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Effects of collagenase on the release of [^{3}H]-noradrenaline from bovine cultured adrenal chromaffin cells

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1 Bovine isolated adrenal chromaffin cells maintained in culture at 37°C for 1–7 days become polygonal and bipolar, with typical varicosity-like extensions. Catecholamine levels and dopamine β-hydroxylase activity decreased after 24–48 h of culture, but recovered to normal levels 3–7 days later.

2 Incubation of 1–7 day-old cells in the presence of increasing concentrations of [^{3}H]-noradrenaline (3.91 to 125 nM) resulted in the retention by the cells of amounts of radioactivity directly proportional to the amine present in the media. One day-old cells took up and retained only one third of the radioactivity found in 2–7 day-old cells.

3 The addition of collagenase to cultured cells caused a decrease in the uptake of tritium. However, the enzyme treatment did not affect the amine taken up by the cell before collagenase treatment.

4 Release of tritium from cultured cells evoked by nicotine, acetylcholine (ACh) or 59 mM K^+ was very poor in 24 h-old cells; the secretory response to nicotine, ACh or K^+ was dramatically increased after 2–7 days of culture. Bethanecol did not cause any secretory response.

5 When treated with collagenase, cultured cells which had recovered fully their secretory response, lost again the ability to release tritium evoked by ACh or nicotine. However, the responses to high K^+, veratridine or ionophore X537A were not affected. The nicotinic response was recovered two days after collagenase treatment.

6 The data suggest that the use of collagenase to disperse the adrenomedullary tissue during the isolation procedure might be responsible for the lost secretory response of young cultured chromaffin cells. Since collagenase specifically impairs the nicotinic cholinoceptor-mediated catecholamine release, it seems likely that the enzyme is exerting its action on the ACh receptor complex. It is unlikely that either voltage-sensitive Na^+ or Ca^{2+} channels are affected by collagenase as the responses induced by high K^+ or veratridine were unaffected by this enzyme.

Introduction

Chromaffin cells isolated from adrenomedullary tissue, have been used extensively in fresh suspensions or monolayer cultures to study their developmental and functional properties (Trifaró, 1982; Livett *et al.*, 1983). This paraneuron model is especially useful and convenient to determine the receptors, ionic channels and other proteic cell molecules involved in the basic release mechanism of catecholamine secretion (Hochman & Perlman, 1976; Brooks, 1977; Schneider *et al.*, 1977; Baker & Knight, 1978; Fenwick *et al.*, Kilpatrick *et al.*, 1980; Aunis & García, 1981).

When, in collaboration with D. Aunis and J. Ciesielski-Treska, we started to set up the procedure to isolate chromaffin cells at our laboratory, we were

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disappointed with the poor catecholamine secretory response obtained in acutely isolated cells; this response was, however, drastically improved in cells cultured for 3–7 days. Trifaró & Lee (1980) also noticed that the responses to acetylcholine (ACh) and depolarizing concentrations of K^+ seemed to be enhanced in cultured cells compared to newly isolated cells.

Since in most of the numerous papers recently published on secretion, chromaffin cells have been isolated by collagenase digestion of adrenomedullary tissues (see Table 1 for references), and this proteolytic enzyme has been shown to inhibit ACh release from synaptosomes (Sgaragli *et al.*, 1977), we decided to perform a systematic study on the effect of the age of the culture and collagenase on [^{3}H]-noradrenaline uptake into bovine cultured adrenal chromaffin cells and on its subsequent release by different secretagogues. A preliminary account of some of these results has been given (Montiel *et al.*, 1982).

Methods

Preparation of cells

Cells were isolated from bovine adrenal medulla according to the method used by Fenwick *et al.* (1978) with the following modifications: (1) the glands were perfused intact, in the presence of the cortex; (2) 0.15% collagenase (Sigma, Type V) in Ca^{2+}-Mg^{2+}-free Krebs solution was used during the initial perfusion (45 min) step; (3) once the adrenomedullary tissue was isolated and finely minced, two 30 min incubation periods in the presence of 0.15% collagenase followed; (4) after filtering, 'crude' chromaffin cells were centrifuged on Percoll gradients to remove contaminating erythrocytes and cell debris (Bader *et al.*, 1981). Chromaffin cells, which sediment at a density of 1.060, were washed from Percoll beads by low-speed centrifugation, and finally collected in Dulbecco's modified Eagle's medium (DMEM). Viability (usually greater than 90%) was determined by mixing an aliquot of the cell suspension with an equal volume of 0.04% trypan blue. The procedure gave approximately 40 million cells per gland. The dispersed cell suspension was diluted to 5×10^5 cells ml^{-1} with culture medium (DMEM supplemented with 20% foetal calf serum, containing 10 $\mu g\,ml^{-1}$ ampicillin and 10 μM cytosine arabinoside). Cells were plated on uncoated, 16 mm diameter, plastic culture wells (500,000 cells per well) and maintained at 37°C in a humidified incubator (Heraeus) under an atmosphere of 5% CO_2 plus 95% air. DMEM was changed 24 h later and every 2–3 days.

Morphological observations

To perform these experiments, cells were grown on collagen-coated coverslips which were placed in Falcon Petri dishes (30 mm in diameter). Living cultures were then examined with an inverted phase contrast light microscope and photographed 1–7 days after plating.

Biochemical assays

For catecholamine assay, cells were collected in 0.4 N perchloric acid, homogenized and centrifuged at 15,000 r.p.m. for 15 min at 2°C. The total catecholamine content (adrenaline + noradrenaline) was directly determined in aliquots of the supernatant, according to the method used by Shellenberger & Gordon (1971), without further purification on Alumina, and expressed as nmol per 5×10^5 cells.

For dopamine β-hydroxylase (DBH) assay, cells were harvested in 5 mM Tris buffer, pH 7.4 containing 0.2% Triton X100, homogenized and centrifuged at 15,000 r.p.m. for 15 min. DBH activity was assayed in aliquots of the supernatant following the spectrophotometric method of Nagatsu & Udenfriend (1972).

