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Catecholamine release from fractionated chromaffin cells

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Abstract

Bovine chromaffin cells were separated by density gradient centrifugation into subfractions. After centrifugation on a self-generating Percoll gradient (42.75% isotonic Percoll, 30000 × *g* for 22 min at 20°C), the chromaffin cells were found in two clearly distinguishable peaks. The peak with the lower density contained most of the noradrenaline-producing cells (~ 80%), whereas the adrenaline-producing cells were equally distributed between the two peaks. After collection of suitable fractions from the gradient, cell cultures were obtained, which were enriched with either > 90% adrenaline- or ~ 65% noradrenaline-producing cells. When stimulated by nicotine or carbachol, the dose–response curves of both cell fractions yielded similar EC_{50}s for the release of adrenaline and noradrenaline. On the other hand, the cells of the less dense fraction released 30% more catecholamines (adrenaline as well as noradrenaline) than the cells of the more dense fraction. It is suggested that there are subpopulations among the adrenaline- and noradrenaline-producing cells with differences in receptor–effector coupling.

Keywords: Chromaffin cell; Cell separation; Noradrenaline release; Adrenaline release; Nicotinic acetylcholine receptor

1. Introduction

Morphological studies indicate that the catecholamines adrenaline and noradrenaline are stored in distinct cells of the adrenal medulla (Hillarp and Hökfelt, 1953; Coupland et al., 1964). Under physiological and pathophysiological conditions, the two catecholamines are released independently, resulting in different ratios of noradrenaline and adrenaline.

Adrenal cells which produce adrenaline can be separated at least partly from those producing noradrenaline (Moro et al., 1990; Moro et al., 1991). The method for separation of the two cell types is based on the fact that adrenaline-containing and noradrenaline-containing chromaffin cells differ slightly in their mean density: ~ 1.056 and 1.065 g/ml, respectively. Although the difference in their densities is relatively small, it can be used to enrich the two types of chromaffin cells by density gradient centrifugation.

We modified Moro's method and separated bovine chromaffin cells into fractions which contained and released preferentially one of the two catecholamines. After separation, the cells were held in primary monolayer culture for 2–4 days and were investigated regarding their response to nicotine and other catecholamine-releasing agonists. Our investigations aimed in comparing the dose–response curves obtained from the two cell populations producing preferentially one of the catecholamines adrenaline or noradrenaline, in order to provide more information about the mechanisms which induce a preferential release of either adrenaline or noradrenaline from the adrenal gland.

2. Material and methods

2.1. Preparation of adrenal medullary cells

Bovine adrenal gland were obtained from a local slaughterhouse and were processed for cell culture within 2 h of slaughter. The chromaffin cells were isolated by digestion with collagenase A as described by Livett (Livett et al., 1987).

The principle steps were as follows: the adrenal glands were trimmed of fat and washed with Ca^{2+}- and Mg^{2+}-free buffer containing 154 mM NaCl, 2.6 mM KCl, 0.85 mM KH_2PO_4, 2.15 mM K_2HPO_4, 10 mM glucose and 12.7

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mM HEPES buffer at pH 7.4. The buffer was injected into the adrenal vein to rinse out the remaining blood from the gland and then the glands were digested with a solution consisting of this buffer containing 0.25% collagenase A + 0.01% DNAase I. Five ml of the collagenase solution was injected into the adrenal vein of each gland. This procedure was repeated every 15 min with the glands incubated at 37°C. After 1 h, the digested medullary tissue was separated mechanically from the adrenal cortex and filtered through a 500-μm nylon mesh. The filtered cells were washed 3 × with Ca^{2+}–Mg^{2+}-free buffer to remove the collagenase/DNAase and then filtered through a 60-μm nylon mesh.

