary tissues of the rat, most of our knowledge about receptor transduction mechanisms comes from bovine chromaffin cells. Considerable biochemical information has been obtained from the pheochromocytoma cell line PC12, but although PC12 are originated from rat medullary tissues, data obtained with PC12 cells needs to be confirmed in the non-tumoral chromaffin cells.

Malhotra *et al*[60] carried out an extensive study identifying the second messengers associated with different secretory stimuli. Splanchnic nerve stimulation resulted in increases in calcium uptake, in intracellular levels of cAMP and IP_3 and in PKC activity. Muscarinic receptors enhanced PKC activity and IP_3 content whereas nicotine only affected Ca^{2+} uptake. Vasoactive intestinal peptide stimulated cAMP, IP_3 and PKC but the splanchnic-mediated stimulus did not modify the intracellular levels of cGMP.

Muscarinic-, bradykinin-, histamine- and angiotensin II-receptors have been included in the group of G-protein associated receptors coupled to IP_3. However, their time course of secretory responses are different (Figure 1) and the participation of PKC in their transduction mechanism is not entirely similar. Direct activation of PKC by phorbol esters potentiated the K^+-elicited release, reduced the secretion induced by histamine, methacholine and angiotensin II, but did not affect the secretory responses induced by bradykinin.[53,56] Taken together these data indicate that PKC appears to be involved in the regulation of, at least, two different sites in the stimulus–secretion coupling process: (i) as a negative regulator of the receptor function of muscarine, angiotensin II and histamine (but not bradykinin), and (ii) facilitating the secretion responses by directly acting on the secretory machinery.[56]

Treatment of adrenal glands with forskolin, an adenylate cyclase activator, produces an increase in the secretion of catecholamines evoked by any agent, suggesting a direct control of PKA at steps beyond external Ca^{2+} entry.[56,60]

Intracellular calcium

Secretory experiments carried out in the absence of external calcium have concluded that rat chromaffin cells possess larger intracellular calcium stores than bovine chromaffin cells. The presence of spontaneous Ca^{2+} oscillations in resting rat adrenal cells has been described in recent years. This is a phenomenon observed in about 70% of the cells.[25,61] The real nature of this phenomenon remains obscure: Malgaroli *et al*[25] proposed an IP_3-independent, caffeine- and ryanodine-sensitive intracellular reservoir, as the source of calcium whereas external Ca^{2+} has been

proposed by others.[27,61] In other work, Finnegan *et al*[29] observed Ca^{2+} oscillations accompanied with secretion only when the cells were placed within the vicinity of a leaking pipette containing bradykinin. Calcium oscillations can be increased by external application of moderate depolarizing solutions (KCl 15 mM) or agonists like muscarine or bradykinin.[27,61]

Rat chromaffin cells are more sensitive to caffeine than bovine cells. Low concentrations of this drug (0.3–3 mM) cause a rise in intracellular calcium and secretion even in the absence of external Ca^{2+}. Moreover, caffeine concentrations necessary to cause similar responses in bovine cells (10–40 mM) caused cell membrane disruption.[29] Intracellular calcium stores mobilized with caffeine seem to be different compared to those activated by IP_3 since ryanodine fails to prevent Ca^{2*} release and secretion evoked by muscarine.[49]

Secretion can be elicited also by lowering extracellular pH. Although the nature of this phenomenon is far from being clear, this secretory response is dependent on external calcium. Calcium-induced internal calcium release could be involved since the initial rapid component can be selectively eliminated with the intracellular calcium antagonist TMB-8.[62]

Calcium channels and secretion

An extensive review of the electrophysiology of chromaffin cells has been recently carried out by Artalejo.[63] We only summarize here some of the aspects related to the participation of calcium channel subtypes in the secretory process.

The resting membrane potential of rat chromaffin cells has been estimated to be in the range of –40 to –75 mV.[51,64] Gandía *et al* found that about 10% of the total calcium current is active at -40 mV.[30,32] This could explain why some cells exhibit spontaneous calcium oscillations. Calcium oscillations could be related to the spontaneous action potentials observed in rat and mouse chromaffin cells, these action potentials not being Na^+-dependent since they are not abolished upon sodium removal or TTX treatment.[65] Using selective ω-toxins and furnidipine at least four components/subtypes of calcium channels in rat chromaffin cells have been identified.[32] Figure 2 shows a comparison of the distribution of the different Ca^{2+} channel subtypes in bovine, cat and rat chromaffin cells. This could have relevance for the control of secretion by different calcium channels in these three animal species.

Due to the high muscarinic component, splanchnic- and acetylcholine-mediated secretion is only weakly affected by nifedipine or inorganic cations.[20,39] The contribution of L- and N-type calcium channels to secretion evoked by membrane depolarization has been studied by Kim *et al*.[31] These authors found that L- and N-type channels account for 60% and 40% respectively of the secretion elicited by membrane depolarization, also the secretion can be completely abolished by a combination of L-type calcium channel

