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Loss of cell-cell and cell-substrate contacts in single pancreatic β-cells divert insulin release to intracellular vesicular compartments

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Running tilte: Development of vacuoles in insulin-producing β-cells

Keywords: vacuoles, pancreatic β-cell, insulin secretion, endocytosis, exocytosis

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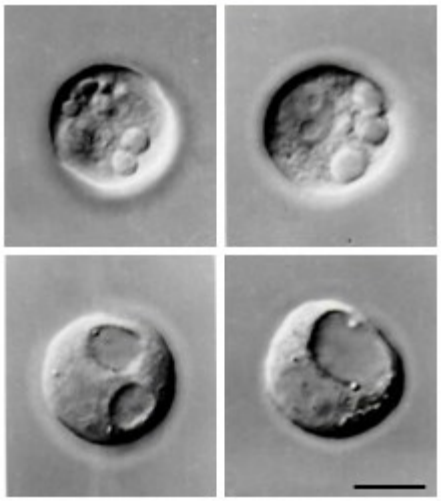
ABSTRACT

Background Information: Cell-cell or cell-substrate interactions are lost when cells are dissociated in culture, or during pathophysiological breakdowns, therefore impairing their structure and polarity, and affecting their function. We show that single rat β-cells, cultured under non-adhesive conditions, form intracytoplasmic vacuoles increasing in number and size over time. We characterized these structures and their implication in β-cell function. **Results**: Ultrastructurally, the vacuoles resemble vesicular apical compartments and are delimited by a membrane, containing microvilli and expressing markers of the plasma membrane, including GLUT2 and actin. When insulin secretion is stimulated, insulin accumulates in the lumen of the vacuoles. By contrast, when the cells are incubated under low calcium levels, the hormone is undetectable in vesicular compartments. Insulin release studies from single cells revealed that vacuole-containing cells release less insulin as compared to control cells. When added to the medium, a non-permeant fluid phase marker becomes trapped within vacuoles. Inhibition of vesicular trafficking and exocytosis as well as dynamin-dependent endocytosis changed the percentage of vacuole-containing cells, suggesting that both endocytic and exocytic track contribute to their formation. **Conclusions**: These results suggest that loss of cell-cell and cell-substrate contacts in isolated β-cells affect normal vesicular trafficking and redirects insulin secretion to intracellular vesicular compartments. **Significance:** Our study reveals for the first time that single β-cells develop vacuolar compartments when cultured in suspension and redirect their insulin secretion to these vacuoles.

This may underlie a compensatory process for cultured cells who lost their interactions with adhesive substrates or neighbouring cells.

When cultured in suspension, dissociated rat islet β-cells progressively develop vacuoles similar to the vesicular apical compartments found in other epithelial cells. These vacuoles have different size and number, depend on vesicular trafficking and endocytosis and disappear or are absent from aggregated β-cells. While insulin is found in the vacuoles, vacuole-containing β-cells have a reduced stimulated insulin secretion as compared to control cells, suggesting that loss of cell-cell and cell-substrate contacts redirects insulin granules to these compartments.



INTRODUCTION

When removed from their original environment, cells encounter different changes, including loss of cell-cell and cell-substrate contacts as well as interaction with their extracellular matrix. In most cases, these changes lead to rearrangement of cytoplasmic and membrane components, that impairs intracellular trafficking and disturbs cell polarity and identity (Honda 2017; Vaidziulyte, Coppey et al. 2019). One of the common traits seen in dissociated epithelial cells with loss of cell-cell and cell-substrate contacts is intracellular vacuolation. Referred by various authors to as *vacuoles, intracellular lumens or vesicular apical compartments*, these structures are

round, have a variable diameter extending from 1 to 6 μm, are bordered by a membrane that often expresses apical plasma membrane markers and usually also contains microvilli. In the past, different techniques were used to observe the strictly intracellular character of these lumens (Kondo, Tamura et al. 1970); (Remy 1986), (Hashieh, Remy et al. 1989), including serial sections of tissues or cells in cultures (Kondo, Tamura et al. 1970, Remy 1986, Achler, Filmer et al. 1989, Hashieh, Remy et al. 1989). In almost all cases, these studies showed that intracellular vacuoles develop when cells fail to form cell-cell and cell substrate contacts and present a disturbed polarity.

The vesicular apical compartment is an organelle originally found and extensively studied in Madin-Darby canine kidney (MDCK) cells maintained in culture with loss of or incomplete intercellular contacts (Vega-Salas, Salas et al. 1987). When conditions become appropriate, *e. g.* after cell-to-cell or cell-to-substrate contact recovery, cells externalize the intracellularly stored membranes by an exocytotic mechanism (Brignoni, Pignataro et al. 1995). The intracellular vesicular compartments are not only restricted to cultured MDCK cells. In Caco-2 intestinal epithelial cell line, disruption of the microtubular network results in the appearance of brush border-containing intracellular vacuoles. It is thought that these compartments are newly synthesised and it has been proposed that they have a similar origin to that observed in Davidson's disease (Gilbert and Rodriguez-Boulan 1991), characterized by the presence of atrophied microvilli. In tumoral cell lines, such as the colonic adenocarcinoma cell line HT-29, these lumens are able to open outside the cell by a process similar to exocytosis, the microvilli becoming the microvilli of the

new apical plasma membrane (Remy and Marvaldi 1985). Moreover, it has been also shown that cultured hepatic cells develop intracellular lumina whose membrane has the morphological appearance of canalicular membrane (Hashieh, Remy et al. 1989). In dispersed thyroid cells, intracellular vacuoles are characterised by the presence of microvilli and resemble follicular lumina. They are thought to represent the site of iodination involved in the thyroid hormone synthesis by dispersed cells (Rousset, Authelet et al. 1986). Intracellular lumens in two adjoining thyroid cells can connect to form a single cavity, thus giving birth to a new follicular structure (Remy, Michel-Bechet et al. 1977). Analogous observations have been made in endothelial cells during angiogenic events *in vitro*; indeed, it was shown that isolated endothelial cells develop intracellular vacuoles that coalesce to form capillary lumens after cell-to-cell adhesion (Davis and Camarillo 1996).