Protein contents were estimated by the Folin method of Lowry *et al.* (1951), using bovine serum albumin as a standard.

[^{3}H]-noradrenaline uptake

[^{3}H]-noradrenaline uptake was performed in the incubator at 37°C, under an atmosphere of 5% CO_2 plus 95% O_2. One–seven day-old cells were incubated for 1 h in 1 ml fresh DMEM containing 3.91–125 nM of (±)-^{3}H-noradrenaline (Amersham, specific activity 27 Ci $mmol^{-1}$). At the end of this incubation period, cells were washed 3 times with fresh DMEM, still at 37°C and finally collected in 0.5 ml 10% trichloroacetic acid; the extract was then added to a vial containing 2.5 ml Instagel (Packard), counted in a liquid scintillation counter ISOCAP 300 and quenching corrected with an automatic external standard. Tritium taken up and retained by cells (uptake) was expressed as d.p.m. h^{-1} per 5×10^5 cells.

[^{3}H]-noradrenaline release

To label the endogenous stores of catecholamines, 1–7 day-old cells were bathed in 1 ml DMEM containing (±)-[^{3}H]-noradrenaline 3.91–125 nM maintained in the incubator for 1 h and subsequently washed 3 times with fresh DMEM for 30 min. The cells were further washed for 1 h at room temperature using 1 ml aliquots of oxygenated Krebs-HEPES

Table 1 Nicotinic receptor-mediated catecholamine (CA) release from freshly isolated or cultured adrenomedullary chromaffin cells

Source of chromaffin cells	*Tissue digestion*	*Cells used*	*Secretory response*	*Reference*
Guinea-pig adrenal	Collagenase 0.1%	Fresh	37°C ACh (200 μM): [^{3}H]-Na release 6 × basal, Nic (50 μM): 2 × basal release	Role *et al.*, 1981
Bovine adrenomedullary slices	4–5 incubations in 0.2% collagenase, 30 min each	Fresh or stored overnight at room temperature	37°C, ACh (100 μM): CA release 7 × basal release	Schneider *et al.*, 1977
Bovine perfused adrenal medulla	0.05% collagenase 30 min perfusion followed by additional 15 min incubation of chopped tissue	Fresh	25°C, ACh (100 μM): CA release 5 × basal release	Aunis & García, 1981
Bovine perfused whole adrenal	0.05% collagenase perfusion for 30 min, followed by 30 and 20 min incubation periods of medullary tissue	Cultured	25°C, ACh (50 μM): CA release 50 × basal; [^{3}H]-NA release 12 × basal	Kilpatrick *et al.*, 1980
Bovine perfused whole adrenal	0.05% collagenase perfusion for 60 min	Cultured 8 days	ACh (100 μM): CA release 33 × basal; [^{3}H]-NA release 8 × basal	Trifaró & Lee, 1980

Evoked release has been expressed as a multiple of the spontaneous release, since experiments from different laboratories considerably differ in experimental conditions. ACh, acetylcholine; [^{3}H]-NA, [^{3}H]-noradrenaline; Nic, nicotine.

