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Morphological aspects of chemically stimulated bovine chromaffin cells

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Summary. Isolated bovine chromaffin cells were chemically stimulated. The resulting appearance of local changes in membrane structure were compared with the integration of granular proteins into the cell membrane as seen after binding of fluorescence labeled anti-dopamine-β-hydroxylase. Protuberances of the cell membrane which can be perceived by Nomarski contrast optics are largely congruent with local tracer accumulations at the cell surface.

In isolated chromaffin cells exocytotic release of catecholamines (CA) can be made visible immunochemically by observing the appearance of membrane vesicular antigens at the surface of the cell membrane by means of fluorescence microscopy[1]. Recently, structural changes were observed at the cell surface of chemically stimulated rat chromaffin cells by means of Nomarski contrast optics[2]. Here we try to correlate these 2 results.

Materials and methods. a) Preparation of cells: Bovine chromaffin cells were isolated as described recently[1]. 2 or 3 drops of cell suspension which contained $0.8\text{–}1.2\times10^7$ cells/ml isotonic standard buffer (i.e. 5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 0.135 M NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$ and 9 mM D-glucose) were dropped on to a slide and incubated for at least 45 min at 36 °C in a moist chamber. After washing out residual suspended cells, the cells adhering to the glass were stimulated at 21 °C with either 3.5 mM Ba^{2+}, with black widow spider venom (BWSV; extract of 1 gland in 2.5 ml standard buffer) or with 1.0 mM La^{3+} in standard buffer. CA secretion of an aliquot of cell preparation was assayed radioenzymatically by a catechol-O-methyltransferase method. During these stimulations, antiserum raised in rabbits against bovine membrane integrated dopamine-β-hydroxylase (amphiphilic DBH) was present in a dilution of 1:20. The antiserum was highly specific for bovine DBH but did not bind to DBH of other species[1]. After repeated washings the cells were incubated with fluorescein-conjugated γ-globulin (20 min at 21 °C, dilution 1:10). Thereafter, cells were washed 3 times.

The living cells were observed in a chamber consisting of 2 circular slides (1 slide used for application of the cells), an aluminium frame and a silicone gasket. The length of the beam through the chamber was approximately 1 mm.

b) Optics: A Zeiss Standard miscroscope was used, equipped with Nomarski contrast optics (objective $\times$ 100) and a condenser for incident fluorescent illumination. Cells which were brought into the chamber were observed and photographed both by means of Nomarski optics and by fluorescence microscopy.

Photographic prints were made on Ilford HP5 400 ASA black/white film. For fluorescence pictures exposure time was 25 sec. Prints were made on hard paper. Nomarski photographs were exposed automatically and printed on normal paper.

Results. By stimulation with Ba^{2+} during 30 min, bovine chromaffin cells can be induced to form several protuber-

Increase in percent of CA secreted by an aliquot of the same cell preparation stimulated for 10 min at 21 °C by various stimuli over non-stimulated controls incubated for 10 min in standard buffer. Control samples of approximately 5×10^5 cells released 3.8 µmoles CA, i.e. 3.3 µmoles adrenaline + 0.4 µmoles noradrenaline + 0.06 µmoles dopamin into the supernatant

	Dopamine	Adrenaline	Noradrenaline
La^{3+}	310 ± 46	567 ± 100	424 ± 78
Ba^{2+}	238 ± 43	474 ± 119	616 ± 91
BWSV	236 ± 58	528 ± 112	493 ± 103

ances (fig. 1). This phenomenon had already been observed with rat cells under similar conditions, and called 'microspikes'[2].

During stimulation with 1.0 mM La^{3+}, after 20 min only a few relatively small protrusions could be seen (fig. 2a and b), whereas stimulation with BWSV leads to quite a similar picture as seen after treatment with Ba^{2+} (fig. 3a and b; fig. 4a and b).

With increasing stimulation times, more pronounced cell-toxic effects were observed. After long-term stimulation (4 h) with Ba^{2+} and BWSV significantly fewer cells survived than with lanthanum. All the stimuli apparently leading to the integration of vesicle membrane components into the cell membrane caused also a release of catecholamines (table). DBH can be traced as specifically located on the protuberances and microspikes.

The emergents recognized by means of Nomarski contrast microscopy are largely congruent with local tracer accumulations on the plasma membrane as seen in the corresponding fluorescence image.

Discussion. Exocytotic integration of vesicular membrane components into the cell membrane can be shown immunochemically by binding of antibodies against a vesicle-specific protein, i.e. DBH[1].