Vacuolation has also been observed *in vivo*. In murine epididyme, ligation induced the formation of unusual cells containing vacuoles with long microvilli on their inner surface (Abe, Takano et al. 1982) and sometimes containing a specific epididymal glycoprotein (Toshimori, Araki et al. 1990). Similarly, in the pancreas of rats with experimental pancreatitis, large vacuoles appear in the Golgi area and contain digestive zymogens (Watanabe, Baccino et al. 1984). The development of vacuoles has been also documented after pathophysiological breakdowns. Indeed, intracellular vesicular compartments have a high occurrence in several carcinomas. In fact, it was shown that the degree of malignancy directly correlates with the number of intracellular lumens. A good example is the invasive breast cancer,

whereby about 50 % of cells display intracellular lumens as compared with only 3.4 % in cases of benign lesions (Tsuchiya 1981).

As described above, vacuoles have been observed mostly in different epithelial tissues or at least in corresponding cells in culture, and in physiopathological conditions. Here, we report that after their isolation from the pancreas, cultured primary β-cells in non-adhesive conditions form apparent intracellular vesicular compartments, with varying size and number. We described the characteristics of the vacuoles developing in islet β-cells and studied their involvement in β-cells function, in particular insulin secretion. We found that vacuoles growth increases with time, non-adhesive conditions and glucose concentrations in the culture media. Interestingly, when tested on single vacuole-containing β-cells, insulin secretion was dampened, while, insulin labeling was still found inside the vacuoles. Noticeably, treatment of cells with an inhibitor of dynamin-dependant endocytosis decreased the number of cells containing vacuoles, while an inhibitor of vesicular trafficking and exocytosis increased the vacuolation in the cells. Taken together, our data strongly suggest that loss of cell-cell and cell-substrate contacts in isolated cultured β-cells divert insulin secretion to intracellular vesicular compartments instead of plasma membrane, a mechanism that could also reveal a loss of polarity in dissociated cultured β-cells.

MATERIALS AND METHODS

Isolation and culture of islet cells. Rat islets were isolated by ductal injection of collagenase and Histopaque purification from the pancreas of male Sprague-Dawley

rats weighing 250-350 g (Sutton, Peters et al. 1986, Lebreton, Lavallard et al. 2019). To prepare single cells, the isolated islets were first incubated for 15 min at room temperature in a Krebs-Ringer bicarbonate (KRB) buffer without added Ca^{2+} and supplemented with 11.2 mM glucose, 0.5% BSA and 3 mM EGTA. They were then disrupted by sequential aspirations through 18-, 21-, and 25-gauge needles (3 aspirations per needle). The cells and clumps obtained at this stage were incubated 3 min at room temperature in a slow rotating spinner flask containing the Ca^{2+}-free Krebs-Ringer bicarbonate buffer supplemented with 11.2 mM glucose, 0.1% BSA and 0.1% trypsin (1:250; Difco Laboratories, Inc., Detroit, MI). The cells were then diluted with sterile RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) containing 11.2 mM glucose, supplemented with 10% fetal calf serum (FCS), 110 U/ml penicillin, and 110 μg/ml streptomycin (hereafter referred to as control RPMI medium). They were centrifuged for 5 min at 130 g, and resuspended in control RPMI medium. This procedure was repeated twice, and aliquots of about 200,000 cells were seeded in 4 ml of the same RPMI medium on 6 cm dishes, a process preventing cell attachment (No. 1007, Falcon Plastics, Oxnard, CA). Where needed, the following treatments were done: FCS was replaced by 0.5% BSA, 2.8 or 16.7 mM glucose-containing RPMI was used, 4 mg/ml Lucifer yellow (LY) (Sigma Chemical Co., St. Louis, MO) or 2 mM EGTA were added to the RPMI medium. Cells were cultured for 2, 5, 6, 8.5 and 24 hours at 37°C, in a humidified incubator gassed with 4% CO_2.

Attachment of cells for vacuoles analysis. For the attachment assay, 50-μl aliquots of cultured cells in RPMI medium were injected into 50-μl glass chambers coated with

0.1 mg/ml poly-L-lysine (mol 150,000-300,000; Sigma Chemical Co., St. Louis, MO) and incubated for 45 min at 37°C to allow cell attachment. Cells were then rinsed with 200 μl of appropriate medium and processed for successive protocols.

Labeling with fluorescent lectins. Cells attached in glass chambers were rinsed with 200 μl control RPMI medium and filled with 100 μl of the same medium supplemented with either 100 μg/ml fluorescein-labeled Wheat Germ Agglutinin (No. L-8131; Sigma Chemical Co., St. Louis, MO) or 100 μg fluorescein-labeled Concanavalin A (No. C-7642; Sigma Chemical Co., St. Louis, MO). The cells were then incubated at 4°C for 1 hour and rinsed 3 times with 200 μl control RPMI medium before being analysed under a microscope fitted with filters for fluorescein detection.

Immunofluorescence. Cells attached in glass chambers were rinsed with 200 μl PBS and filled with 100 μl appropriate fixative. For insulin labeling, the chambers were incubated for 16-20 h at room temperature, either with Bouin's fixative (if chambers were processed before for reverse haemolytic plaque assay (RHPA)) or with a solution of 2 % paraformaldehyde in PBS supplemented with 17 % (w/v) BSA. The chambers were then dismantled and slides were rinsed with PBS, passed through a series of graded ethanols, rinsed again with PBS and incubated for 2 h at room temperature with monoclonal anti-insulin antibodies (Ventrex Laboratories, Portland), diluted 1:200 in PBS. After rinsing with PBS, the slides were incubated at room temperature for 1 h with a fluorescein-labeled serum against mouse immunoglobulin G. For GLUT2 and actin labeling, the chambers were filled with cold acetone or ethanol and incubated 3 min at -20°C. The chambers were then dismantled and the

slides were rinsed with PBS and incubated 2 h at room temperature with rabbit anti-GLUT2 (kindly provided by Bernard Thorens, Lausanne) or rabbit anti-actin antibodies diluted 1:100 and 1:300 in PBS, respectively. After rinsing, the slides were incubated 1 h at room temperature with a fluorescein-labeled serum against rabbit immunoglobulin G, diluted 1:200 in PBS. Finally, the slides were rinsed again with PBS and covered with 0.02% paraphenylenediamine in PBS-glycerol (1:2, v/v) and analysed on a fluorescence microscope fitted with filters for fluorescein detection.