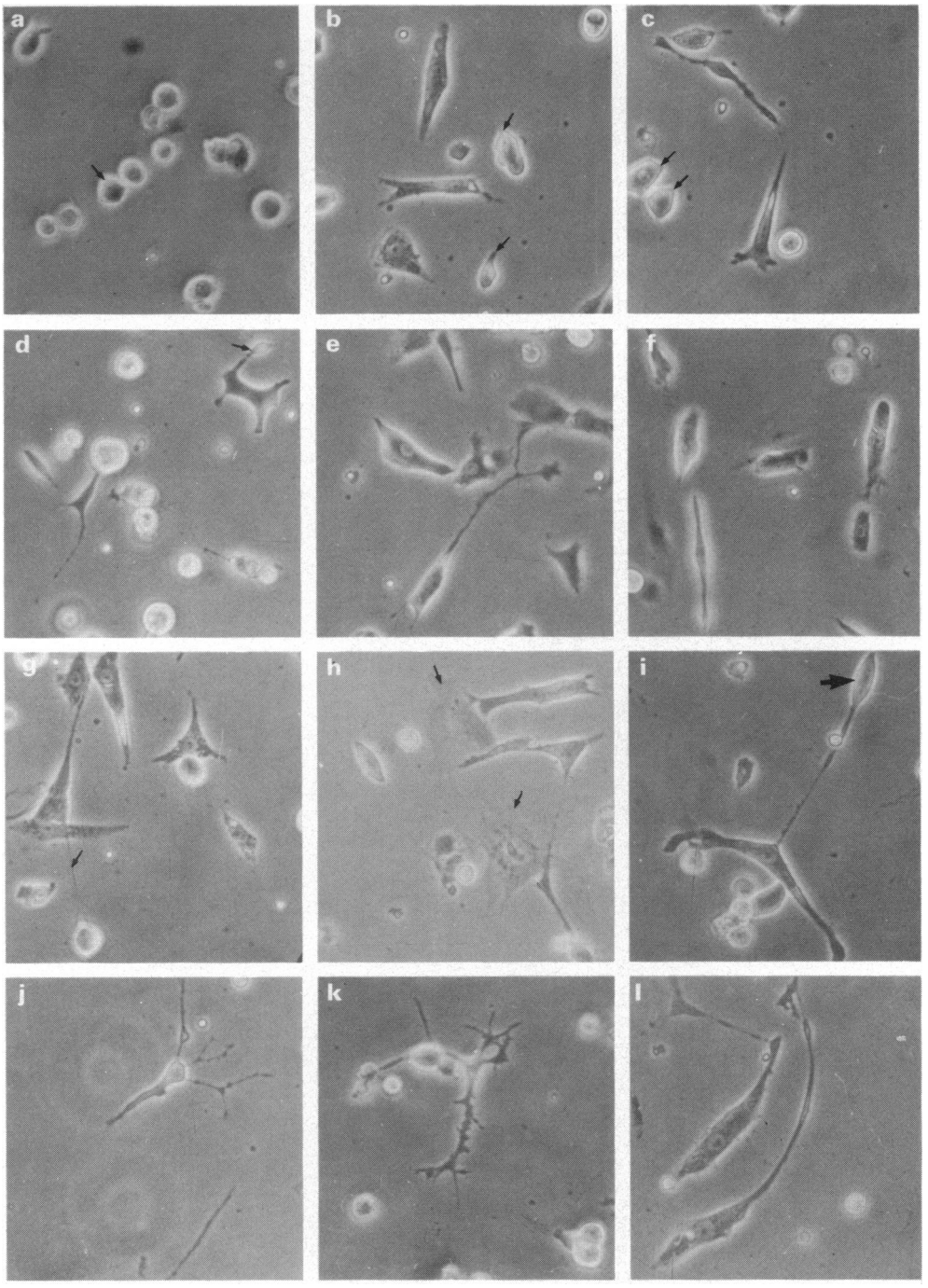

Figure 1 Phase-contrast micrographs of isolated bovine adrenal chromaffin cells maintained in cultures for increasing periods of time up to one week. (a) Chromaffin cells in culture 12 h after plating. The arrow indicates a chromaffin cell changing its morphology from round to polygonal, with a diameter of around 20 μm. (b and c) Chromaffin cells in culture 24 h after plating. In (b) arrows indicate chromaffin cells starting to grow neurite-like structures; note also the presence of some bipolar cells, with typical cytoplasmic extensions. In (c) arrows show polygonal cells. (d, e and f) Chromaffin cells in culture 48 h after plating. About 50% of cells are still round, but many others have prolongations (arrow in (d) indicate a growth cone-like, bulbous tip), which were 50 μm long, as shown in (e). In (f) many cells remained bipolar. (g and h) Chromaffin cells in culture 72 h after plating. Note the long, thin extension at the arrow in (g). In (h) arrows indicate fibrillar fibroblasts easily recognizable with phase-contrast optics. (i and j) Chromaffin cells in culture 96 h after plating. In (i) a single chromaffin cell with typical bulbous terminal (arrow) appears. Note ramifications of neurite-like structures of the cell shown in (j). (k and l) Chromaffin cells in culture one week after plating. Note single cell with spike-like structures extending from ramifications (k) and a cell with unique, long extension (l).

solution which was replaced at 10 min intervals. This solution had the following composition (mM): NaCl 140, KCl 4.7, KH_2PO_4 1.2, $MgSO_4 7H_2O$ 1.2, $CaCl_2$ 2.5, 4-(2-hydroxyethyl)-l-piperazine-ethanesulphonic acid (HEPES) 15 and glucose 11. The solution was equilibrated with pure O_2 and the pH adjusted to 7.4.

At the end of the washing period, always at room temperature, the cells were incubated for 5 min with 0.5 ml fresh Krebs-HEPES solution and the media collected to estimate the amount of tritium released spontaneously. Then evoked release was studied by incubating the cells with 0.5 ml fresh medium containing ACh (10 μM), nicotine (10 μM), bethanecol (10 μM) or 59 mM K^+ solution (53.1 mM NaCl substituted by KCl).

The radioactivity present in all incubation media and the collected cells was counted as above. Release (spontaneous and evoked) was expressed as d.p.m. per 5 min per 5×10^5 cells; net evoked release was calculated by substracting spontaneous from evoked release. In another group of experiments, release was expressed as the ratio evoked: spontaneous. Data are usually given as mean ± s.e. Only total ^3H release was measured; no attempt to measure intact [^3H]-noradrenaline release was made, but Kilpatrick *et al.* (1980) showed that release of ^3H from bovine cultured adrenal chromaffin cells prelabelled with [^3H]-noradrenaline was parallel to the release of endogenous catecholamines.

Results

Morphological characteristics of cultured chromaffin cells

Since a major objective of this investigation was to see how the release of [^3H]-noradrenaline was affected by the amount of time the chromaffin cells had been in culture, it seemed appropriate to study the evolution of their morphological characteristics during the first week of culture.

The main characteristics that we found agree with those previously observed by other authors (Trifaró & Lee, 1980; Unsicker *et al.*, 1980; Bader *et al.*, 1981). Initially, the cells are round and have a diameter of 20 μm (Figure 1); neurites start to grow 24 h later; cells become polygonal and mainly bipolar with typical cytoplasmic extensions. After 2–7 days in culture, the extensions increase in number and many varicosities are seen along them.

Effects of the culture time on the content of proteins, catecholamines and dopamine β-hydroxylase activity of chromaffin cells

Total catecholamine (adrenaline + noradrenaline) content of the cells was decreased from 12.5 nmol per 5×10^5 cells found in freshly isolated cells (mean of 18 determinations), to 10 nmol per 5×10^5 cells found 24 h after plating (Figure 2). Catecholamine levels were decreased further 48 h later, but recovered to values similar to those of fresh cells after 3 and 7 days of culture. DBH activity followed a pattern very similar to that found for catecholamines. The protein content decreased slightly the second day of culture but recovered to above control values 3 and 7 days later (Figure 2). These catecholamine and protein values are similar to those obtained by Kilpatrick *et al.* (1980) in bovine cultured adrenal chromaffin cells. However, our DBH values are about 10 times higher, probably because we used a different DBH assay method with saturating concentrations of the substrate tyramine.

