Various fluorescent probes were assessed for investigating intact islets of Langerhans using two-photon excitation imaging. Polar fluorescent tracers applied on the outside rapidly (within 3 min) penetrated deep into the islets via microvessels. Likewise, an adenovirus carrying a Ca\(^{2+}\)-sensitive green fluorescent protein mutant gene, yellow cameleon 2.1, was successfully transfected and enabled ratiometric cytosolic Ca\(^{2+}\) measurement of cells in the deep layers of the islets. Interestingly, FM1-43, which is lipophilic and does not permeate the plasma membrane, also rapidly reached deep cell layers of the islets. In contrast, lipophilic fluorescent probes that permeate the plasma membrane (for example, fura-2-acetoxymethyl and BODIPY-forskolin) accumulated in the superficial cell layers of the islets, even 30 min after application. Thus, two-photon excitation imaging of pancreatic islets is a promising method for clarifying signaling mechanisms of islet cells, particularly when it is combined with membrane-permeable probes. In addition, our data suggest that membrane-permeable antagonists may affect only the superficial cell layers of islets, and so their negative effects should be interpreted with caution. *Diabetes* 51 (Suppl 1):S25–S28, 2002

The two-photon excitation microscope uses a near-infrared ultrashort-pulse laser for the excitation of fluorescent probes rather than the visible continuous laser used in the confocal microscope (1). Because two-photon excitation only occurs at the focal plane of the objective lens (1), tomographic images can be obtained without the use of a confocal pinhole in front of the photodetector. This imaging technique is particularly suited for investigating living tissue preparations. First, phototoxic effects are markedly reduced because the excitation occurs only at the focal plane. Second, the effects of light scattering are minimized by the use of near-infrared light for excitation and by the absence of confocal pinhole for photodetection. In fact, two-photon excitation imaging has been applied to various neuronal preparations (1–3) and exocrine tissue (4) and has proven to be a promising method for the analysis of cellular mechanisms in intact tissues, which preserve their original structures. Such imaging has also been conducted in pancreatic islets, but only with autofluorescence (5,6).

Here, we have assessed various fluorescent indicators for investigating intact islets of Langerhans using two-photon excitation imaging. We have found that hydrophilic probes are particularly useful because they readily penetrate into the deep cell layers of islets via microvessels. In contrast, membrane-permeable substances tend to accumulate in the superficial cell layers.

**RESULTS**

Hydrophilic probes. When acutely isolated islets were perfused with a solution containing hydrophilic tracers, such as lucifer yellow (Fig. 1A) or sulforhodamine (data not shown), highly fluorescent areas (Fig. 1A, the white arrow and arrowheads) and puncta (Fig. 1A, the blue
arrow and arrowheads) quickly appeared within 30 s (Fig. 1B), which apparently reflected the major blood vessels and microvessels in the islets, respectively. In addition, we noted all the interstitial spaces of islets were subsequently filled with tracers (Fig. 1A, the green arrow and arrowheads; Fig. 1B). These structures were evenly stained between the inner as well as outer layers of islets, when images were obtained at planes within 20–50 μm of the bottom of an islet on a glass coverslip. Thus, two-photon excitation imaging can visualize deep cell layers (down to the third or fifth layers) in the islet. These staining patterns were preserved in islet preparations cultured for >6 days.

The adenovirus vector can be transfected into deep cell layers of an islet. Figure 1C shows an islet transfected with an adenovector carrying the gene encoding yellow cameleon 2.1. Fluorescent expression was detected in the cells deep within the islet (Fig. 1C). The fact that expression levels of cameleon 2.1 greatly differ from cell to cell indicates that cameleon cannot penetrate the gap junctions (12). Furthermore, synchronous increases in the fluorescence ratio at 480 nm/530 nm were induced in many cells in the islet by application of a high-concentration potassium solution.