Cell apoptosis. Cells attached in glass chambers were rinsed with 200 μl PBS and filled with 100 μl appropriate fixative. They were permeabilized with Triton X-100 supplemented with 0.1 % sodium citrate for 2 min on ice, washed with PBS and cell apoptosis was assessed using the In Situ Cell Death Detection Kit TMR Red (Sigma).

Labeling with Lucifer yellow. Isolated islet cells were incubated 3 and 16-18 h in the presence of control RPMI medium supplemented with 4 mg/ml Lucifer yellow or LY (Sigma Chemical Co., St. Louis, MO). The cells were then diluted with control RPMI medium and centrifuged 5 min at 130 g. The cell pellet was resuspended in control RPMI medium and centrifuged again 5 min at 130 g. The last operation was repeated twice. The cells were then injected into a glass chamber and analysed on a fluorescence microscope fitted with filters for fluorescein detection.

Treatment with drugs. After isolation and dissociation of rat islets, resulting isolated islet cells were distributed in non-adherent petri dishes and cultured in islet cells medium containing either 10 μM cycloheximide (CHX) (Sigma) that inhibits protein

synthesis, 50 ng/ml brefeldin A (BFA) (Sigma) or 10 μg/ml dynasore-hydrate (DYN) (Sigma) supplemented with 4 mg/ml LY to track endocytosis of vacuoles. DMSO was used as a control for BFA and DYN treatment. After 16-18h of treatment, cells were rinsed, injected into 50-μl glass chambers coated with 0.1 mg/ml poly-L-lysine as previously described and incubated for 45 min at 37°C to allow cell attachment, cell fixation and immunofluorescence to distinguish insulin-producing β-cells from non-β-cells. The chambers were then analysed with a microscope allowing visualization of fluorescence and vacuole-containing cells were counted.

Cell Imaging. During the course of the study, when indicated, the cells were imaged using a Zeiss Microscope equipped with light microscopy, phase contrast microscopy or differential interference contrast microscopy (DIC). For electron microscopy, after 24h of culture in control RPMI medium, cells were centrifuged 5 min at 130 g, and the pellet was fixed in a 2.5% solution of glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), post-fixed in 1% phosphate-buffered osmium tetroxide, dehydrated in graded ethanols, and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and examined with a Philips 301 electron microscope (Philips, Mahway, NJ).

Reverse haemolytic plaque assay. Insulin secretion from β-cells was followed using a RHPA, as previously described (Parnaud, Lavallard et al. 2015). Briefly, cultured β-cells were diluted in a KRB buffer supplemented with 0.1% BSA, 0.1% ascorbic acid and 2.8 mM glucose. 4% (v/v) of packed sheep red blood cells were then mixed with the islet cells and attached to glass chambers coated with poly-L-lysine. After a 45-

min incubation at 37°C, the chambers were rinsed with KRB buffer containing either 2.8 mM or 16.7 mM glucose, and then filled with same buffer supplemented with a heat-inactivated anti-insulin antibody diluted 1:50. After a 30-min incubation at 37°C, the chambers were rinsed, filled with guinea pig complement (Behring Institut, Marburg, Germany) diluted 1:50 in KRB buffer and incubated 60 min at 37°C. The chambers where then exposed to 0.04% Trypan blue for 2 min at room temperature, rinsed, filled with Bouin's fixative, and finally processed for insulin immunostaining.

Cell counts and area measurements were restricted to immune-identified and Trypan blue excluding islet β-cells. Under a microscope equipped with a 40x objective and a phase-contrast illumination we first examined for each β-cell, the presence of intracytoplasmic vacuole(s) and the presence of a surrounding haemolytic plaque. The individual areas of these plaques were then measured using a calibrated grid inserted in the ocular of the microscope. Total plaque development for both vacuole-containing cells and control cells was then calculated by multiplying the percentage of secreting cells by the average area of haemolytic plaques.

Insulin content analysis. Isolated β-cells were identified by immunostaining and pictures were taken with a Zeiss Axio Observer Z1 microscope. The intensity of fluorescence of the insulin staining in the cytoplasm was measured using Zen software (Zen 3.1 blue edition) on more than 80 β-cells.

Membrane potential measurement. To assess the membrane potential, aliquots of cultured islet cells were plated on 35-mm dishes and incubated 2-3 h at 37°C to allow cell attachment. The dishes were then transferred to an inverted ICM405 Zeiss

microscope. Individual cells were impaled with a glass microelectrode (150-200 MΩ) pulled from borosilicate glass tubing and filled with a solution containing 150 mM LiCl, buffered to pH 7.2 with 10 mM Hepes. Immediately before use, the body of the electrode was filled with 3 M KCl. The microelectrode was connected to a pulse generator for passing current and recording membrane potential (Meda, Bruzzone et al. 1986).

Statistical analyses. Our data sets were analysed for statistical significance using PRISM (GraphPad, San Diego, CA) for a two-tail unpaired Student's *t*-test when two groups were compared or one-way ANOVA (Tukey's post-test) when more than two groups were compared.