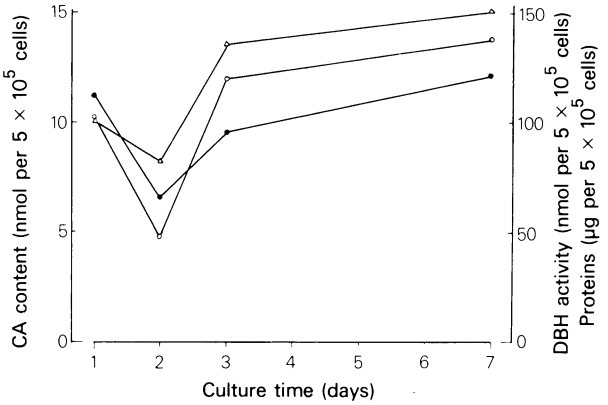

Figure 2 Catecholamine (CA) (○), protein contents (△) and dopamine β-hydroxylase (DBH) activity (●) of bovine adrenal chromaffin cells maintained in cultures for 1–7 days. One, 2, 3 and 7 days after plating cells were collected and their protein contents, the total CA (adrenaline + noradrenaline) and DBH activity determined as described in Methods. Left ordinate shows CA contents and the right ordinate displays the values of proteins and DBH activity. Data shown are means of a duplicate experiment.

Effects of the culture time on [^3H]-noradrenaline uptake into bovine adrenal chromaffin cells

Initial experiments were designed to test the effects of the culture time on the uptake of [^3H]-noradrenaline by chromaffin cells. Incubation of 1–7 day-old cells in the presence of increasing concentrations of this amine (3.91 to 125 nM) resulted in retention by the cells of amounts of radioactivity which were directly proportional to the concentration of [^3H]-noradrenaline present during the 1 h incubation period (Figure 3). The uptake process did not saturate, probably because the highest concentration of the amine used was only 125 nM.

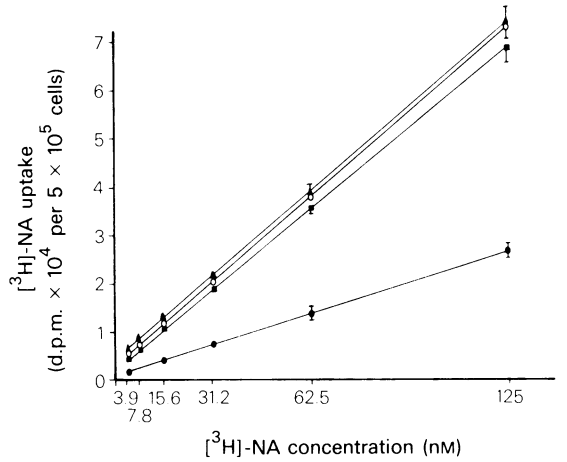

Figure 3 [^{3}H]-noradrenaline ([^{3}H]-NA) uptake into bovine cultured adrenal chromaffin cells. One to seven day-old cells were incubated for 1 h in 1 ml fresh Dulbecco's modified Eagle's medium (DMEM) containing 3.91–125 nM (abscissa) of (±)-[^{3}H]-NA, at 37°C in the incubator under an atmosphere of 5% CO_2 plus 95% air. After washing with DMEM, radioactivity present in the cells (ordinate) was determined. Data shown are means ± s.e. of 4 different batches of cells ($n = 13–21$ individual wells). At all [^{3}H]-NA concentrations, uptake by 2, (○) 3 (▲) and 7 (■) day-old cells was significantly higher ($P < 0.001$) than 1 (●) day-old cells; s.e. not drawn are smaller than the symbol.

At all concentrations used, the uptake of [^{3}H]-noradrenaline was considerably lower in 1 day-old cells; after 2, 3 or 7 days in culture, the cells retained similar amounts of the amine, although the retention was 3 times higher when compared with 1 day-old cells ($P < 0.001$; $n = 21$).

Effects of collagenase on [^{3}H]-noradrenaline uptake

The smaller quantitative uptake of tritiated noradrenaline by 1 day-old cells could be due to collagenase treatment during the isolation procedure. Therefore, we decided to study the radioactivity retained by 3 day-old cells, which had a fully 'restored' uptake capability, when re-exposed again, while in culture, to collagenase. A group of cells was incubated with [^{3}H]-noradrenaline (125 nM) for 1 h as above and washed; then, they were exposed to collagenase (0.15%) in DMEM for 1 h in the incubator, washed with fresh DMEM, kept 24 h in the incubator and finally collected as above to measure their content of radioactivity (Figure 4a). A second group of cells was treated first with collagenase, then after washing with fresh DMEM, maintained in the incubator for 24 h and finally incubated with [^{3}H]-noradrenaline as above (Figure 4b). The amount of tritium retained by cells incubated first with [^{3}H]-noradrenaline was similar to that obtained by normal cells (Figure 3), indicating that once the amine is taken up collagenase does not affect it. However, pretreatment with collagenase 24 h before, caused a 60% diminution of retention (Figure 4), suggesting that collagenase certainly impairs the noradrenaline uptake and/or the storage process.

Influence of the culture time on [^{3}H]-noradrenaline release evoked by several secretagogues

To test whether the time of culture affected the amount of tritium released evoked by different secretagogues, cells were prelabelled with increasing concentrations of the amine. ACh or nicotine (10 μM) failed to release an appreciable amount of [^{3}H]-noradrenaline compared to spontaneous release from 1 day-old cells. In fact, the release evoked by ACh dramatically increased in 2–3 day-old cells, being maximal on the seventh day (Figure 5). In the case of nicotine, peak release was reached on the third day of culture.

Bethanecol (10 μM) did not evoke a secretory response in any of the cells studied (Figure 5). As far as high K^+ (59 mM) is concerned, the behaviour was identical to that seen in the case of nicotine, release being maximal in 3 day-old cells (Figure 5).

