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SHORT COMMUNICATION

NON-INVASIVE SINGLE CELL pH MEASUREMENTS IN THE ISOLATED PERFUSED PANCREAS

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SUMMARY

1. A new method was developed for non-invasive investigations of intracellular pH (pH_i) regulation in different cell types of the isolated perfused pancreas using a confocal laser scanning technique.

2. After removal of the rat pancreas the coeliac artery was cannulated and the splenic segment of the pancreas was perfused with dextran (5%)–Ringer solution at a constant flow rate of 2 mL/min. In a temperature-controlled (37°C) chamber, pH regulation was studied using the pH-sensitive fluorescent dye 2′,7′-bis-(2-carboxyethyl)-5-(-6)-carboxyfluorescein (BCECF) with a confocal microscope (MRC-600; Bio-Rad, Hercules, CA, USA).

3. Image analysis permitted the identification and comparison of different cell types with a pH_i of 7.26 ± 0.1 in acinar cells and of 7.02 ± 0.1 in endothelial cells. Increasing P_{CO_2} from 5 to 20% resulted in a rapid decrease in pH_i. Omission of sodium from the perfusate resulted in a smooth decline in pH_i. Both decreases were found to be fully reversible. Increasing P_{CO_2} under sodium-free conditions also resulted in a drop of pH_i that was, however, not fully reversible, suggesting involvement of the Na^+/H^+ exchanger in the regulation of pH_i in the intact organ.

4. The above method completely preserves tissue integrity and, therefore, allows the study of pH regulation in different cell types of the pancreas simultaneously and without interference with their functional arrangement. The technique should be of specific value to investigate experimental disease states of the pancreas.

Key words: 2′,7′-bis-(2-carboxyethyl)-5-(-6)-carboxyfluorescein, confocal microscopy, image analysis, intracellular pH, isolated perfused pancreas, organ architecture.

INTRODUCTION

The mechanisms that regulate the intracellular and extracellular pH in the tissue microenvironment of every organ system are of critical importance for various pathophysiological conditions, such as hypoxic damage,[1] inflammation[2] and malignant tumour invasion.[3] To address the fundamental issues relating to pH_i regulation, a number of techniques have been developed. These include pH-sensitive electrodes that predominantly measure extracellular pH in the interstitium,[4] magnetic resonance spectroscopy, which is a non-invasive method that cannot, however, distinguish between different cell types within an organ,[5] and fluorescent pH-sensitive dyes that have been used predominantly to study pH in freshly isolated or cultured cells.[6]

The pancreas is a notoriously inaccessible organ and studies addressing its pH_i regulation in health and disease have used either pH-sensitive electrodes that were inserted into the surgically exposed organ[7] or had to rely on isolated cell preparations that inherently have to exclude the tissue microenvironment and lack intact gap junctions and cell–cell contacts.[8] To overcome the limitations of these techniques, we have developed a non-invasive method that uses the pH-sensitive fluorescent probe 2′,7′-bis-(2-carboxyethyl)-5-(-6)-carboxyfluorescein (BCECF) and allows us to study intracellular pH alterations by laser confocal microscopy in different cell types of the isolated perfused pancreas with only minimal impairment of organ structure.

METHODS

The pancreas was removed from male Wistar rats under urethane anaesthesia (1.4 g/kg, i.p.), as reported previously,[9] and, after ligation of the side branches of the superior mesenteric artery, the organ was fixed in a coverslip chamber under constant perfusion via the coeliac artery (flow rate 2 mL/min). The perfusate was continuously gassed with 95% O_2 and 5% CO_2 at 37°C and contained (in mmol/L): NaCl 117; KCl 5.6; $NaHCO_3$ 25; NaH_2PO_4 1; $MgCl_2$ 1; $CaCl_2$ 2.5; glucose 5; dextran T70 (5% wt/vol). The perfusion medium was buffered to pH 7.4 with 10 mmol/L *N*-2-hydroxyethylpiperazine-*N*-ethanesulphonic acid. For experiments in which sodium-free buffer was required, NaCl and $NaHCO_3$ were substituted by *N*-methylglucamine and choline carbonate, respectively, while NaH_2PO_4 was omitted. For intracellular pH measurements using the pH-sensitive BCECF (Molecular Probes, Eugene, OR, USA), the pancreas was loaded with the membrane permeant ester of BCECF (AM) by either topical or intravascular (coeliac artery) application (10 μmol/L) for 20 min. The requirement for cleavage of the AM-ester by intracellular esterases before pH-dependent fluorescence