RESULTS

Identification and morphological characterisation of intracellular vacuoles

When maintained in culture, isolated β-cells, detected by insulin immunolabeling, had one or several intracellular lumens resembling to intracellular vesicular compartments (**Fig. 1A**). Using electron microscopy, we found that these compartments represented spherical cavities of various sizes and were delimited by a membrane, often containing microvilli (**Fig. 1B**). When freshly isolated, β-cells did not contain vacuolar compartments. Formation of these structures was observable in β-cells after 2 hours of culture and their number and size increased with time culture (**Fig. 1C**). After 18 hours of culture, few aggregated β-cells (12.88 ± 0.43 %, n=4)

contained vacuoles as compared to single cells (40.38 ± 2.41 %, n=4) (**Fig. 2A** and **B**), suggesting that these compartments are absent or are exocytosed from β-cells aggregates. In order to assess the impact of cell attachment on vacuole formation, freshly isolated single β-cells were incubated 18 hours either in non-adherent or laminin-coated petri dishes. After incubation under this last condition, cells resulted either round or spread. We found that a higher percentage of single cells maintained in suspension developed vacuoles (28.70 ± 2.05 %, n=5) as compared to the round and attached single cells on laminin (11.75 ± 3.06 %, n=4). None of the spread single cells developed vacuoles on laminin (n=3) (**Fig. 2C**). These results suggest that cell-to-cell contact and cell attachment to its extracellular matrix prevents vacuole formation in β-cells.

To test the possibility that vacuoles represent an invagination of the cell plasma membrane rather than an intracellular organelle, we incubated the cells in the presence of different fluorescent lectins. We found that concavalin A and wheat germ agglutinin labeling was easily detected only on the plasma membrane, but not on the intracellular lumina membrane of these cells (**Fig. 3A** and **B**). Therefore, no path connecting the lumen to the outside of the cell exists, indicating that these vacuoles constitute a real intracellular compartment rather than an invagination of the plasma membrane.

Intracellular vacuoles share common markers with the β-cell plasma membrane

To study whether the membrane of the vacuoles could contain typical plasma membrane markers of primary β-cells, we tested the presence of the glucose transporter 2 (GLUT2) in vacuole-containing cells. By immunofluorescence, we detected GLUT2 at the plasma membrane as well as at the vacuoles' membrane of these cells (**Fig. 3C** and **D**). Furthermore, we looked at the presence of actin, which is known to be expressed at the sub-plasma membrane of β-cells. By immunolabeling, both the plasma and vacuoles' membranes were stained, indicating that actin is associated with these structures (**Fig. 3E** and **F**). Of note, actin and GLUT2 staining (**Fig. 3C** and **3E**) is stronger in vacuole-containing membrane as compared to plasma membrane. This may be due to the higher density of microvilli on vacuolar compartments as compared to plasma membrane. The vacuoles containing-cells excluded Trypan blue at a membrane potential of -49.6 ± 3 mV (n=13), similar to the one measured in control islet cells (-50.7 ± 3 mV, n=13). Also, after quantification of apoptosis in our cell population with or without vacuoles, no differences were found, suggesting that there is no correlation between vacuoles and cell death or decreased cell viability (data not shown). To analyse whether the vacuole compartments were formed by endocytosis of the plasma membrane, a fluid phase marker (LY), was added to the cell culture medium. After 1 hour of incubation in the presence of this fluorescent tracer, small LY-containing vesicles were sparsed into the cytoplasm of all primary islet cells. After a prolonged period of time (18 hours), LY was also found to be present in the vacuoles (**Fig. 3G** and **H**) suggesting

that the former vesicles fused together. These observations show that the vacuole-containing cells are viable, not damaged and suggest that the membrane surrounding the vacuoles shares similar features with the cell plasma membrane.

To test the involvement of newly synthesized proteins in the formation of vacuoles, we used CHX, a drug that inhibits protein biosynthesis (Dai et al., 2013), and followed vacuoles development during time. Cells treated with 10 μM CHX developed vacuoles (37.17 ± 1.42, n = 3) at the same extent as control cells (27.50 ± 5, n = 3) after 18 h of culture. Thus, our results suggest that the vacuoles formation does not require newly synthesized proteins (**Fig. 2D**).

The ability of isolated cells to spontaneously form intracellular lumens could result from the presence of hormones in the FCS used in the culture medium. To test this possibility, we incubated the cells in absence of serum. We found that, even under these conditions, islet cells formed vacuoles (data not shown), suggesting that their formation is serum-independent.

Insulin secretion is redirected to isolated vacuole-containing β-cells

Immunoreactive insulin was detected inside the vacuoles of β-cells by immunolabeling performed on islet cells incubated in the presence of 11.2 mM glucose for 18 hours (**Fig. 4A** and **B**). However, no staining was observed when the cells were incubated either in the presence of 2.8 mM glucose, a condition that does not stimulate insulin secretion, or in 11.2 mM glucose supplemented with 2 mM EGTA, a condition which reduces Ca^{2+} intracellular levels and thus inhibits insulin

release (**Fig. 4C** and **D**). We then compared insulin secretion between vacuole-containing and control β-cells using a reverse haemolytic plaque assay, allowing the quantification of insulin release from single cells. After a 30 min stimulation by 16.7 mM glucose, 26 ± 6.79 % of vacuole-containing single β-cells formed a haemolytic plaque with an average haemolytic plaque area of 918 ± 159 μm^2. In control β-cells, values were significantly higher, with 55.18 ± 3.62 % of single β-cells forming a haemolytic plaque with an average of 1404 ± 164 μm^2 (**Fig. 4E** and **F**) (n=4 for both control and vacuole-containing cells). As a result, total plaque development, which reflects the global secretory contribution of 100 cells, was 4-fold lower for vacuole-containing single β-cells when compared to control single β-cells (**Fig. 4G**). An example of islet β-cell with or without apparent vacuoles in haemolytic plaque assay is represented in **Supp Figure 1**. The insulin contents resulting from vacuole-containing cells (3732 ± 336.7, n=3) were not significantly different from control cells (3128 ± 328.8, n=3) (**Supp Figure 2**). These experiments suggest that, when insulin secretion is stimulated, the hormone is partly released into the vesicular compartment.

Development of vacuoles in cultured β-cells is glucose-dependent

To assess whether glucose can modulate vacuole formation, we incubated islets cells in the presence of different concentrations of glucose during 18 h. We found that in the presence of 16.7 mM glucose, the number of β-cells containing vacuoles is twofold higher (n=4) as compared to the one observed in the presence of 2.8 mM

glucose (**Fig. 5**). In non β-cells, only a low percentage (2-8 %, n=2) of the cells could form vacuoles regardless of the glucose concentration (**Fig. 5**). This observation suggests again that vacuoles develop preferentially in conditions when insulin secretion is stimulated in the β-cell.