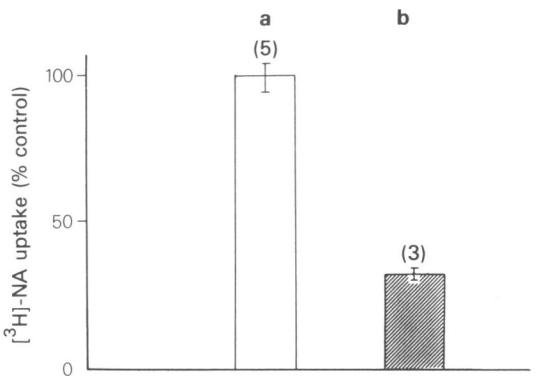

Figure 4 Effects of collagenase on [^{3}H]-noradrenaline ([^{3}H]-NA) uptake into chromaffin cells. In (a) (open column) 3 day-old cells were incubated first with [^{3}H]-NA (125 nM for 60 min), subsequently treated with collagenase (0.15%) for 1 h, and kept in the incubator for 24 h. In (b) (hatched column) cells of the same age were treated first with collagenase (0.15%) for 1 h, then kept in the incubator for 24 h and finally incubated with [^{3}H]-NA as in (a). Radioactivity present in the cells was then determined after washing. Data shown are means ± s.e. of the number of experiments shown in parentheses.

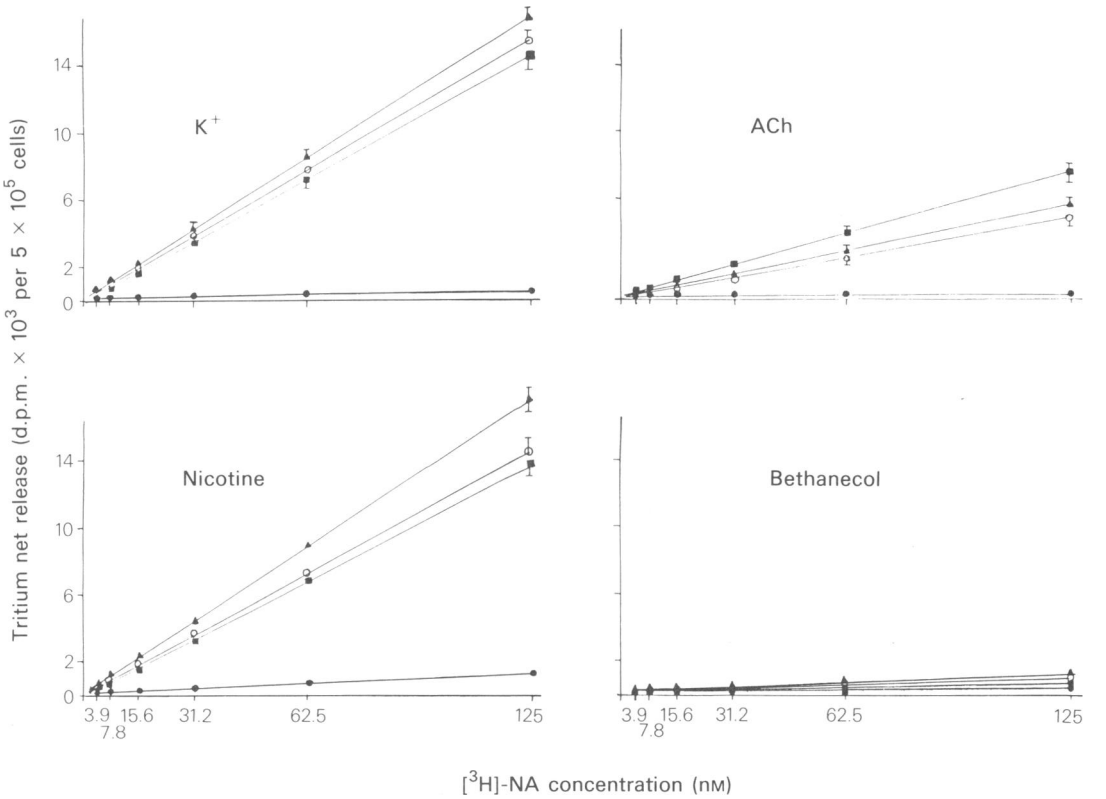

Figure 5 Effects of the culture time on tritium release evoked by 10 μM acetylcholine (ACh), nicotine, bethanecol or high K^+ (59 mM) from chromaffin cells. One–seven day-old cells ((●) day 1, (○) day 2, (▲) day 3 and (■) day 7) were prelabelled with increasing concentrations of [^{3}H]-noradrenaline (3.91–125 nM) as in Figure 2. After a 1 h washing period, spontaneous and evoked release of tritium were determined as shown in Methods. Ordinate shows net release which was obtained by subtracting spontaneous from evoked release. Data shown are means ± s.e. of 4 experiments from different cell batches. Data on bethanecol are from a single experiment made in duplicate.

Effects of collagenase on the release of [^{3}H]-noradrenaline induced by high K^+ or nicotine

Impairment of the secretory response in 1 day-old cells could be due to the collagenase treatment during the dispersion step of the cell isolation procedure. Therefore, the acute effects of collagenase in cells which had fully recovered their secretory response were studied.

After loading with [^{3}H]-noradrenaline in the usual way, 7 day-old cells were incubated at 37°C for 60 min in the presence of collagenase (0.01, 0.05 or 0.15%). After the wash out period, release of tritium evoked by K^+ (59 mM) was similar in control and in collagenase-treated cells (Figure 6). In contrast, the secretory response to nicotine (5 μM) was gradually impaired as the concentration of collagenase increased.

Selectivity of the collagenase effects on the secretory response

Since secretion of noradrenaline evoked by several secretagogues involves different underlying mechanisms, it seemed of interest to explore further the degree of selectivity of the collagenase inhibitory effects using 5 different agents to evoke the release of tritium from 3–7 day-old chromaffin cells.

Cells were preloaded with [^{3}H]-noradrenaline and subsequently treated with collagenase (0.15%) as in the previous section. Only the nicotinic receptor-mediated release was clearly impaired in collagenase-treated cells (Figure 7). The secretory responses to direct depolarization with K^+, veratridine or the ionophore X537A were not affected by the enzyme pretreatment.