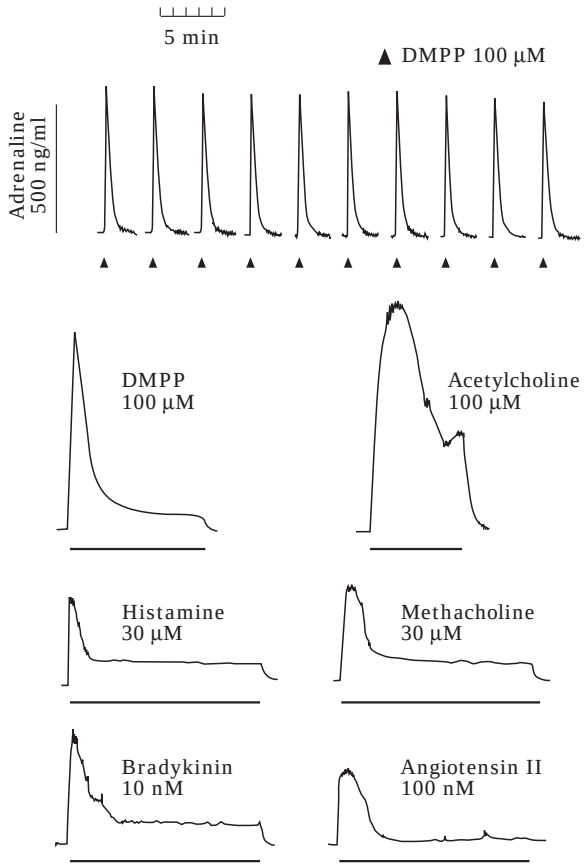

Figure 1. On-line measurement of catecholamine release from isolated-perfused rat adrenal glands. Catecholamine secretion was continuously measured by electrochemical detection. Upper traces were obtained by short-pulse application of the nicotinic agonist DMPP, for 10 s every 8 min (filled triangles). Lower traces are representative experiments, from different glands, obtained by continuous application of drugs, at the indicated concentrations. Solid horizontal lines indicate the period of drug application. The vertical bar indicates the oxidation current corresponding to that elicited by 500 ng/ml of adrenaline. Time (5 min) is indicated by the upper horizontal line.

blocker (nicardipine) and an N-type blocker (ω-conotoxin-GVIA). In our hands, ω-conotoxin -GVIA does not inhibit K^+-evoked catecholamine release in perfused adrenal glands although it reduced splanchnic- and acetylcholine-mediated responses. We have also found that ω-agatoxin-IVA (a P-type calcium channel blocker) and ω-conotoxin-MVIIC (a Q-type calcium channel blocker) are more potent blockers of secretion than ω-conotoxin-GVIA (P. Michelena *et al*, manuscript in preparation). The physiological role of the different subtypes of calcium channels is far to be established although it is possible that these channels could be used in other cellular functions like calcium-dependent phosphorylation or vesicle traffic.[32]

Final steps of exocytosis

The use of amperometry with microelectrodes has revealed that the catecholamine content of chromaffin granules of the rat is very similar to bovine.[29] The distribution of granule content of catecholamines followed a distribution which resembles granule sizes described by electron microscopy observations.[66] From the analysis of the onset time of different secretagogues to trigger secretion, amperometry will contribute to the clarification of the proteins implicated in the transduction pathway and in the secretory machinery. As was mentioned before, a preliminary study was published recently by Wakade's group comparing the time-course distribution of secretory spikes elicited by cholinomimetic drugs and peptides.[28] In the near future more information about the role of proteins directly implicated in the docking and fusion of chromaffin granules (see Morgan and Burgoyne, this issue) will emerge from amperometric recordings of single granule exocytosis in cultured rat chromaffin cells.

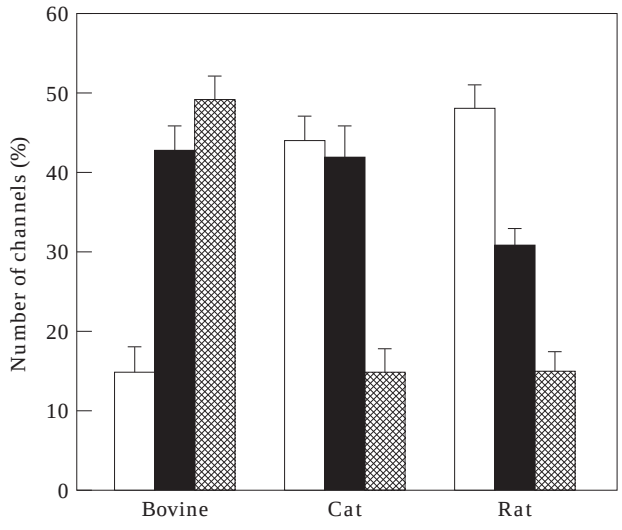

Figure 2. Distribution of calcium channel subtypes in three different species. Average data of blockade of calcium currents upon treatment of cells with furnidipine (□) (L-type), ω-conotoxins GVIA (■) and IVA (▩) (N- and P-type respectively). Data from Gandía *et al.* [32]

Conclusions

Chromaffin cells of the rat offer an excellent model to study all aspects of the secretory phenomena of the adrenal medulla. Studies have been done analysing the role of the CNS in the control of catecholamine release. Rat adrenal glands have been used to understand how splanchnic nerves promote the secretion and how cell membrane receptors transduce the signal to trigger or modulate the secretory response. Single-cell studies have shown the importance of the intracellular calcium stores and the contribution of membrane ionic channels and receptors to promote exocytosis. Rat chromaffin cells are not affected by feeding, hormone treatment, variations in the killing procedures, uncontrolled age, race and sex as frequently occur with bovine cells. When a small number of cells is not the limitation, cultured rat chromaffin cells are cheap and easy to prepare and provide an excellent model for investigation of catecholamine release by exocytosis.

Acknowledgements

I would like to thank Dr Antonio G. García and Dr Luis Gandía (Universidad Autónoma de Madrid) for their helpful comments in the preparation of this manuscript. This work was supported by a grant from Spanish Ministerio de Educación y Ciencia DGICYT PB95-0540.

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