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Immuno-localization of sulphonylurea receptor 1 in rat pancreas

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Abstract

Aims/hypothesis. A sulphonylurea receptor, SUR1, and an inward rectifier potassium channel, Kir6.2, reconstitute the ATP-sensitive K^+ channel that mediates glucose-induced insulin secretion in pancreatic beta cells. We reported previously that Kir6.2 were localized at insulin-, glucagon-, and somatostatin-producing cells. In this new study we aimed to determine the distribution of SUR1 in rat pancreatic islets and to suggest the location of the ATP-sensitive K^+ channels in the islet.
Methods. Western blot analysis was carried out using two anti-SUR1 antibodies, which had been raised against different portions of rat SUR1. SUR1, Kir 6.2, and islet hormones were then localized by indirect immunofluorescence staining of the cryosections of rat pancreas.
Results. In Western blot analysis, each of the anti-SUR1 antibodies detected a band at 140 kDa, which is close to the predicted molecular weight of SUR1, in the homogenate of isolated pancreatic islets. Double immunofluorescence staining of cryosections showed that SUR1 occurred all over the islets, and that SUR1 colocalized with insulin, glucagon, somatostatin, and pancreatic polypeptide. Kir6.2 was also shown to be present in pancreatic polypeptide cells.
Conclusion/interpretation. Together with our previously reported data, the above findings indicate that K_{ATP} channels comprising SUR1 and Kir6.2 occur not only in beta cells but also in the alpha, delta, and pancreatic polypeptide cells of the pancreatic islets, suggesting that therapeutic sulphonylureas could act on these cells directly. [Diabetologia (1999) 42: 1204–1211]

Keywords Sulphonylurea receptor, ATP-sensitive potassium channel, SUR1, Kir6.2, insulin, glucagon, somatostatin, pancreatic polypeptide, pancreas.

Sulphonylureas such as tolbutamide and glibenclamide are hypoglycaemic drugs widely used in the treatment of non-insulin-dependent diabetes mellitus [1]. So far two sulphonylurea receptors have been cloned: SUR1 with a high affinity for glibenclamide [2] and SUR2 with a low affinity [3–5].

Recent genetic and electrophysiological studies have determined that ATP-sensitive potassium (K_{ATP}) channels comprise two subunits, a SUR subunit of the ATP-binding cassette (ABC) superfamily and an inwardly rectifying K^+ channel subfamily (Kir6.0) member [6–8]. Coexpression of hamster SUR1 and mouse Kir6.2 in COS-1 or COSm6 cells reconstitutes K^+ channel currents with properties similar to those of the K_{ATP} channels found in pancreatic beta cells [6].

In the current model of glucose-induced insulin secretion, the metabolism of glucose in pancreatic beta cells increases the intracellular ATP/ADP ratio, clos-

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Abbreviations: ABC, ATP-binding cassette; K_{ATP} channel, ATP-sensitive K^+ channel; Kir, inward rectifier potassium channel; NBF, nucleotide-binding fold; NDP, nucleoside diphosphates; PP, pancreatic polypeptide; SUR, sulphonylurea receptor.

ing the K_{ATP} channels and depolarizing the beta-cell membrane to open the voltage-dependent Ca^{2+} channels and thus raise the intracellular Ca^{2+} level. This triggers exocytosis of the insulin secretory granules [7, 9]. It is thought that sulphonylureas stimulate insulin secretion by inhibiting the activity of the K_{ATP} channel complex of SUR1 and Kir6.2. Northern blot analysis has found SUR1 mRNA to be expressed mainly in the pancreatic islets in vivo [6], and SUR1 mRNA has been localized to mouse pancreatic islets by in situ hybridization [10]. Which type of islet cells produce SUR1 in vivo has, however, not been determined. Detailed localization of SUR1 is necessary to fully understand the effects of the sulphonylureas and the secretory mechanism of islet hormones. In this study, we have examined the distribution of SUR1 in rat pancreatic islets at the cellular level by immunohistochemistry.