2.2. Separation of adrenaline- and noradrenaline-producing cells

~ 4×10^8 cells were resuspended in 84 ml of the Ca^{2+}–Mg^{2+}-free buffer and mixed with 76 ml isotonic Percoll (68.4 ml Percoll + 7.6 ml 10-fold concentrated Ca^{2+}–Mg^{2+}-free buffer). The cell–Percoll suspension with a final pH of 7.4 was then added to four polycarbonate centrifuge tubes each of 40 ml. The tubes were centrifuged at 30000 × *g* (= 16000 rpm in a Sorvall centrifuge model RC 5C Plus with rotor SS-34) for 22 min at 22°C to produce a self-generating Percoll gradient.

These parameters of centrifugation (30000 × *g* for 22 min) differ considerably from those published by Moro et al. (1991), who centrifuged in a same type of centrifuge/rotor at 13000 rpm (= 20000 × *g*) for 20 min. When using the protocol of Moro under our conditions, we did not obtain a sufficient separation of the two types of chromaffin cells.

After centrifugation, the content of each tube was fractionated by aspirating the gradient from the bottom of the tubes using a peristaltic roller pump. In order to monitor the distribution of the two cell types, the Percoll gradient containing the cells was fractionated and of each fraction washed 3 × in buffer to remove the Percoll.

After the last wash, the cells were pelleted by centrifugation (50 × *g* for 10 min), the supernatants were discarded, the cell pellets lysed by addition of 0.8 ml perchloric acid, and centrifuged at 20000 × *g* for 8 min to precipitate proteins. The supernatants were analysed for catecholamines by high-performance liquid chromatography (125 mm × 3 mm i.d. column, packed with Nucleosil 100C18; 3 μm particle size) and electrochemical detection (model Waters 460). The mobile phase consisted of 5% acetonitril, 10 g/l citric acid, 4 g/l KH_2PO_2, and 0.17 g/l octanesulfonic acid; pH = 3.0.

2.3. Cell culture

Cell fractions containing preferentially one of the catecholamines (adrenaline or noradrenaline) were collected from the gradient, washed free of Percoll as described above, and transferred into culture medium consisting of 45% Dulbecco's modified Eagle's medium, 45% nutrient mixture F-12 Ham, and 10% foetal calf serum. Additionally, the medium contained antibiotics (penicillin and streptomycin; 100 μg/ml of each), antimitotics (fluorodeoxyuridine, cytosine arabinoside and uridine; 2.5 μg/ml of each) and glutamine (0.365 g/l). The cell density was adjusted to 1000 cells/μl culture medium and 400 μl were plated into each well of a 24-well plate and incubated at 37°C in an atmosphere consisting of 5% carbon dioxide in air for 3–5 days before use in functional experiments. After that time, > 90% of all cells were viable as tested by staining with trypan blue.