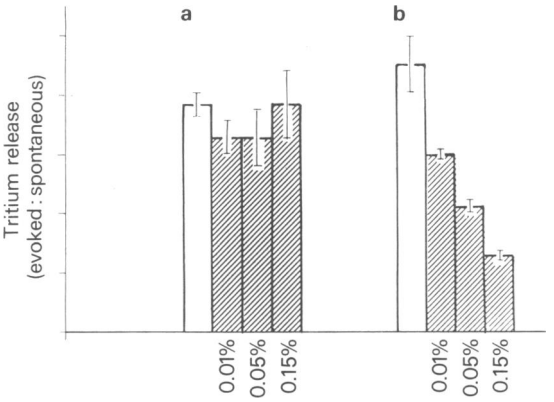

Figure 6 Effects of collagenase on the release of tritium evoked by (a) high K^+ or (b) nicotine from 7 day-old chromaffin cells. After loading with [^{3}H]-noradrenaline cells were treated for 60 min with increasing concentrations of collagenase (hatched columns) at 37°C for 60 min. The secretory response to high K^+ (59 mM for 5 min) or nicotine (5 μM for 5 min) was studied following the usual washing period. Ordinate shows the released tritium expressed as the ratio evoked: spontaneous. Spontaneous release amounted to about 800 d.p.m. per 5×10^5 cells. Data shown are means ± s.e. of 3 experiments.

Time course of the restoration of the secretory response in collagenase-treated cultured chromaffin cells

In view of the fact that the acute treatment of cultured cells with collagenase inhibited tritium release evoked by nicotine, it was desirable to know the time course of such an effect. So, experiments were designed in which 3 day-old cells from the same batch were treated with 0.15% collagenase for 1 h at 37°C; then the cells were washed with fresh DMEM and kept in the incubator. One–seven days later they were prelabelled with [^{3}H]-noradrenaline as usual and after washing subjected to the release protocol.

Twenty four hours after collagenase treatment the release evoked by nicotine (10 μM for 5 min) was greatly inhibited (Figure 8). However, the nicotinic response was restored to levels comparable to control cells 3 and 7 days after the enzyme treatment (Figures 9 and 10). Once more, the secretory responses to high K^+ (59 mM for 5 min) or the ionophore X537A (50 μM for 15 min) were not affected by collagenase.

It is curious to note that the uptake and retention of [^{3}H]-noradrenaline were comparable in normal and collagenase-treated cells. One, 3 and 7 days after collagenase treatment, control cells retained 111.425 ± 6.054, 77.588 ± 3.412, and 33.089 ± 1.441 d.p.m. per 5×10^5 cells, respectively (n= 11); retentions by collagenase-treated cells were very similar (Figures 8, 9 and 10).

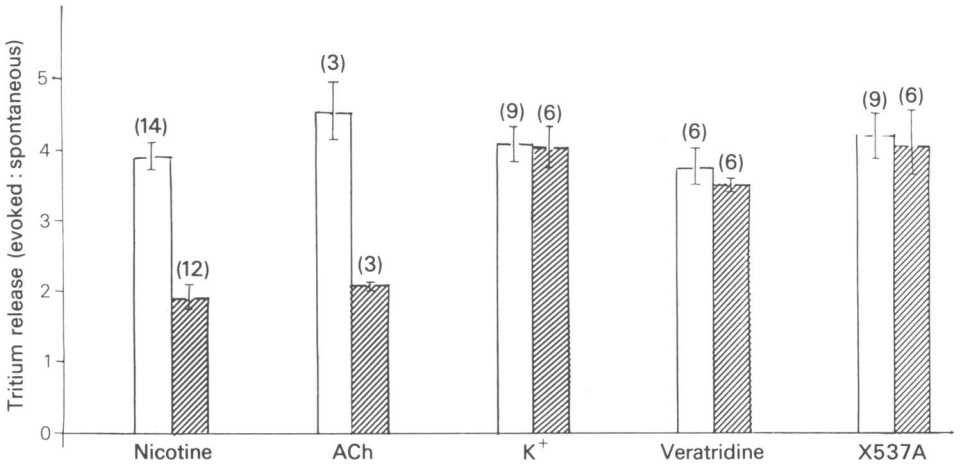

Figure 7 Effects of collagenase on the release of tritium evoked by different secretagogues. Three day-old cells were preloaded with [^{3}H]-noradrenaline and treated with 0.15% collagenase at 37°C for 1 h. After the washout period, tritium released by subjecting the cells for 5 min to nicotine (10 μM), ACh (10 μM), X537A (10 μM), veratridine (10 μM) or K^+ (59 mM) was determined. Secretagogue alone, open columns; collagenase + secretagogues, hatched columns. Release of tritium is expressed as the ratio evoked: spontaneous. Data are means ± s.e. of the number of experiments shown in parentheses. Nicotine- and ACh-evoked release were significantly reduced with respect to control release ($P < 0.01$).

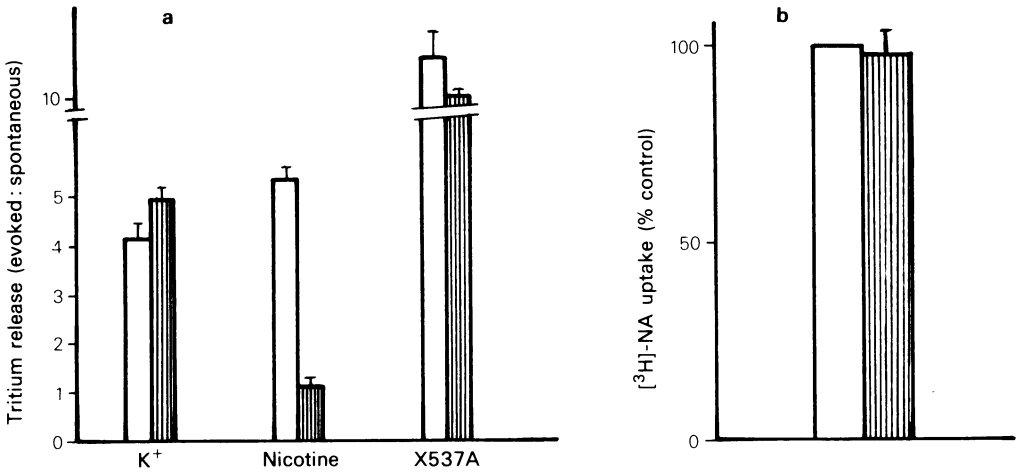

Figure 8 Release of tritium from chromaffin cells pretreated with collagenase 1 day before. Three day-old cells from the same batch were treated with collagenase (0.15%) for 1 h at 37°C; then, the cells were washed with fresh DMEM and kept in the incubator. Twenty four hours later they were prelabelled with [^{3}H]-noradrenaline in the usual way, and after washing, exposed to K^+ (59 mM, 5 min), nicotine (10 μM) or X537A (50 μM, 15 min). (a) Tritium released as ratios evoked: spontaneous; (b) represents [^{3}H]-NA taken up and retained by control (open columns) and collagenase-pretreated (hatched columns) cells. Data shown are means ± s.e. of 4 (release) and 11 (uptake) experiments.