Vacuolation is dependent on vesicular trafficking, exocytosis and endocytosis

As described before, the uptake of the fluorescent marker LY from the culture medium is able to reach the vacuoles, suggesting an influence from the endocytic pathway in their formation. To further understand the origin of vacuoles development in isolated islet β-cells, we inhibited both the endocytic and the exocytic pathways using the drugs DYN and BFA, respectively, and measured the percentage of vacuole-containing cells in these conditions. DYN is an inhibitor of dynamin-dependent endocytosis (Wen et al., 2012), while BFA is used to inhibit vesicular trafficking by blocking the transfer of vesicular cargo from the ER to the Golgi and exocytosis of vesicles (Cunha et al, 2017). For this purpose, isolated islets cells were maintained overnight in non-adherent petri dishes in a medium containing either 4 mg/ml LY with or without 10 μg/ml DYN or 50 ng/ml BFA. As shown in the **Fig. 6A**, DYN was able to decrease the percentage of isolated islet β-cells containing vacuoles labeled with LY (0.29 ± 0.015, n=4) as compared to control cells (0.43 ± 0.04, n=3), suggesting that the endocytic pathway, represented by the entry of LY in

the cells was inhibited and depends partly on dynamin. In contrary, BFA enhanced the vacuolation (0.55 ± 0.056, n=4) as compared to control cells only treated with DMSO (0.39 ± 0.0296; n=4) (**Fig. 6B**). By contrast, neither DYN (0.057 ± 0.027, n=3) nor BFA (0.22 ± 0.038, n=3) had a significant effect on vacuolation in aggregated islet β-cells as compared to their respective controls (LY, 0.1 ± 0.019, n=3; DMSO, 0.22 ± 0.04, n=3).

DISCUSSION

The results reported in the present study indicate that β-cells plated after trypsin dissociation under conditions that abolish or reduce cell-cell and cell-substrate interactions form intracellular compartments, which share morphological, biochemical, and physiological properties with elsewhere described vacuoles developing in different epithelial cell types (Vega-Salas, Salas et al. 1987, Gilbert and Rodriguez-Boulan 1991, Honda 2017). We showed that aggregated β-cells or cells attached to laminin-coated petri dish have a reduced tendency to develop vacuoles when compared to isolated β-cells and cultured in suspension. At present, it is unclear if these vacuoles are exocytosed, as in MDCK cells, either after β-cell aggregation or after β-cell re-establishment with an extracellular matrix, or whether aggregated or attached β-cells have a lower capacity to form vacuoles.

There are at least two possible origins for the development of vacuolar structures. One is internalization of the plasma membrane; another is *de novo* membrane synthesis. Both of these hypotheses have been experimentally confirmed in previous studies (Vega-Salas, Salas et al. 1987, Bailey and Kirk 1991, Gilbert and

Rodriguez-Boulan 1991, Utech, Ivanov et al. 2005). In some cases as well, vacuoles were shown to contain specific apical plasma membrane proteins and to exclude basolateral antigens suggesting that these vacuoles could represent an intracellular storage compartment of apical plasma membrane (Vega-Salas, Salas et al. 1987). Using DYN and BFA we showed that the percentage of vacuole-containing cells was affected, suggesting that endocytosis and exocytosis/vesicular trafficking pathways contribute to the formation of vacuoles. Interestingly, we observed that BFA increased vacuolisation, in agreement with the hypothesis that a perturbation of vesicular trafficking and exocytosis may induce intracellular fusion of vesicles and secretory granules to the nascent vacuoles. The vacuoles may originate from a defection fate of endocytic vesicle fusing together to form intracellular lumen. This hypothesis is consistent with our observation that LY, a fluid phase marker which cannot cross the plasma membrane, was found localised in the cells and, particularly, in the vacuoles. Before the formation of these compartments, LY labeling was restricted to the cytoplasm and appeared as discrete little points, which could correspond to small endocytic vesicles. These vesicles may fuse together to form large LY-containing vacuoles, which is consistent with our observation that the number and the size of the vacuoles increase in a time-dependent manner. When isolated β-cells already labeled with LY were maintained in the presence of an inhibitor of dynamin-dependent endocytosis, the entry of the fluorescent marker was found to be restricted, suggesting that this endocytic pathway is partly responsible for the vacuole formation. However, we do not exclude that other types of endocytic pathways such as pinocytosis may also participate in this process.

In the contrary, using an inhibitor of vesicular trafficking and exocytosis, we observed increased vacuolation in the islet cells, suggesting that exocytosis pathway is indeed involved in the development of vacuoles. In addition, the presence of insulin inside the lumen of vacuoles suggests that the exocytosis pathway can also compete with the growth of vacuoles. Two lines of evidence indicate that insulin is secreted into the vacuoles. Firstly, insulin appears into the vacuoles only in the presence of normal Ca^{2+} levels and elevated glucose concentrations, two conditions known to sustain insulin secretion. In this regard, it is interesting to note that using RHPA we showed that total insulin release of vacuole-containing β-cells is lower to that of control β-cells. This result suggests that isolated vacuole-containing β-cells present an impaired trafficking affecting their function: the insulin-containing secretory granules do not reach the plasma membrane and are not ordinarily exocytosed, but rather fused with the membrane delimiting the vacuoles. In *in vitro* conditions, vacuoles formation may reflect a malfunction of the secretory system, which may occur because of a loss of apical differentiation and polarity (Honda 2017). In fact, it has been proposed that, in isolated cells with a loss polarity, newly-formed granules are prevented from reaching the cell surface and are fused together to form a vacuolar compartment with characteristics of plasma membrane (Adler and Gerhards 1981, Remy 1986, Vega-Salas, Salas et al. 1987, Gilbert and Rodriguez-Boulan 1991, Vega-Salas, San Martino et al. 1993). Secondly, these observations are emphasized by the fact that in some cells, vacuoles development is associated with supramaximal stimulation of exocytosis (Garrett and Thulin 1975, Garrett, Thulin

et al. 1978, Adler and Gerhards 1981, Saluja, Saito et al. 1985) and that vacuoles can contain exocytotic materials (Watanabe, Baccino et al. 1984). Several studies also suggest that vacuoles originate from modifications of Golgi apparatuses, such as dilatation and fusion of the cisternae. For example, in HT 29 colonic adenocarcinoma cell line, vacuoles arise from dilatation and fusion of the Golgi apparatur stacks (Remy and Marvaldi 1985, Lebivic, Hirn et al. 1988). This observation was also made for the vacuoles developing in exocrine pancreas (Watanabe, Baccino et al. 1984) and in cultured hepatic cells (Hashieh, Remy et al. 1989). At present, no evidences show that such mechanism could account for vacuole formation in isolated β-cells.