**FIG. 1.** Two-photon excitation imaging of islets of Langerhans with hydrophilic substances. A: An image of an islet immersed in lucifer yellow (1 mmol/l) for 30 s. The XY scanning was made at the plane 25 μm above the glass surface. B: Time courses of fluorescence intensity at four distinct areas: extra-islet (the red arrow and circle in A), major blood vessels (the white arrow and arrowheads in A), microvessels (the blue arrow and arrowheads in A), and interstitial spaces (the green arrow and arrowheads in A). Fluorescence values were obtained at the areas with the same size as the red circles and normalized with those at 150 s after the application of lucifer yellow. Each color of the traces reflects one of four distinct areas in A depicted by arrows. C: An image of an islet that was transfected with adenovirus carrying yellow cameleon 2.1. D: Time courses of fluorescence ratios for the two emission wavelengths (480 nm/530 nm) acquired at six areas depicted in C (a: red; b: orange; c: green; d: cyan; e: blue; f: black). The hatched bar indicates the period of application of 150 mmol/l KCl from a glass pipette to the islet.
from a glass pipette, indicating that Ca\(^{2+}\) signaling is preserved after the virus infection. Thus, GFP-based Ca\(^{2+}\) indicator can monitor the cytosolic Ca\(^{2+}\) concentration of cells in deep layers of islets, unlike acetoxymethyl esters of organic Ca\(^{2+}\) indicators (Fig. 2E).

**Lipophilic probes.** FM1-43 is a lipophilic yet membrane-impermeable tracer (13). We found that FM1-43 also brightly stained the islet blood vessels and the plasma membrane facing the interstitial space throughout the islet preparations within 3 min (Fig. 2A). The fluorescent intensity of blood vessels in the middle of the islets was as strong as those in the superficial layers (Fig. 2B), and there was little cytosolic staining within 20 min after application. In contrast, BODIPY-forskolin and fura-2-acetoxymethyl, which are lipophilic and membrane-permeable tracers, were accumulated in the superficial cell layers of the islets (Fig. 2C and E) and did not penetrate into deeper cell layers even 30 min to 2 h after the application (data not shown) (14). This result indicates that, even though these dyes are dissolved into solution by DMSO, they probably stick to the plasma membrane of cells in the superficial cell layers, are internalized, and do not readily leave the superficial cell layers. Therefore, the concentrations of substances that can reach the deeper cell layers can be very low. Lipophilic probes that have unusually high membrane permeability, however, were found to penetrate into deeper cell layers but over a longer period of time. For example, TMRE stained mitochondria throughout the islet 30 min after application.
DISCUSSION
We have demonstrated that various fluorescence probes can be successfully applied for two-photon excitation imaging of the whole-islet preparations. The rapid penetration of hydrophilic substances via blood vessels is consistent with nearly synchronous increases in NADH autofluorescence throughout the islet during high glucose stimulation (5). Direct visualization of islet interstitial space with either polar tracers or FM1-43 will provide a promising methodology for the direct imaging of insulin exocytosis in the whole-islet preparations.

On the other hand, membrane-permeable fluorescence probes tend to get trapped in the superficial layer of the islets and exhibited steep concentration gradients along the depth axis of a single islet (Fig. 2D and F). Thus, the most efficient method for cytosolic loading of the cells in the islet seems to involve virus-mediated gene transfer. In fact, we have shown that an adenovirus carrying the cameleon 2.1 gene can induce expression of cameleon proteins in deep cell layers of islets and enable the detection of increase in cytosolic Ca2+ by depolarization (Fig. 1C), which was impossible with commonly used acetoxyethyl esters of organic Ca2+ indicators (Fig. 2E).

It has been reported that insulin exocytosis from single β-cells shows high sensitivity to cytosolic cAMP (12,15). In contrast, cytosolic cAMP was claimed to play a less significant role in the whole-islet preparation because the membrane-permeable antagonist of cAMP, Rp-cAMP (16, 17), or inhibitors (protein kinase inhibitors [PKIs]) of cAMP-dependent protein kinase (PKA) (9,10) showed relatively weak effects on glucose-induced insulin secretion. It needs to be noted, however, that Rp-cAMP did not sufficiently reach the deep cell layers of islets (18,19) and that the myristoylated PKI peptide (18) or PKI peptide in liposome (19,20) was trapped in the most superficial layers of islets. Direct evidence for the involvement of cAMP/PKA in insulin exocytosis will be gained either by using adenoviruses carrying cAMP/PKA inhibitory peptides or proteins or by using two-photon excitation imaging of insulin exocytosis in whole-islet preparations.

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