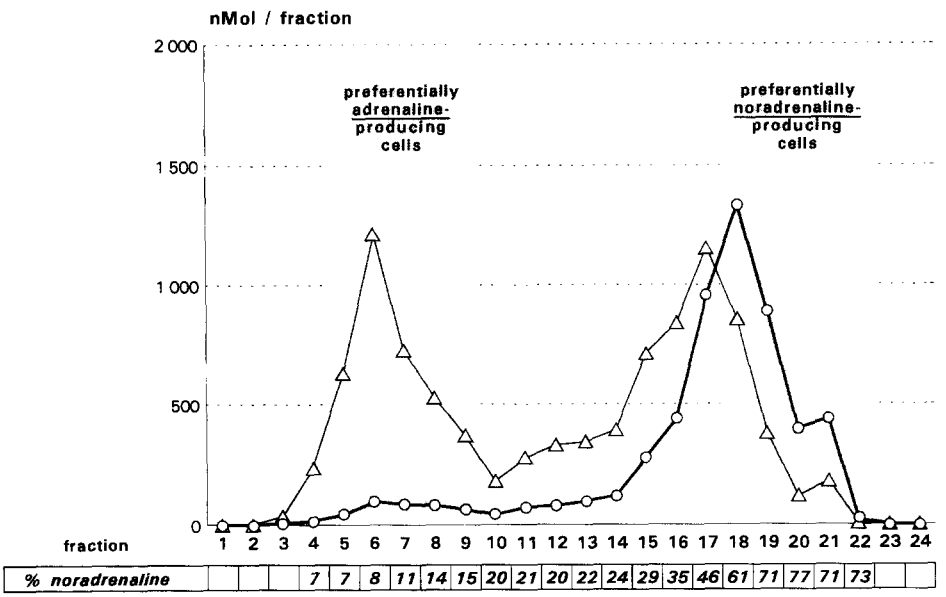

Fig. 1. Density gradient centrifugation of chromaffin cells in 42.75% Percoll at 30000 × *g* for 22 min. Distribution of adrenaline (△) and noradrenaline (○) in 24 fractions (each 1.6 ml) collected from the gradient.

2.4. Functional studies: release of catecholamines

The cells were washed twice with buffer [154 mM NaCl, 2.6 mM KCl, 2.15 mM K_2HPO_4, 0.85 mM KH_2PO_4, 1.18 mM $MgSO_4$, 2.2 mM $CaCl_2$ 10 mM D-glucose, 0.5% (w/v) bovine serum albumin (Serva, fraction V), pH 7.4] and then incubated with the appropriate agonist for 5 min. All incubations were at room temperature (20–22°C). After the incubation, the buffer was collected, acidified to 0.4 M with perchloric acid and catecholamines assayed as described above.

3. Results

Fig. 1 shows the distribution of adrenaline and noradrenaline in cells on the Percoll gradient. The first two fractions near the bottom of the gradient were comprised of erythrocytes, visible by eye, but no catecholamine-producing cells were detectable. The next region (fractions 5–9) contained preferentially adrenaline-producing cells (ratio adrenaline : noradrenaline, 10 : 1). This fraction was plated for functional experiments and named 'adrenaline fraction'.

There was a bimodal distribution of adrenaline-containing cells on the gradient. In contrast, the noradrenaline-containing cells were distributed more continuously from bottom to top of the gradient (compare last line in Fig. 1) with an enrichment in noradrenaline toward the top of the gradient. At the top of the gradient (from fraction 15 up; see Fig. 1), the cell number increased again. In the upper part of the Percoll gradient, the number of noradrenaline-containing cells increased continuously reaching a maximum of 70% noradrenaline cells and 30% adrenaline cells in the top layers. Cells of these layers were plated and used as a model for testing the properties of noradrenaline-producing cells ('noradrenaline fraction'). Cell fractions containing < 60% noradrenaline-producing cells were not plated.

The yield of cells for plating was lower in comparison to the method for isolation of chromaffin cells without separation of subfractions. In the first step of isolation, the digestion of one bovine adrenal gland of mean size yielded in 100–150 millions of chromaffin cells. After Percoll centrifugation and separation of the gradient, each of the two fractions suitable for plating contained ~ 20% of the cells which had been applied to the Percoll. 50–60% of all cells were lost since that portion of the gradient was discarded which contained > 10% adrenaline-producing and < 60% noradrenaline-producing cells (fractions 10–17 in Fig. 1).

In a second series of experiments, cells of the two fractions were stimulated with nicotine. Fig. 2 shows that the cells of the adrenaline fraction (with a ratio of adrenaline : noradrenaline of 10 : 1; upper part of Fig. 2) released ~ 10 × more adrenaline than noradrenaline, while