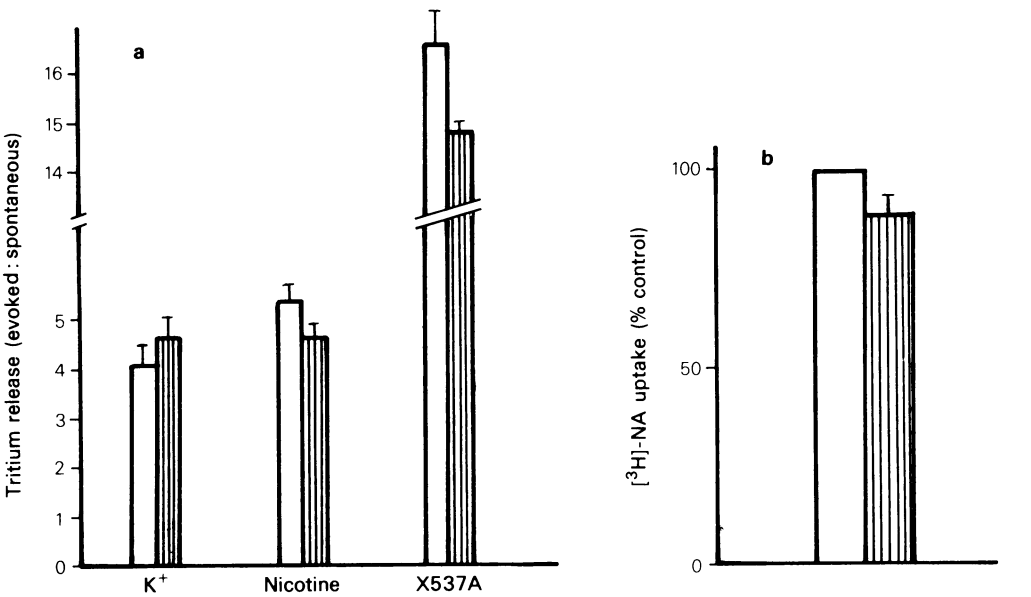

Figure 9 Release of tritium from chromaffin cells pretreated with collagenase 3 days before (hatched columns). Experimental protocol identical to that of Figure 8, but here the release and uptake studies were performed 3 days after collagenase treatment. Control cells, open columns. Data shown are means ± s.e. of 4 (release) and 11 (uptake) experiments.

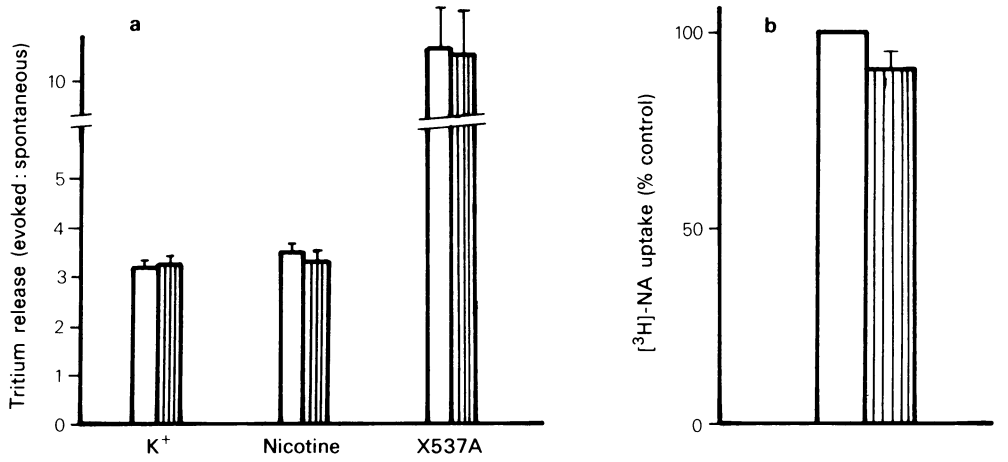

Figure 10 Release of tritium from chromaffin cells pretreated with collagenase 7 days before (hatched columns). Experimental protocol as in Figure 8, but here the release and uptake studies were performed 7 days after collagenase treatment. Data shown are means ± s.e. of 4 (release) and 11 (uptake) experiments.

Discussion

Discussion of this investigation will be focussed on the following aspects: (a) the effect of the amount of time the chromaffin cells are in culture on the uptake of [^{3}H]-noradrenaline; (b) whether collagenase is responsible for these effects; (c) why at early culture times the secretory response is impaired, but later is restored; and (d) what might be the underlying mechanism implicated in the variability of the secretory response with the age of the cultures and in the collagenase inhibitory effects on secretion?

How the culture time and collagenase affects the uptake of [^{3}H]-noradrenaline into chromaffin cells

Chromaffin cells maintained in culture for 1–7 days accumulated [^{3}H]-noradrenaline in a concentration-dependent manner; the retention showed strict linearity and did not saturate probably because the highest concentration of the amine used was only 125 nM. If higher concentrations are used a saturation curve is seen (Kenigsberg & Trifaró, 1980). Since the uptake of [^{3}H]-noradrenaline is blocked by cocaine and tricyclic antidepressants, is saturable and Na^+-dependent (Kenigsberg & Trifaró, 1980; Ceña *et al.*, 1983), it seems that the cultured chromaffin cell contains an uptake$_1$ (U_1) carrier system for noradrenaline similar to that described for sympathetic nerve terminals (Iversen, 1965).

