Biochemical Pharmacology, Vol. 34, No. 9, pp. 1497–1500, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

DECLINING CATECHOLAMINE SECRETION IN ADRENAL MEDULLA ON PROLONGED STIMULATION WITH ACETYLCHOLINE

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(*Received* 10 *May* 1984; *accepted* 13 *November* 1984)

Abstract—Perfused pig adrenal glands and cortex-free ox adrenal medullae were stimulated by continuous infusion of 10^{-4} M acetylcholine (ACh). Secretion of adrenaline rose to a maximum in approximately 5 min but, after a further 15 min, declined to 36 ± 19% (± S.D.) of maximum for pig (N = 5) and 27 ± 10% of maximum for ox (N = 3), in spite of continued infusion of ACh. After 20 min, no further significant decline was detectable. Nevertheless, in ox medullae, oxygen consumption measured after stimulation showed no significant change relative to the pre-stimulation value, indicating that the decline in secretion did not arise from a failure of oxidative energy metabolism. In 4 pig adrenal glands subjected to a 1 hr infusion of ACh, adrenaline secreted in the last 20 min was only 52 ± 10% of that secreted in the first 20 min but, after a 2 hr rest interval, recovered to 74 ± 18% ($P < 0.05$) in the first 20 min of a second 1 hr stimulation. In the same glands, no reproducible recovery was detectable for noradrenaline and, by the last 20 min of the second 1 hr stimulation, noradrenaline secretion had declined to 36 ± 20% of the initial value, even though only 15% of the noradrenaline originally in the gland had been secreted. It is concluded that, while decline and recovery of adrenaline secretion may, in part, have arisen from desensitization and resensitization of the ACh receptor; the decline in noradrenaline secretion arose mainly from depletion of a readily secreted pool which was considerably smaller than the total in the gland.

Stimulation of adrenal medulla *in vitro* with a cholinergic agonist causes a rapid increase in the rate of catecholamine secretion which subsequently declines in spite of the continued presence of the agonist [1, 2]. Recent work with ox adrenal chromaffin cells in monolayer culture [3] showed that a component of this decline required extracellular calcium, and hence may have been caused by depletion of cellular stores of catecholamine. However, the remainder of the effect was calcium-independent and may have arisen from desensitization of the cholinergic receptor [4].

Depletion of cellular metabolites other than the secretion products may also be important, however, as experiments of this type are often performed in simple media containing only salts and glucose which may be inadequate for maintenance of secretion [5]. For example, glucose alone may not be the appropriate energy source [6] to maintain the cytoplasmic production of adenosine 5′-triphosphate which is required for exocytosis [7].

Therefore, in this paper, two aspects of the possible link between metabolite depletion and declining catecholamine secretion have been investigated. Firstly, in perfused cortex-free ox adrenal medulla [8], oxygen consumption and catecholamine secretion have been measured in parallel, to test the possibility that oxidative energy metabolism (and hence tissue viability) declines following prolonged stimulation. Secondly, in perfused pig adrenal glands, catecholamine release during stimulation, and catecholamine remaining in the tissue after stimulation, have been measured in order to relate the amount secreted to depletion of the total cellular pool. Unlike earlier studies [9], all stimulations were performed with the physiological cholinergic agonist acetylcholine.†

MATERIALS AND METHODS

For oxygen consumption measurements, it was necessary to use the cortex-free ox adrenal medulla preparation [8] in order to eliminate the contribution from adrenal cortex. In this preparation, efficiency of perfusion was assessed by perfusing with medium containing 0.005% w/v Evans Blue (Gurr) and the resultant staining was found to be patchy. Nevertheless, the perfused portion of the tissue probably received adequate oxygen and glucose, as oxygen consumption was approximately constant for flow rates above 8 ml/min, and net glycogen synthesis was shown to occur during recovery from ischaemia. However, the heterogeneous perfusion precluded use of this preparation for studies on catecholamine remaining in the tissue after stimulation. For these experiments, whole pig adrenal glands were used, as these showed more even perfusion of the medulla [10] and could be perfused without any need to cut the adrenal cortex. All perfusions described in this paper were retrograde perfusions cannulated via the adrenal vein.

Ox adrenal glands were obtained from the local

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† Abbreviations: ACh, acetylcholine; Adr, adrenaline; NAdr, noradrenaline; Q_{O_2}, rate of oxygen (O_2) consumption.

slaughterhouse and a series of lateral slits, spaced 2–3 mm apart, perpendicular to the axis of the adrenal vein, were made through the adrenal cortex. Each gland was then flushed with 60 ml of cold perfusion medium (about 4°) in approximately 3 min. In the laboratory, the strips of cortex were excised while the medulla (4–6 g) perfused at 37° under a pressure of 40 mm Hg at 12–13 ml/min. The oxygen consumption and spontaneous release of catecholamine were then allowed to stabilise for at least another 60 min before commencing experiments.