Non-stimulated chromaffin cells essentially do not bind antiserum against amphiphilic DBH, as this enzyme is not a constitutive component of the outer cell membrane. During exocytotic CA release, vesicle membranes are at least transiently integrated into the cell membrane. Only under these particular circumstances can amphiphilic-DBH, which faces the interior of the granula, be reached from the outside of intact living chromaffin cells.

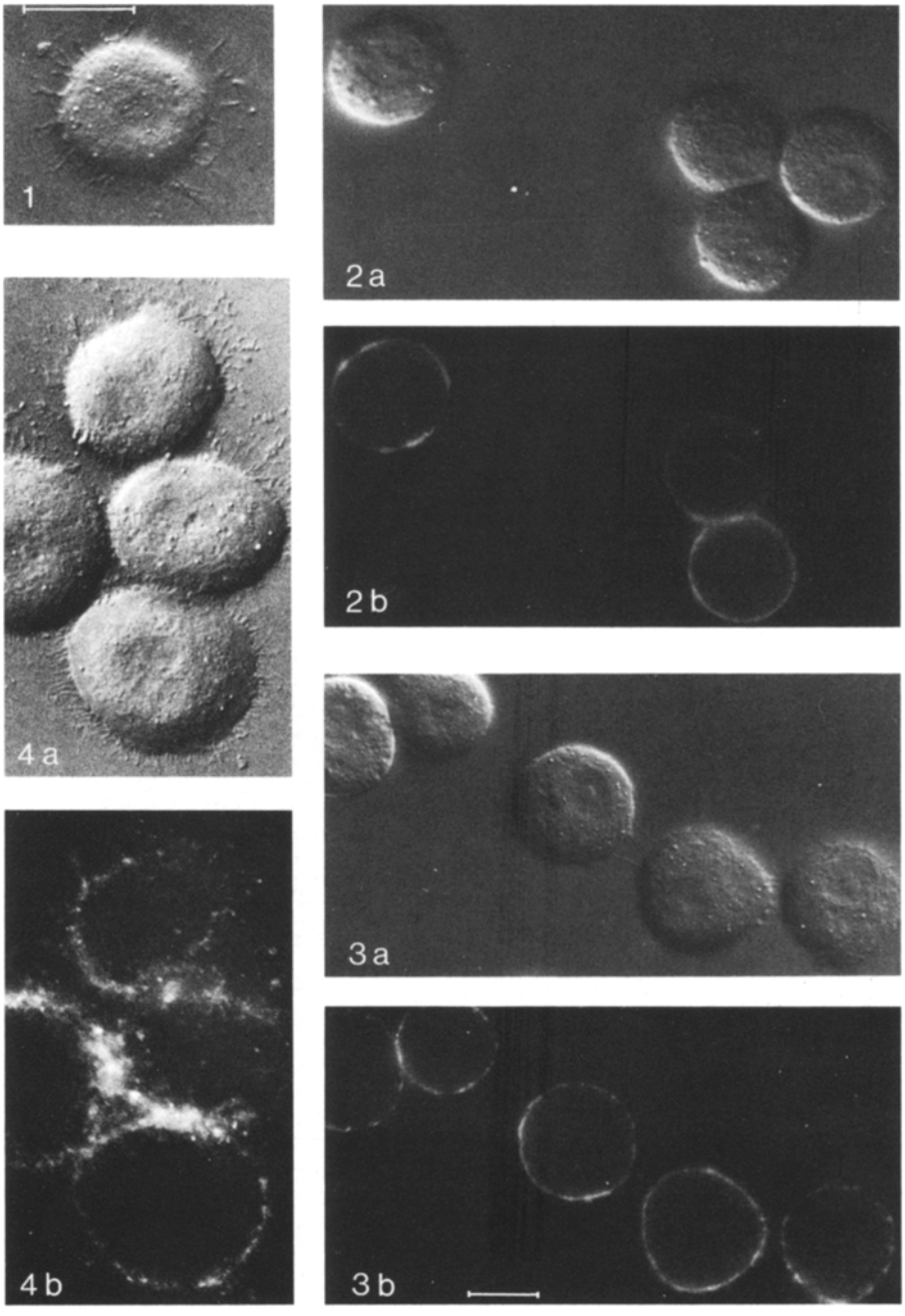

Figure 1. Formation of microspikes on an isolated bovine chromaffin cell after a 20 min exposure to 3 mM Ba^{2+}.

Figures 2–4 show in comparison Nomarski (a) and fluorescence (b) micrographs of identical cell groups after application of different stimuli.

Figure 2a and b. 1 mM La^{3+} for 20 min: One of the cells remains completely unlabeled under those conditions.