In vivo, intracellular lumens formation have been reported by a great number of authors in a variety of cancerous as well as normal adult or fetal epithelia (for a review Remy 1986). In endocrine pancreas, whether vacuoles development also occurs *in vivo* has not been yet explored. It would be particularly interesting to determine whether the development of vacuoles could account for a defect in insulin secretion from islet cells in animal models of β-cell dysfunction.

Altogether, our study may represent a primer to a new research field focusing on the role of vacuolation in pancreatic β-cells. The intracellular vesicular compartments may represent a storage unit for membrane proteins or they may be part of an endosomal system, which develops in isolated cells, in conditions of cell-to-cell and cell-to-substrate loss. They may also correspond to an abnormal secretory process that enables the cell to re-establish its lost polarity (Spriggs and

Meek 1961) by a stimulation of surface structure and thus to preserve cell identity. The vacuole could also represent a metabolic organelle, where metabolites are stored after endocytosis of the extracellular milieu. This is the case for the unicellular fungi, where the vacuole is a well-characterised compartment and is involved in degradative processes, osmoregulation, and plays a role in the precise regulation of cytosolic ion concentration and pH (Klionsky, Herman et al. 1990, Yang, Zhang et al. 2020). Future research will give evidences whether such functions are also related to the vacuoles developing in the epithelial cells.

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ETHICS DECLARATION

The authors declare no competing interests.

AUTHOR CONTRIBUTION:

Concept and design of the research: DB; carrying out the experimental work: DB, SL, DCD; data analysis and interpretation: DB, SL, DCD; writing of the article: DB, SL, DCD.

Abbreviations: MDCK: Madin-Darby canine kidney; FCS: Fetal Calf Serum; LY: Lucifer Yellow; DIC: Differential Interference Contrast; RHPA: reverse haemolytic plaque assay; KRB: Krebs-Ringer bicarbonate; BFA: brefeldin A; DYN: dynasore; GLUT2: glucose transporter 2; CHX: cycloheximide

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FIGURE LEGENDS

Figure 1. Identification and characterization of intracellular vacuoles in cultured islet β-cells. A. Images of dissociated β-cells cultured for 16-18 hours using differential interference contrast microscopy (DIC). The first image on the left shows a typical β-cell without vacuoles. The following images show intracellular vacuoles with varying size and number. **B**. Electromicrograph (EM) of a β-cell showing the ultrastructure of an intracellular vacuole, delimited by a membrane. The white arrows show the microvilli. The bar represents 10 μm in A and 0.5 μm in B. **C**. Time points. ***Top Panel***: Representative experiment of the percentage of β-cells with apparent cytoplasmic vacuoles just after isolation (0 h), or 2, 6, 8.5 and 24 h after culture in non-adherent petri dishes. ***Bottom panel:*** Representative experiment of the respective mean diameter of the vacuoles formed inside the β-cells over time. The number of β-cells analysed is shown in parentheses.