Pig adrenal glands were handled in a similar way [10] but no attempt was made to remove the adrenal cortex.

The perfusion medium was a modified Krebs–Henseleit of the following composition: NaCl 118 mM, $NaHCO_3$ 25 mM, $CaCl_2$ 2.0 mM, $MgSO_4$ 1.2 mM, KCl 6.0 mM, D-glucose 10 mM, and was equilibrated with 95% O_2/5% CO_2 throughout the perfusion.

Fluorometric catecholamine assays, and extractions of catecholamine from whole pig adrenals, were performed by the method of Anton and Sayre [11] with the modifications previously described [10]. Differential analysis of adrenaline and noradrenaline was achieved by measuring the intensity of trihydroxyindole fluorescence at two sets of excitation and emission wavelengths [12].

Oxygen consumption by ox adrenal medulla was calculated from the decline in dissolved oxygen concentration in the perfusion medium, measured before and after it had passed through the tissue. Dissolved oxygen was measured polarographically in the perfusion line [13]. Glycogen was extracted and measured as described in [14].

The amount of catecholamine present in each pig adrenal gland at a specified time in the perfusion was estimated by measuring the amount present in the tissue at the end of the perfusion, and adding to this the total released into the perfusion medium between the specified time and the end of the perfusion. This included spontaneous (non-stimulated) catecholamine release. Net biosynthesis of catecholamine was assumed to be negligible, as no synthetic precursors were present in the perfusion medium [9].

All catecholamine secretion results reported here refer to net secretion stimulated by infusion of acetylcholine chloride at a final concentration of 10^{-4} M. Spontaneous catecholamine release was measured before and after each bout of stimulated secretion, and a baseline was interpolated and subtracted from the stimulated values.

All other perfusion details and reagents were as described previously [10].

Numerical results have been quoted as mean ± 1 S.D. Statistical significance of changes was evaluated by paired Student's t analysis.

RESULTS

Normalized time courses of adrenaline secretion during prolonged stimulation with acetylcholine are plotted in Fig. 1. In both ox and pig, secretion rate rose rapidly to its maximum value after 5 min, but after a further 15 min, this had declined to 27 ± 10% of maximum for ox ($P < 0.01$, $N = 3$) and 36 ± 19% for pig ($P < 0.002$, $N = 5$). No further significant decline in secretion was detected after 20 min, in agreement with earlier work on perfused cat adrenals stimulated with histamine [9]; indeed, in some preparations, transient increases were observed.

Ox adrenal medullae were also stimulated intermittently with 4 min pulses of 10^{-4} M acetylcholine at 30 min intervals. Adrenaline secreted in the fourth stimulation was only 48 ± 21% of the amount secreted during the first ($P < 0.05$, $N = 3$). The corresponding values for noradrenaline were 42 ± 12% ($P < 0.02$, $N = 3$) in the same medullae.

Oxygen consumption results from experiments of this type are presented in Fig. 2. Within 15 min of the end of each stimulation, oxygen consumption stabilized at a value which was close to the pre-stimulation value. A transient increase in oxygen consumption was observed during the acetylcholine infusions, and has been described in detail elsewhere [1, 15].

In Fig. 3a, the percentage decline in the amount of adrenaline secreted over 20 min intervals has been compared with the corresponding percentage decline in adrenaline remaining in the tissue. Each gland was subjected to 1 hr of stimulation, followed by a 2 hr