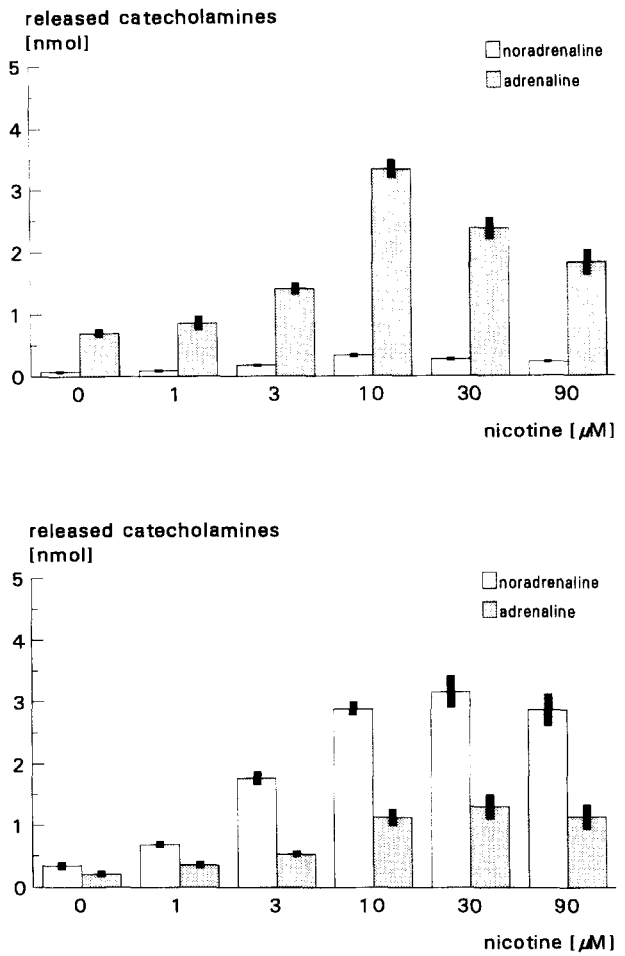

Fig. 2. Dose–response curves of nicotine tested on fractions of chromaffin cells producing preferentially adrenaline (top) or noradrenaline (bottom). Absolute release of adrenaline and noradrenaline from 400000 cells/5 min (mean ± S.E.M., $n = 4$).

the cells of the noradrenaline fraction (with a ratio of adrenaline : noradrenaline of 1 : 2) released 2–3 × more noradrenaline than adrenaline. Both cell fractions were stimulated half-maximally by 5 μM nicotine. This concentration of nicotine compares favourably to that required for half-maximal stimulation of a mixed adrenaline–noradrenaline cell population (see Livett et al., 1983). A half-maximal quantity of adrenaline as well as noradrenaline was released from both fractions by stimulation with the same concentration of nicotine. However, there was a significant difference in the amount of released catecholamines (compared to the total cell content at the beginning of the stimulation) which was generally by ~ 30% higher from the noradrenaline fraction than from the adrenaline fraction. The maximal release of catecholamines was also higher from the noradrenaline fraction. In response to maximally active doses of nicotine, the cells of the preferentially noradrenaline-producing fraction released 31.4 ± 1.5% of their total noradrenaline and 20.2 + 1.6% of their total adrenaline, whereas those of the preferentially adrenaline-producing fraction released only 21.2 ± 0.9%

Table 1
Release of adrenaline and noradrenaline induced by a maximally active concentration of carbachol (10^{-3} M) from two different fractions of chromaffin cells

Fraction containing preferentially	Cell content of adrenaline/noradrenaline	Release of total cell content	
		Adrenaline	Noradrenaline
Adrenaline cells	84%/16%	9.0 ± 0.3%	14.4 ± 0.3%
Noradrenaline cells	17%/83%	14.2 ± 1.0% [a]	17.8 ± 0.7% [a]

Mean ± S.E.M. after subtraction of basal release ($n = 4$). [a] Significantly different to the fraction containing preferentially adrenaline cells; $P < 0.05$.

of their total noradrenaline and 14.9 ± 0.6% of their total adrenaline.