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develops makes interference by either extracellular BCECF-AM with the pH signal or non-specific binding of BCECF unlikely.[8] The entire pancreas in the coverslip perfusion chamber was placed under a confocal laser scanning microscope (argon and helium–cadmium laser; MRC-600; Bio-Rad, Hercules, CA, USA) and fluorescence emission was recorded at 535 nm after excitation at 440 nm (the BCECF pH-independent excitation wavelength) as well as at 490 nm (the BCECF pH-dependent excitation wavelength; Fig. 1a,b). Calculation of the ratio intensities at the two excitation wavelengths of 440 and 490 nm allowed for intrapancreatic pH measurements that were independent of possible intensity losses due to photobleaching (Fig. 1c,d). To calibrate pH_i measurements in this optical system, we used 1 mm glass capillaries that were placed in the coverslip chamber and filled with a buffer solution that largely reflects the cytosolic ion composition (in mmol/L: KCl 110; NaCl 5; Hepes 10; $MgCl_2$ 1) and that was adjusted to different pH values ranging between 6 and 8 in the presence of BCECF. This calibration technique resulted in a linear correlation between the pH and the 490/440 nm emission ratio (Fig. 1e). A signal calibration using the nigericin-ionophore method, which is the preferred method for isolated single cells, proved inappropriate in the perfused organ model because it requires increasing and depolarizing potassium concentrations, which, in turn, induce profound blood vessel contractions and a very inhomogeneous perfusion of pancreatic tissue.