Materials and methods

Generation of anti-SUR1 antibodies. The study was carried out in accordance with the guide lines of the Animal Care Commitee of Gunma University, Japan. Anti-SUR1 antibody was raised in a rabbit against the synthetic peptide KPETLLSQKDSVFASFVRADK corresponding to the 21 C-terminal amino acid residues of rat SUR1 [2]. The antibody was purified by immunoaffinity column chromatography as in our previous report [11]. Another antibody against SUR1 was produced in a guinea pig by multiple injections of the synthetic peptide KNWPDQGKIQIQNLSV at positions 1336 to 1351 of rat SUR1 (Accession number L40624).

Transfection and preparation of membrane fractions. COS-1 cells were transfected with a mammalian expression vector pCMV6b (2 μg), or with pCMV6b vector containing rat SUR1, SUR2A, or SUR2B (2 μg each) using the lipofectamine method, as described previously [6, 11]. The cells were washed three times three days after transfection, with PBS and suspended in a buffer consisting of 50 mmol/l Tris-HCl (pH7.5), 1 mmol/l EDTA, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μmol/l pepstatin A, and 100 μmol/l 4-amidinophenylmethanesulphonyl fluoride hydrochloride (PMSF). The cells were then sonicated and centrifuged at 100 000 *g* for 1 h. The pellet was resuspended in 500 μl of the same buffer and stored at –80 °C.

Preparation of pancreatic cells. Male Wistar rats of 12 weeks of age (supplied from the Animal Breeding Facility, Gunma University, Gunma, Japan) were anaesthetized with ether, and islets of Langerhans were isolated from their pancreas, basically according to the method of Pipeleers et al [12]. Islets were detached from exocrine cells by partial digestion with collagenase P (Boehringer Mannheim, Mannheim, Germany), and both isolated islets and exocrine cells were manually collected under a dissecting microscope. They were suspended in a buffer containing 50 mmol/l Tris-HCl (pH7.5), 1 mmol/l EDTA, 2 μg/ml leupeptin, 0.1 MIE aprotinin, 2 μmol/l pepstatin A, and 2 mmol/l PMSF, and homogenized by sonication.

Western blot analysis. Proteins were denatured in a modified sample buffer [13] and electrophoresed on a 7 % SDS-polyacrylamide gel. Electroblotting and signal detection were carried out as described previously [11, 14, 15]. Blotted polyvinylidene fluoride membranes (Immobilon, Millipore,Tokyo, Japan) were incubated with rabbit anti-SUR1 antibody at a concentration of 0.19 μg/ml, with guinea pig anti-SUR1 antibody at a concentration of 0.76 μg/ml or with anti-glucose transporter 2 (GLUT2) antibody (1:5000 dilution; Biogenesis, Poole, Britain).

Tissue preparation for immunohistochemistry. We anaesthetized 1-month-old male Wistar rats with ether or pentobarbital sodium. The pancreata were dissected out, immediately embedded in Tissue-Tek OCT compound (Miles, Inc., Elkhart, Ind., USA), and frozen in liquid nitrogen. Frozen sections (5 μm) were cut with a Jung CM 3000 cryostat (Leica, Wien, Austria), mounted on poly-L-lysine-coated slides, and fixed in acetone at –15 °C for 5 to 10 min.