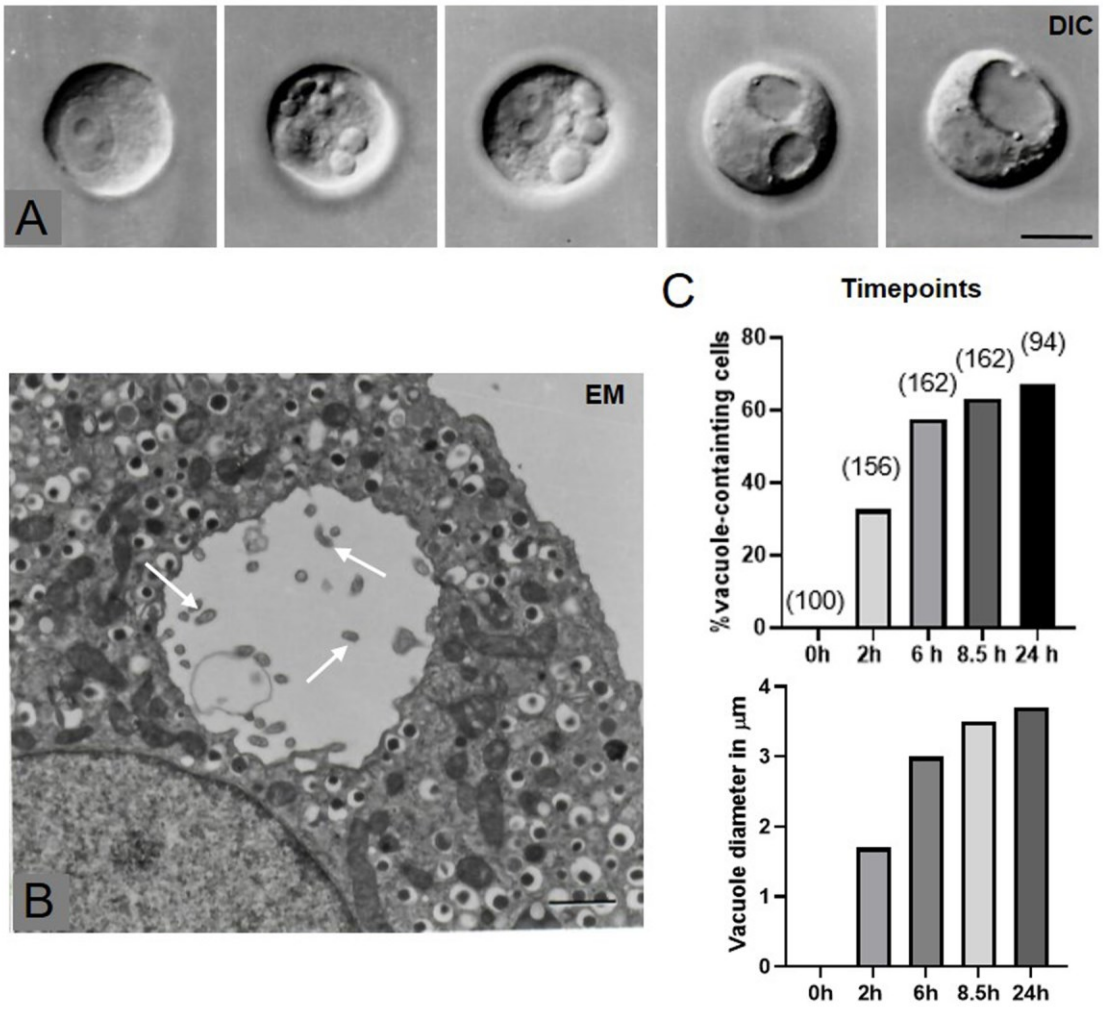

Figure 2. Effect of cell-cell contact and cell attachment in the development of vacuoles. A. Phase-contrast image of a single β-cell with a vacuole and a β-cell aggregate without vacuoles. **B**. Percentage of single or aggregated β-cells containing vacuoles after 24 h in culture. **C**. Percentage of isolated β-cells containing vacuoles maintained 24 h in culture either in suspension (**1**), round and attached to laminin (**2**) or spread and attached to laminin (**3**). **D**. Percentage of single β-cells containing vacuoles after 24 h in culture with or without CHX. Data are represented as means

+/-SEM. *p<0.05, **p<0.01 and ****p<0.0001. Statistical analyses were performed using one-way *ANOVA*.

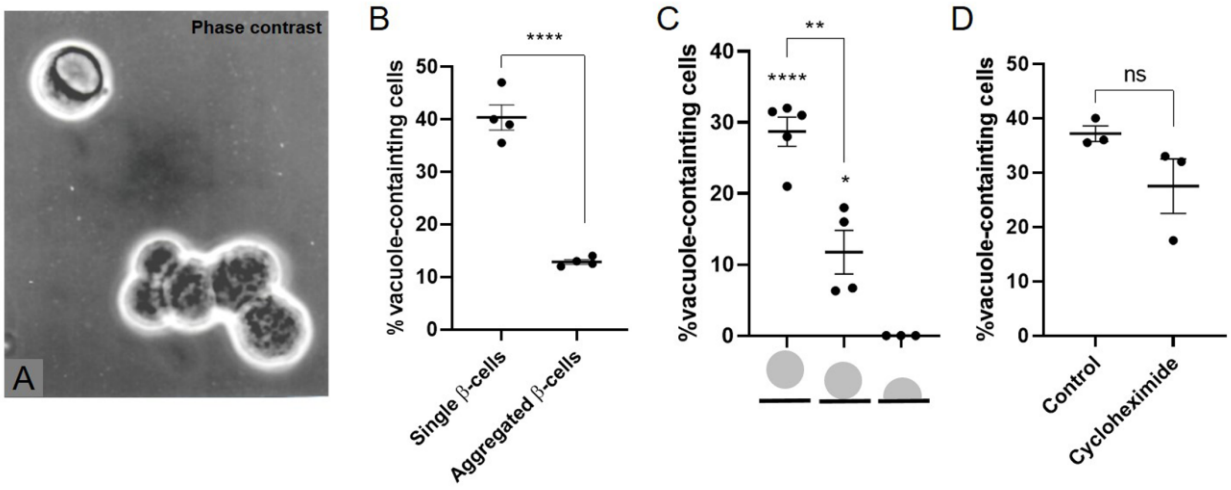

Figure 3. Intracellular vacuoles share common markers with β-cell plasma membrane. Phase-contrast (**A**, **C**, **E, G**) and corresponding immunofluorescence pictures (**B**, **D**, **F, H**) of β-cells containing vacuoles labeled with antibodies against concavalin A (**A**, **B**), GLUT-2 (**C**, **D**), actin (**E**, **F**) or incubated for 16-18h in complete RPMI media in the presence of Lucifer yellow (**G**, **H**). The bar represents 10 μm in **A-D** and 30 μm in **G-H**.