In a third series of experiments, we compared the reaction of the two fractions of chromaffin cells on stimulation with carbachol and methacholine. Moro et al. (1990) considered that adrenaline-producing cells could differ from noradrenaline-producing cells regarding the specificity of their cholinoreceptors and suggested that adrenaline-producing cells have more muscarinic receptors than noradrenaline-producing cells which have preferentially nicotinic receptors. We found that the EC_{50} concentration of carbachol was the same in both adrenaline- and noradrenaline-enriched fractions (50 μM), i.e. 10 × higher than that for nicotine. Again, the maximal release of adrenaline and also of noradrenaline was significantly higher from the noradrenaline cell fraction (Table 1). Methacholine, on the other hand, was a very weak agonist for the release of catecholamines from adrenaline fraction as well as from the noradrenaline fraction. The release induced by even high concentrations of methacholine (up to 10^{-3} M) did not exceed twice basal release. Methacholine at a concentration of 10^{-4} M or higher partially inhibited the catecholamine release induced by 5 μM nicotine (data not shown). In all these experiments, methacholine did not significantly change the ratio of adrenaline to noradrenaline released. These results do not support a different specificity of the cholinoreceptors on the two cell types.

The nicotinic receptor antagonist hexamethonium (100 μM) attenuated the catecholamine release from chromaffin cells of both fractions. The release induced with 5 μM nicotine was reduced by > 80%, but that of an equieffective concentration of carbachol (50 μM) by < 70%. On the other hand, the muscarinic receptor antagonist atropin (1 μM) was without any significant effect on nicotine-induced catecholamine release whereas it inhibited the carbachol-induced release by 22%. Again, the release of adrenaline and noradrenaline was inhibited by the same percentage of the total cell content.

In Ca^{2+}-free medium, the secretion of catecholamines induced by nicotine and by carbachol was reduced (Table 2). The reduction was less pronounced for carbachol-induced release than for nicotine-induced release of catecholamines.

4. Discussion

The results show that the adrenaline or noradrenaline chromaffin cells of the adrenal gland can be separated by density gradient centrifugation into a predominantly adrenaline-containing fraction and a noradrenaline-enriched fraction. Considering the distribution of the two cell types, the distribution of adrenaline cells on the gradient was bimodal. The adrenaline-producing cells were found to be nearly equally distributed between the two peaks evoked by density gradient centrifugation. In the middle region of the gradient (fraction 10–14 in Fig. 1), there were few cells. This was obvious in all gradients in which we have

Table 2
Calcium dependency of the release of adrenaline (A) and noradrenaline (NA) induced by nicotine or carbachol from two different fractions of chromaffin cells

Fraction containing preferentially	Release of total cell contents into buffer containing		Ratio release without Ca^{2+}/release with Ca^{2+}
	1.5 mM Ca^{2+}	No Ca^{2+}	
(a) Stimulation with 5 μM nicotine			
Noradrenaline cells	13.6 ± 0.2% NA	2.2 + 0.1% NA	0.16
	8.3 ± 0.2% A	1.6 ± 0.1% A	0.19
Adrenaline cells	10.0 ± 0.4% NA	1.5 + 0.1% NA	0.15
	6.4 ± 0.3% A	1.0 ± 0.1% A	0.16
(b) Stimulation with 100 μM carbachol			
Noradrenaline cells	15.7 ± 0.4% NA	4.3 ± 0.2% NA	0.27
	8.8 ± 0.2% A	2.0 ± 0.1% A	0.23
Adrenaline cells	10.6 ± 0.1% NA	3.3 ± 0.4% NA	0.31
	7.0 ± 0.1% A	1.9 ± 0.1% A	0.27

Percentage of total contents/5 min; mean ± S.E.M. ($n = 4$).

analysed the catecholamine content. It indicates that the population of adrenaline cells is not homogenous and has two density peaks.

Most of the noradrenaline-producing cells (~ 80%) were found in the peak representing the lower density cell peak. Nevertheless, the noradrenaline-producing cells were also found in two peaks, although the first peak was relatively low, i.e. they are distributed over the gradient in a principally analogous way.