Immunohistochemistry. Double-immunostaining was done against SUR1 and one of the pancreatic islet hormones. Sections were washed in PBS, and pretreated with 5 % normal goat serum-PBS for 10 min. They were then incubated for 1 h with a mixture of rabbit anti-SUR1 antibody at a 1:200 dilution and one of the following antibodies: guinea pig anti-insulin antibody (a gift from Dr. K. Wakabayashi [16, 17] at a 1:500 dilution, rabbit anti-glucagon antibody (K30 [18]) at a 1:250 dilution, rat monoclonal anti-somatostatin antibody (Chemicon International, Inc., Temecula, Calif., USA) at 1:10 dilution, or guinea pig anti-pancreatic polypeptide antibody (Linco, St. Charles, Mo., USA) at 1:500 dilution. After washing in PBS, the sections were incubated for 1 h in a mixture of Cy3 (indocarbocyanine)- or LRSC (lissamine rhodamine sulphonyl chloride)-labelled donkey anti-rabbit IgG antibodies and either FITC (fluorescein isothiocyanate)-labelled donkey, anti-guinea pig IgG antibody or Cy2-labelled goat anti-rat IgG antibody, and washed in PBS. Other sections were double-immunostained against Kir6.2 and either insulin or pancreatic polypeptides. A rabbit anti-Kir6.2 antibody was used at a 1:200 dilution, as described previously [11]. As immunohistochemical controls, the primary antibody was replaced with normal goat IgG or serum, or applied with SUR1 oligopeptides. These control sections did not show positive staining, confirming the specificity. Immunofluorescent images were recorded with an Olympus AX70 epifluorescence microscope (Olympus, Tokyo, Japan) equipped with a PXL 1400 cooled-CCD camera system (Photometrics, Tucson, Ariz., USA), which was operated with IP Lab Spectrum software (Signal Analytics, Vienna, Va., USA).

Results

A polyclonal antibody was generated in a rabbit against the C-terminal domain of rat SUR1 and affinity-purified to investigate the distribution of SUR1 protein in rat pancreas. The specificity of the antibody was assessed by Western blot analysis. The anti-SUR1 antibody recognized a prominent protein at 140 kDa in the membrane fraction of COS-1 cells transfected with rat SUR1 cDNA (Fig. 1A, lane 1), but no signal was detected in COS-1 cells transfected with rat SUR2 A (Fig. 1A, lane 2), 2B (Fig. 1A, lane 3), or the pCMV vector alone (Fig. 1A, lane 4), corroborating the specificity of the antibody. Both pancreatic islets and exocrine acini were isolated from