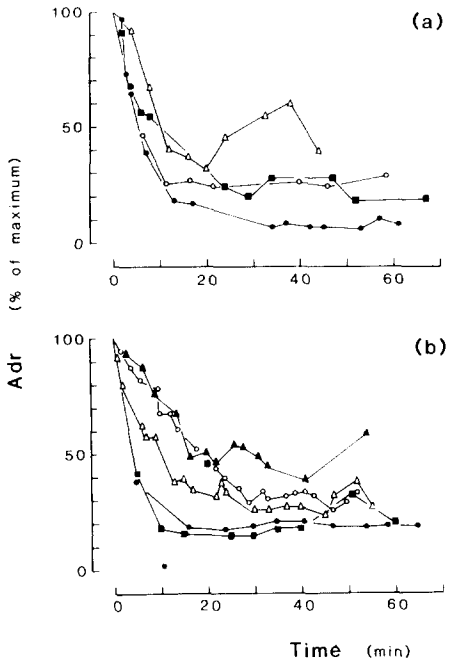

Fig. 1. Normalized time courses of the decline in adrenaline secretion during continuous infusion of ACh at a final concentration of 10^{-4} M, (a) in cortex-free ox adrenal medullae and (b) in whole pig adrenal glands. Adrenaline concentration (nmoles/ml) in samples of perfusion effluent was multiplied by the corresponding perfusion rate (in ml/min) to yield a secretion rate (nmoles/min). Spontaneous adrenaline secretion rate was subtracted (see Methods), and the resulting net secretion rates were normalized by taking the highest observed value in each perfusion to be 100%. The mean 100% value was 370 nmoles/min/medulla for ox and 40 nmoles/min/gland for pig. This maximum value has been plotted at zero on the time axis. Stimulation with ACh commenced at approximately minus 5 min. Each different symbol represents a separate perfusion.

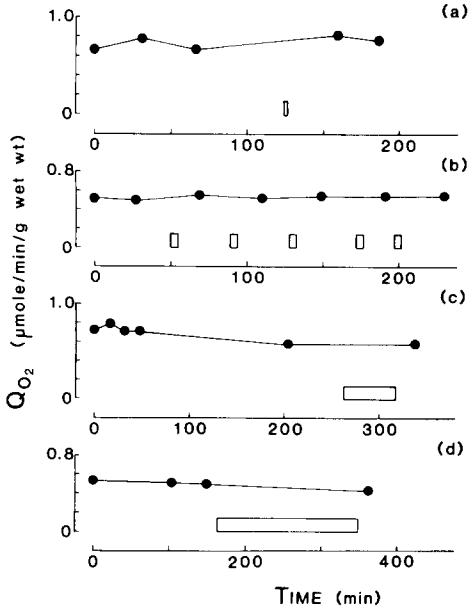

Fig. 2. Long-term stability of oxygen consumption (Q_{O_2}) in four cortex-free ox adrenal medullae. White bars denote infusions of 10^{-4} M ACh of (a) 2 min, (b) 5 × 4 min, (c) 50 min and (d) 180 min duration. In (b) the net amount of adrenaline secreted during the fifth 4 min stimulation was only 52% of that in the first. In (c) and (d), the net stimulated rates of adrenaline secretion, at the end of the two stimulations, were 40% and 2.0% respectively of the maxima observed just after stimulation commenced.

rest and then a further 1 hr of stimulation. During the first 1 hr stimulation, the amount of adrenaline secreted in the last 20 min was only 52 ± 10% of that secreted in the first 20 min ($P < 0.01$, $N = 4$). However, the percentage of the original adrenaline remaining in the glands after 60 min was still 86 ± 3% of the initial value. During the first 20 min of restimulation after the rest interval (Fig. 3a), adrenaline secretion recovered to 74 ± 18% of the initial value ($P < 0.05$ relative to the value in the last 20 min of the first stimulation). This recovery occurred even though the total adrenaline remaining in the gland was approximately 10% lower than at the end of the first stimulation, owing to spontaneous release of adrenaline during the rest interval.

For noradrenaline in the same 4 glands (Fig. 3b), no significant recovery of secretion rate was observed at the start of the second stimulation. Hence the amount of noradrenaline secreted in the second stimulation was only 57 ± 18% of that secreted in the first ($P < 0.02$, $N = 4$).

DISCUSSION

In perfused adrenal glands, two lines of evidence suggest that the decline in secretion rate, on prolonged stimulation, is not exclusively a receptor desensitization phenomenon analogous to that described at neuromuscular junction [4]. Firstly, we have observed a significant decline in secretion on intermittent stimulation, in agreement with earlier reports [1, 2]. Perfusion without ACh, between the stimulations, should allow time for removal of ACh from the receptors and hence resensitization of the tissue as, in cultured ox chromaffin cells, resensitization occurs within a matter of minutes [3]. Secondly, the agonists veratridine [15, 16], barium [17] and intermittent potassium depolarization [18] also elicit a declining response, even though these agents by-pass the acetylcholine receptor.

Measurement of oxygen consumption in cortex-free ox adrenal medulla (Fig. 2) showed no evidence for declining tissue viability or a failure of oxidative energy metabolism after prolonged stimulation. Indeed, it has been shown previously that this preparation can sustain rates of respiration which are even higher than the basal values reported here [1, 15]. It remains to be shown, however, that secretion rate is not limited by depletion of some other metabolite which was not detected by these oxygen consumption measurements.