At early stages of the culture the uptake process seems to deteriorate. So, in 1 day-old cells the amount of [^{3}H]-noradrenaline taken up was one third of that found at 2–7 days. The uptake is fully restored the second day of culture. It could be of interest to see how the uptake process recovers between 12 and 48 h of culture.

Impairment of the uptake process for noradrenaline might be due to the collagenase treatment (used to disperse the chromaffin cells) of the adrenomedullary tissue. In fact, when collagenase was added to cultured cells which had recovered fully their uptake capacity, retention of [^{3}H]-noradrenaline was again depressed by 60% (Figure 4). However, in the experiments shown in Figure 8 retention of [^{3}H]-noradrenaline by control cells and cells treated with collagenase 24 h previously was similar, suggesting that collagenase effects on the uptake process are mild and erratic.

In any case, it is clear that collagenase is selectively affecting the uptake process, but not the storage and/or intracellular degradation processes for noradrenaline, since once taken up the cellular levels of the amine were not modified by the enzyme treatment.

The age of the culture, a critical factor for the catecholamine secretory response

The main observation which motivated this study was that after a short time of culture, the catecholamine secretory response to nicotine or ACh was severely impaired. Because [^{3}H]-noradrenaline retained by 1 day-old cells was only one third of that found later on it might be argued that the secretory response is weak because retention of the amine is poor. If this were the case secretion should be diminished, but not abolished as shown in Figure 5. In addition, it should be noted that 4 day-old cells treated with collagenase 24 h previously, retained normal amounts of [^{3}H]-noradrenaline yet the secretory response to nicotine

was decreased by over 80%. Therefore, it seems that there is no correlation between the uptake-storage capacity of the cell and its secretory mechanism, a statement further corroborated by the fact that even though 2 day-old cells contain lower endogenous catecholamine levels (Figure 2), they retain and release [^{3}H]-noradrenaline normally (Figures 3 and 5).

Selectivity of the collagenase effects on the secretory response

Collagenase-inhibition of [^{3}H]-noradrenaline release could be a non-specific phenomenon as suggested by initial experiments in which ACh, nicotine or high K^+ were used as secretagogues. Failure of K^+ to increase tritium release from 1 day-old cells can, however, be explained if an additional effect is implicated, i.e. cell depolarization caused by the isolation procedure.

Subsequent experiments aimed at studying the effects of collagenase on different secretagogues clearly demonstrated that only the nicotinic cholinoceptor-mediated tritium release was depressed by the enzyme treatment. Neither high K^+, veratridine or X537A-evoked secretory responses were affected, which excludes an effect of collagenase on membrane voltage-dependent Na^+ or Ca^{2+} channels or on some intracellular component of the secretory machinery. Thus, the action of collagenase seems to be selective, and restricted to the nicotinic receptor macromolecular complex.

Since the collagenase type V preparation used had characteristically low tryptic activity, it seems that the specific inhibitory effects on nicotinic-evoked secretion may be ascribed to collagenase itself and not to non-specific proteases, clostripain or trypsin. Additionally, because trypsin is known to remove Na^+ channels in squid axons (Carbone, 1982), if it were present in our collagenase preparation in substantial amounts it should also abolish the veratridine-evoked release.

What are the underlying mechanisms implicated in the lost secretory response in acutely isolated cells, its restoration with the time of culture and the collagenase inhibitory effects on secretion?

The present experiments, and those presented in Table 1 collected from recent literature, suggest that the catecholamine release response to nicotinic cholinoceptor activation is poorer in freshly isolated chromaffin cells than the release obtained in cultured cells. In addition, collagenase substantially inhibits such a response. These observations can be explained by the following conjectures. (a) Impairment of the secretory response in recently isolated cells is most probably related to the collagenase digestion step of adrenomedullary tissue during the cell isolation procedure. (b) As shown by Sgaragli *et al.* (1977) in synaptosomes, collagenase could block Ca^{2+} transport into chromaffin cells. Because K^+-or veratridine-evoked releases were not modified by collagenase in our experiments, it seems that voltage-sensitive Na^+ or Ca^{2+} channels are unrelated to the enzyme effects. (c) Collagenase seems to act selectively on the nicotinic cholinoceptor molecule. The receptor may be anchored to the plasma membrane matrix through a filamentous, collagen-like tail in a manner similar to the tailed forms of acetylcholinesterase from mammalian brain (Rodriguez-Borrajo *et al.*, 1982). During the cell isolation procedure, or in cultured chromaffin cells treated with collagenase, the enzyme will release the majority of the nicotinic receptors and the secretory response will be decreased or abolished until new receptors have been synthesized and incorporated into the plasma membrane. (d) With the time of culture, the synthesis of receptors may be induced in these denervated cells; newly synthesized cholinoceptors might then spread out around the entire surface of the cell. The increased secretory response to ACh or nicotine with the time of culture might thus be explained by a process of denervation supersensitivity similar to that seen in denervated skeletal muscle (Green *et al.*, 1975).

In conclusion, the results show: (1) that chromaffin cells isolated with procedures involving collagenase digestion of adrenomedullary tissues have a poor secretory response immediately after isolation or at early stages of culture; (2) the secretory response is fully restored 2–3 days after cell plating; (3) collagenase selectively inhibits the nicotinic-mediated catecholamine release but not the release evoked by other secretagogues, suggesting that collagenase might be responsible for the lost secretory response in young chromaffin cell cultures; and (4) since collagenase effects are directed specifically towards the nicotinic response it seems likely that the nicotinic cholinoceptor of the bovine adrenal chromaffin cell contains in its proteic macromolecular complex a collagen-like tail through which the receptor might be anchored to the plasma membrane.

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