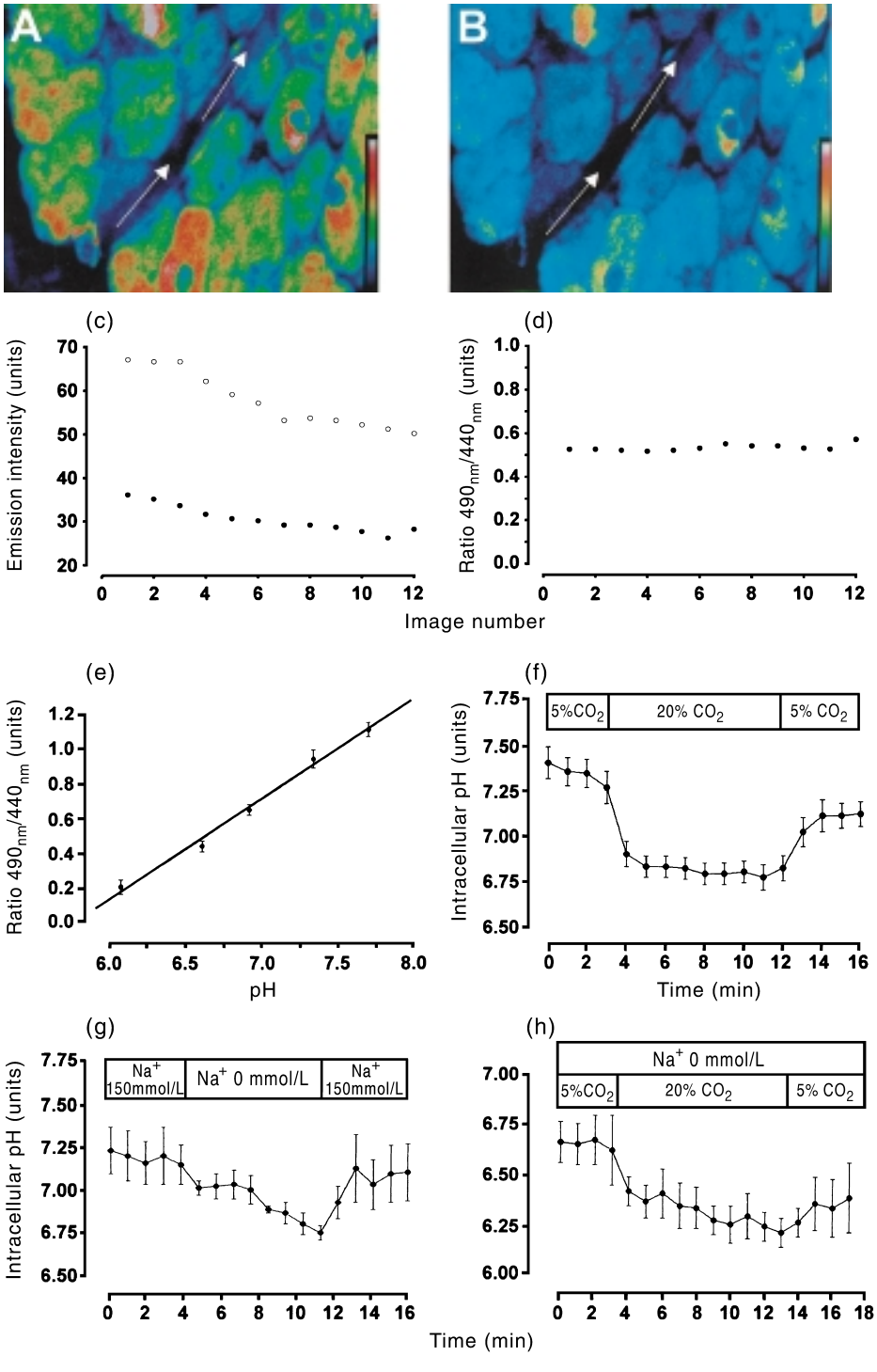

Fig. 1 (a,b) The isolated perfused pancreas in the coverslip chamber was placed under the confocal fluorescence microscope and pH alterations in the surface tissue were recorded (1 image/min over 15 min) after intravascular 2′,7′-bis-(2-carboxyethyl)-5-(-6)-carboxyfluorescein (BCECF) loading and calculated as the ratio between the 490 nm excitation (a) and 440 nm excitation (b). The confocal optical resolution permits a distinction between acinar cells and the endothelial cells lining a surface capillary (arrows). (c) A representative experiment demonstrates the laser-induced decrease in emission intensity at the wavelengths of 490 and 440 nm during a standard recording procedure (rate 1 image/min). (d) During the same experiment as in (c), the 490/440 nm ratio measurements, which were used to study pH regulation, remained stable. (e) *In vitro* pH calibration using BCECF-adjusted (10 µmol/L) glass capillaries in the perfusion chamber. (f) Increasing the perfusion buffer CO_2 to 20% results in a rapid decrease in pH_i in individual acinar cells that is reversible upon returning the CO_2 pressure to 5%. (g) Replacing sodium in the buffer reduces acinar cell pH_i in a slower fashion than CO_2 increases, but this acidification remains reversible upon sodium replacement. (h) In the absence of sodium, a CO_2-induced acidification is not reversed after the return of the CO_2 pressure to 5%. (c–f) Data are the mean±SD of ≥18 or more individual measurements.

RESULTS

By using the high optical resolution of the confocal pinhole technique and the fluorescent signals from the single wavelength lasers, we were able to not only perform pH_i measurements in individual cells at the organ surface but were also able to differentiate between different cell types (Fig. 1a,b). Surprisingly, the cytosolic pH in pancreatic acinar cells was found to be consistently higher (7.26 ± 0.10) than the pH_i in the endothelial cells of surface capillaries (7.02 ± 0.10; $n = 20$; $P < 0.05$). Although the high-energy laser induced a considerable decrease in fluorescence intensity at both wavelengths (Fig. 1c), the use of the 490/440 nm ratio measurements for pH_i calculations indicated that the intracellular pH remained stable over the entire observation period (Fig. 1d). To test the sensitivity of the system, we increased the P_{CO_2} from 5 to 20%. Within minutes, the pH_i in pancreatic acinar cells decreased to 6.75 and recovered promptly after the return to 5% CO_2 (Fig. 1f). Similarly, the omission of sodium in the perfusion buffer by replacing NaCl and $NaHCO_3$ resulted in a continuous decline of the intra-acinar cell pH that was, again, fully reversible after sodium replacement (Fig. 1g). However, when CO_2 was raised to 20% in the absence of sodium, the resulting acidification to pH_i 6.25 was not reversible to normal levels when the CO_2 was returned to 5% (Fig. 1h).

DISCUSSION

Using confocal laser microscopy in conjunction with pH-sensitive probes to study intracellular pH regulation in an intact perfused organ has several advantages.

1. Cells at the organ surface can be studied not only at the level of single cells, but different cell types can be easily identified and differentiated according to their morphological phenotype. A pH_i heterogeneity, as described earlier, has previously also been found using nuclear magnetic resonance techniques (J Gronczewski, unpubl. obs., 1999). Our approach demonstrates that this heterogeneity is due to the different basal pH_i of endothelial and acinar cells, suggesting different basal activities of the pH_i regulatory systems for both cell types.
2. Individual cells can be studied with respect to their pH_i regulation within the context of the tissue microenvironment (i.e. without the need to disrupt cell–cell adhesions and gap junctions or the need to resort to cell culture systems).
3. The system responds in a highly dynamic manner to external stimuli that affect intracellular pH, it is stable and it yields highly reproducible results regardless of whether the pH-sensitive probe is applied topically or into the vascular perfusate. Our data strongly suggest that pH_i regulation in the intact pancreas is sodium dependent, suggesting the presence of either a Na^+/H^+ exchanger or a Na^+/HCO_3^- cotransporter or both.[8]

The advantages of the method reported here are found in neither of the alternative approaches, including magnetic resonance spectrosopy, tissue electrodes or electrophysiological single cell measurements. Although we have demonstrated the efficiency of this system in the isolated perfused pancreas, other organ systems, such as the perfused kidney,[10] the gut[11] or malignant tumour grafts[12] are as easily accessible to studies addressing pH regulation under physiological and pathological conditions.

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