If the declining secretion rate in Fig. 3a arises from depletion of cellular adrenaline stores, the secretion rate and the amount of adrenaline remaining in the tissue might be expected to show a comparable percentage decline. However, in Fig. 3a, during the first 1 hr stimulation, adrenaline secretion rate halved, while the total adrenaline in the tissue only declined to 90% of its initial value. This disparity could simply arise from receptor desensitization [4]. This is consistent with the increase in secretion rate at the start of the second stimulation, which may have arisen from resensitization during the 2 hr rest interval.

For noradrenaline however, a role for receptor

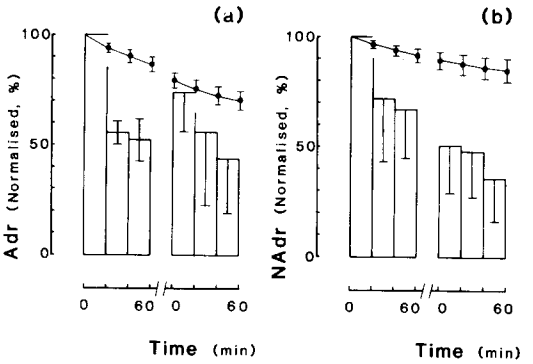

Fig. 3. Normalized comparison of stimulated catecholamine secretion (histogram) with catecholamine remaining in the tissue (continuous line) during infusion of 10^{-4} M ACh in four pig adrenal glands (a) for adrenaline and (b) for noradrenaline. Each gland was subjected to 1 hr of stimulation, followed by 2 hr of perfusion without ACh and then a further 1 hr of stimulation. The amount of stimulated secretion in each 20 min interval was normalized by taking the value for the first 20 min to be 100%. The mean 100% value was 330 nmoles/gland for adrenaline and 480 nmoles/gland for noradrenaline. The corresponding amounts of catecholamine remaining in the same four glands, at specified times, were estimated as described in Methods, and were normalized by taking the values at the start of the first stimulation to be 100%. The mean 100% value was 7300 nmoles/gland for adrenaline and 13400 nmoles/gland for noradrenaline. Note that spontaneous (non-stimulated) catecholamine secretion has also contributed to the decline in the amount of catecholamine remaining in the tissue.

desensitization was not apparent. In spite of the 2 hr rest interval, no reproducible resensitization was detected at the start of the second stimulation (in marked contrast with the rapid resensitization observed in cultured ox chromaffin cells [3]). Hence the total secreted in the second stimulation was only 57% of that secreted in the first. Nevertheless, the percentage decline in noradrenaline secretion was still more rapid than the percentage decline in total tissue noradrenaline (Fig. 3b). This disparity is consistent with the idea that a large fraction of the noradrenaline is in a form which cannot be secreted immediately on stimulation. Perfusion of pig adrenal glands with dyes (see Methods) suggested that this could not be explained by heterogeneous perfusion, although microscopic heterogeneity could not be ruled out by this method. A more likely explanation is that, on prolonged stimulation *in vitro*, the rate of exocytosis may be determined not simply by the size of the total cellular pool of catecholamine, but by the rate of transport of chromaffin granules from the cytoplasm to the plasma membrane [9]. A transport process of this kind, replenishing a pool of readily secreted granules at the plasma membrane, may also have contributed to the partial recovery of adrenaline secretion at the start of the second stimulation (Fig. 3a); and has been postulated to explain a similar recovery in cat adrenals stimulated with histamine [9].

The mechanism of transport of chromaffin granules from the cytoplasm to the plasma membrane is not fully understood, but probably involves the cytoskeleton [19]. Hence drugs such as the cytochalasins and colchicine, which disrupt the cytoskeleton, may be of use in distinguishing the contributions of receptor resensitization and chromaffin granule transport to the recovery of secretion observed after resting the tissue.

Hitherto, experiments with these drugs have used high doses during short cholinergic stimulations [20, 21]. The short stimulations may have tapped only the readily secreted pool of catecholamine, and the high doses of cytoskeleton disrupting agent may have shown poor selectivity for the cytoskeleton [20, 22]. Therefore, in future, it may be of considerable interest to use lower doses of the drugs during prolonged cholinergic stimulations, in which there is a greater chance of cytoskeletal transport of granules being a rate-limiting factor in secretion.

Acknowledgements—This work was supported by the Science Research Council. A. B. thanks the Medical Research Council and Exeter College, Oxford for financial support.

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