The distribution of the cells in two peaks suggests distinct functional properties, but up to now this has not been investigated in detail. The subpopulation of chromaffin cells with a relatively lower density released a significantly higher percentage of the stored cellular catecholamines. The higher release was not dependent on the type of the catecholamine produced by the cells. Adrenaline as well as noradrenaline were released in larger amounts from cells having a lower density than from those of the higher density.

The dose–response curves of nicotine and carbachol did not differ significantly with regard to their EC_{50} values (concentration which induced a half-maximal catecholamine release) when the cells of the two density peaks were compared. This was seen for both adrenaline and noradrenaline release. In contrast to the similar sensitivity of both cell groups to agonists as indicated by similar EC_{50} values, the maximal amounts of noradrenaline released (in relation to the total cell content) were higher than those of adrenaline. Moreover, this difference was seen when the release of noradrenaline and adrenaline from cells of the same density peak was compared.

The higher release of noradrenaline in comparison to adrenaline could be explained by a higher number of nicotinic receptors. But this is less probable since several non-cholinergic agonists also induce a higher release of noradrenaline, as we have found after application of 57 mM K^+ and histamine (data not shown). Again, the cells of the noradrenaline fraction released more catecholamines than those of the adrenaline fraction. These results suggest that there are quantitative differences in the receptor–effector coupling rather than a different receptor distribution in the two fractions of chromaffin cells, since agonists with different sites of actions induced nearly similar effects. Another possible explanation of the higher quantities of catecholamines, which were observed in all release experiments with cells of the noradrenaline fraction, is a more avid catecholamine reuptake. The less release of adrenaline detected in both fractions in comparison to that of noradrenaline could also be based on a higher reuptake of adrenaline. This mechanism remains to be proved or excluded in future experiments with uptake inhibitors.

The nicotinic antagonist hexamethonium attenuated the carbachol-induced release of adrenaline and noradrenaline considerably, but less than that of an equieffective concentration of nicotine. In addition, the activity of carbachol was partially inhibited by the muscarinic antagonist atropin at a concentration which was without any effect on the nicotine-induced release of adrenaline or noradrenaline. This is valid for the cells from both density peaks. In conclusion, the carbachol-stimulated catecholamine release is preferentially (but not completely) mediated via nicotinic receptors.

Stimulation of muscarinic receptors by methacholine produced only a small release of catecholamines. There was no preferential release of one of the two catecholamines after stimulation of muscarinic receptors. This is consistent with earlier reports that in the bovine adrenal gland muscarinic receptors are present but have little or no function with respect to the release of catecholamines in this species (summarized by Livett et al., 1983).

Based on the different pharmacological activities of adrenaline and noradrenaline a preferable release of one of the two catecholamines could be physiologically important. For example, in response to stressor exposition a preferable release of noradrenaline as a short-term effect followed by a decreased noradrenaline : adrenaline ratio induced by long-lasting stress could contribute to the adaptive response of the organism. Nevertheless, the pathophysiological consequences of variations in the quantitative relation of adrenaline and noradrenaline are not well-studied and little is known about the mechanisms which induce a preferable release of one of the two catecholamines. Choi et al. (1993) have shown that histamine is a more effective secretagogue for adrenaline than for noradrenaline. Recent communications indicate that noradrenaline-producing cells respond more sensitively to La^{3+} (Powis and Clark, 1995), bradykinin and angiotensin II (De la Fuente et al., 1995).

Our results suggest that the nicotinic response which mimics the physiological release of catecholamines from bovine adrenal glands is not significantly different in adrenaline- and noradrenaline-producing cells. Other mechanisms, which could mediate a preferable release of noradrenaline or adrenaline could be based on a diverse receptor–effector coupling or the influence of paracrine mediators. Among the modulators, which are assumed to act in a paracrine manner in the adrenal gland, the most studied is substance P (Nieber and Oehme, 1987; Khalil et al., 1988). Work is in progress to compare the modulation of the catecholamine release by substance P in these two catecholamine-containing cell populations.

Acknowledgements

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