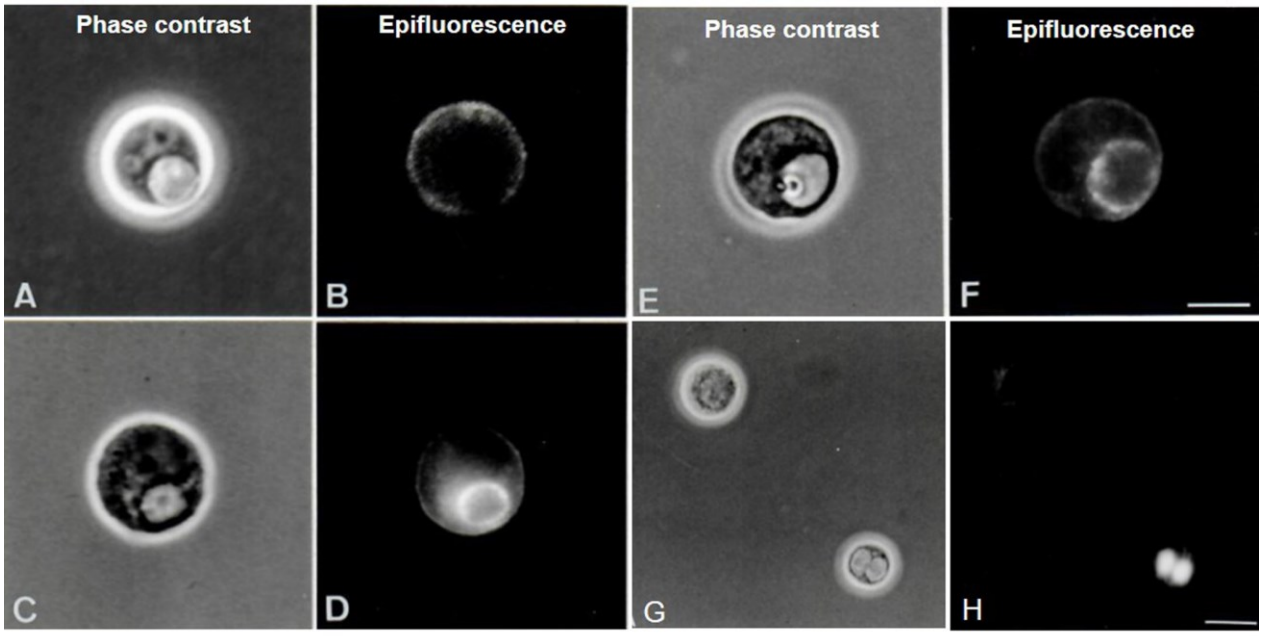

Figure 4. Insulin secretion is diverted from plasma membrane to vacuoles in single β-cells. Phase-contrast (**A**, **C**) and corresponding immunofluorescence pictures (**B**, **D**) of β-cells containing vacuoles labeled with antibodies against insulin after 16-18h of culture in complete RPMI medium supplemented with 11.2 mM glucose (**A**, **B**) or EGTA (**C**, **D**). The bar represents 10 μm. **E-G.** Insulin secretion evaluated by the reverse haemolytic plaque assay. Percentage of cells forming vacuoles (Vac) in single β-cells surrounded by a haemolytic plaque as compared to control cells present in the same preparation (**E**). Plaque area of vacuole-forming β-cells as compared to control cells (**F**). Total plaque development of vacuole-forming cells as compared to control cells (**G**). Data are represented as means +/- SEM. *p<0.05; **p<0.01; ns = non-significant. Statistical analyses were done using two-tailed unpaired *Student t-test*.

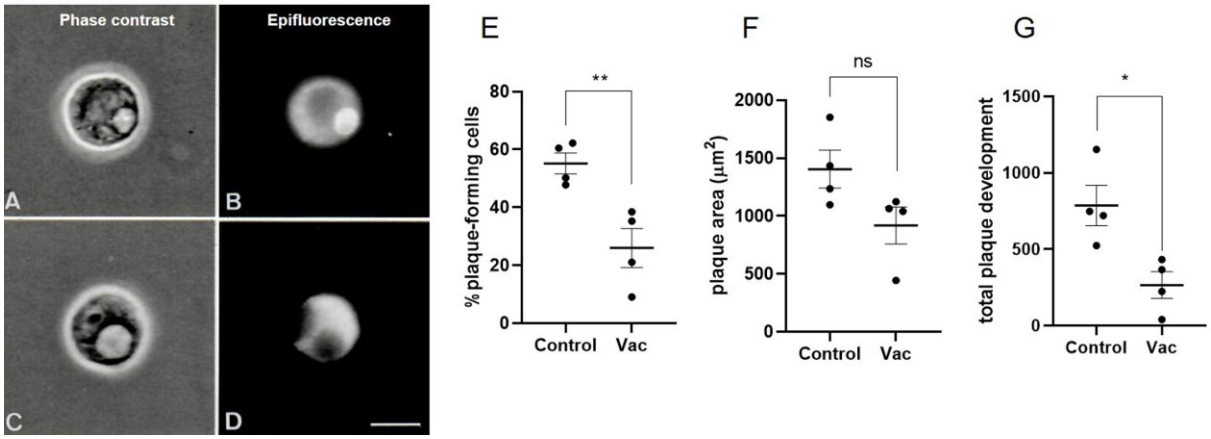

Figure 5. Presence of vacuoles in cultured β-cells is dependent on glucose concentration. The graph represents the percentage of isolated β-cells and non-β-cells containing vacuoles cultured for 18 h in a medium supplemented with 2.8, 11.2 and 16.7 mM glucose. Data are represented as means +/-SEM. **p<0.01 and ***p<0.001. Statistical analyses were done using one-way *ANOVA test*.

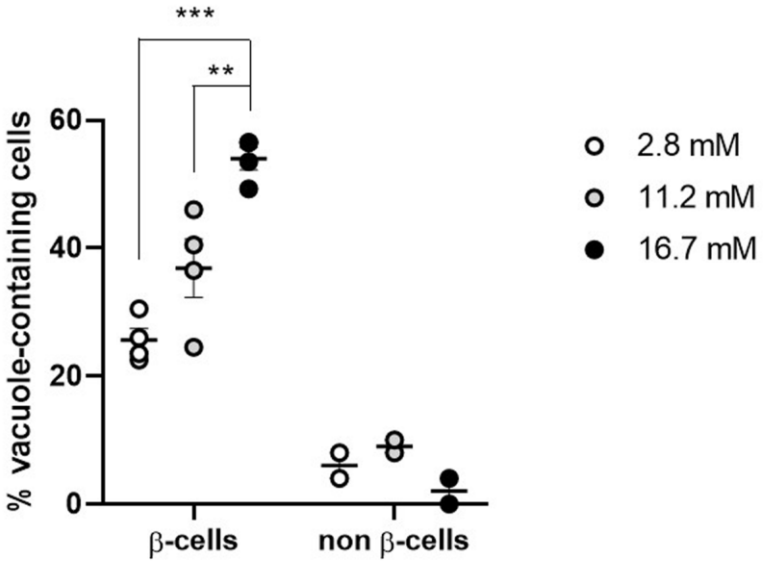

Figure 6. Treatment of dynasore or brefeldin A on vacuoles development in β-cells. The graphs represent the percentage of isolated or aggregated β-cells with apparent cytoplasmic vacuoles 18h after culture in different treatments in non-adherent petri dishes. (**A**) Islets cells were treated with 4 mg/ml Lucifer yellow (LY) and with 10 μg/ml dynasore (DYN). (**B**) Islets cells were cultured in a medium containing 50 ng/ml brefeldin A (BFA) or DMSO (Control). Data are represented as means +/-SEM. *p<0.05. Statistical analyses were done using two-tailed unpaired Student t-*test*.