Figure 3a and b. 3 mM Ba^{2+} for 10 min: Protuberances and a small amount of microspikes are to be seen.

Figure 4a and b. BWSV for 20 min: Fluorescence labeling on the cell periphery derives to a great extent from emergents of the cell membrane. Bars: 10 µm.

With regard to the high proportion of DBH the outer membrane structure of the protuberances resembles the inner side of the granular membrane. Thus, protuberances or microspikes might be interpreted as local enlargements of the cell surface, which occur especially after extensive stimulations leading to numerous inside-out integrations of granular membrane particles per an individual release spot. Normally the vesicle membrane may be quickly recycled. A continuous addition of membrane from integrated secretion organelles to the plasma membrane will lead to a localized enlargement of the cell surface (growth of protuberances), if the mechanism of membrane retrieval is interfered with. This seems to be the case with Ba^{2+} and BWSV but not, or to a lesser extent, with La^{3+}. BWSV and barium are powerful stimulants of CA extrusion by acting directly on the chromaffin cell. Lanthanum in low concentrations facilitates CA release but blocks secretion at higher concentrations[4].

In frog neuromuscular junctions treatment with BWSV was followed by a depletion of synaptic vesicles, which was explained by a blockade of vesicle recycling[5].

Using Ba^{2+} as a stimulant the formation of microspikes was more intensive when compared with K^+ given in high doses, although K^+ is a powerful stimulant of CA release. Also, in contrast to observations with potassium, they did not disappear after removal of the stimulus[2].

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Neonatal masculinization affects maternal behavior sensitivity in female rats[1]

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Summary. The present results indicate that maternal behavior in adult neonatally androgenized female rats is significantly diminished when compared to females at oestrus and intact males. In androgenized females cannibalism was detected.

It is a well established fact that the hormone environment during the early perinatal period determines whether a brain will function in a male or female fashion[3]. In this context a number of brain functions have been suggested to be the result of this process of sexual differentiation[4]. However, no attention has been payed to the possibility that neonatal masculinization may affect some behavior-patterns such as maternal behavior.

Female Wistar Holtzman rats were injected at day 5 of age with 1.25 mg testosterone propionate (androgenized rats) or oil (controls). Testosterone was administered s.c. dissolved in 0.1 ml of olive oil. Males from the same litter were used as normal males. 90 days after treatment the androgenized and control rats at oestrus were tested for maternal behavior after being caged together with 3 foster pups. The observation cages were made of glass and had a rectangular shape measuring 44 cm in length, 21 cm in width, and 20 cm in height. Animals were allowed 2-3 h of habituation to the observation cages before experiments were begun.

The following aspects of maternal behavior during a 2-h observation period were recorded:

A) Licking: the animal must lick one or more pups for at least a total of 1 min.

B) Grouping: the animal must carry in her mouth 3, 2-7-day-old pups and put them together in a corner.

C) Crouching: all pups must be grouped before crouching can be recorded. The animal must have its hind legs spread, its back arched, and its ventrum high enough off the cage floor to accommodate pups beneath her. There must be at least 1 pup beneath her during a crouch. This position must be displayed for at least a total of 1 min.

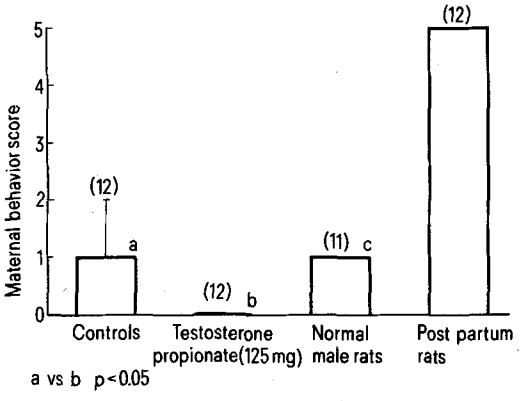
D) Nest building: the animal must pull more than half the 100 paper scraps into a corner where at least 1 pup has been or eventually will be carried (100 scraps were put in each cage during the habituation period).

E) Retrieval: any time within the 2-h observation period after grouping has taken place, 1 pup was separated by the observer to a distance of 15 cm from the female. The carrying of the pups back to the group within 15 min of separation constitutes retrieval.

Incidence of cannibalism in androgenized female rats

	No. of rats	No. of rats presenting cannibalism
Masculinized female rats	18	10
Normal male rats	11	0

$p \ll 0.005$.



Effect of neonatal androgenization on maternal behavior Data are expressed as the median of maternal scores. The vertical lines is the corresponding range. Numbers in parentheses indicate the number of animals per group.