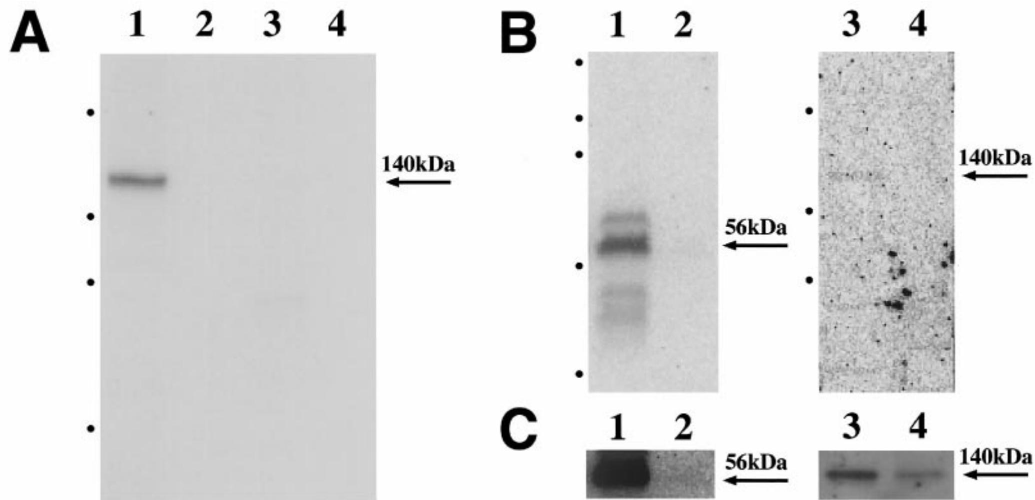

Fig. 1A–C. Western blot analysis against SUR1. **A** Plasma membrane vesicles were prepared from the COS-1 cells transfected with SUR1 (lane 1), SUR2A (lane 2), SUR2B (lane 3), and pCMV vector alone (lane 4). These proteins (8 μg) were separated on 7% SDS-PAGE, and immuno-blotted with the rabbit anti-SUR1 antibody. This antibody does not seem to cross-react with SUR2A or 2B. **B** Total cell homogenates were prepared from isolated pancreatic islets (lanes 1 and 3) and exocrine glands (lanes 2 and 4), and 7 μg of the proteins were run on both 10% (lanes 1 and 2) and 7% SDS-PAGE (lanes 3 and 4). Immuno-detection was done with anti-GLUT2 antibody (lanes 1 and 2) and the above anti-SUR1 antibody (lanes 3 and 4). GLUT2 and SUR1 are detected as bands at 56 kDa and at 140 kDa, respectively, in the lane of isolated islets. **C** Different antibody against SUR1 was used for immuno-blot analysis, and detected the band of 140 kDa in the islets (lane 3). Longer exposure showed faint bands for both GLUT2 and SUR1 in the lanes of exocrine cells (lanes 2 and 4), in addition to the distinct bands in the islets (lanes 1 and 3). The molecular weights of markers are indicated as dots; 208 kDa, 115 kDa, 79.5 kDa, 49.5 kDa, and 34.8 kDa from the top

rat pancreas with collagenase, and the occurrence of SUR1 in these cells was determined by Western blot analysis. Isolated islets did not seem to be completely intact but still usable because an anti-GLUT2 antibody detected a major band at 56 kDa in the total cell homogenate of islets in spite of the occurrence of a few lower faint bands (Fig. 1B, lane 1), consistent with a previous report [19]. Using the anti-SUR1 antibody, a band of 140 kDa was detected in the islets (Fig. 1B, lane 3). We also found two lower bands in the same lane but this was presumably due to partial degradation of SUR1. Compared with GLUT2, SUR1 appeared to be more sensitive to proteases contaminating collagenase P or digestive enzymes which leak out of exocrine cells in preparation or both. Another anti-SUR1 antibody, which had been raised in a guinea pig against a different portion of SUR1, was used for Western blot analysis. A band of 140 kDa protein was also detected in the pancreatic islets (Fig. 1C, lane 3), confirming that this band was composed of SUR1. As to exocrine cells, anti-GLUT2 and anti-SUR1 antibodies detected faint signals at 56 kDa (Fig. 1C, lane 2) and at 140 kDa (Fig. 1C, lane 4), respectively, in longer exposure of the blotted membranes.

Using the rabbit anti-SUR1 antibody, we carried out immunofluorescence staining against SUR1 on 5 μm frozen sections of rat pancreas. SUR1 immunoreactivity was detected all over the islets of Langerhans (Fig. 2, A). Double-immunofluorescence staining against SUR1 and insulin showed that the insulin-positive cells were positive for SUR1 (Fig. 2, A-C). Labeling of an adjacent section for Kir6.2 indicated that SUR1 colocalized with Kir6.2 over the pancreatic islets (Fig. 2). Double-immunofluorescence staining with the rabbit anti-SUR1 antibody and either the guinea pig anti–glucagon or rat anti-somatostatin antibodies showed that SUR1 was present in both alpha and delta cells (Fig. 3). SUR1 was also found in pancreatic polypeptide (PP) cells by double-immunostaining (Fig. 4, A-C). Further Kir6.2 was colocalized with PP (Fig. 4, D-F), suggesting the existence of beta-cell-type K_{ATP} channels in PP-cells. The results were corroborated by immunostaining with the guinea pig anti-SUR1 antibody, which accordingly localized SUR1 over the pancreatic islet (Fig. 5). Significant signals for SUR1 were undetectable in rat soleus muscle which produces SUR2 (data not shown), confirming the specificity of the staining. Taken together with our previous findings [11], all the alpha-, beta-, delta-, and PP-cells were immuno-positive for both SUR1 and Kir6.2.

Discussion

In this study, we found that SUR1 occured not only in beta cells but also in the alpha, delta, and pancreatic polypeptide (PP)cells of rat pancreatic islets. Electrophysiological investigation showed that SUR1, coex-

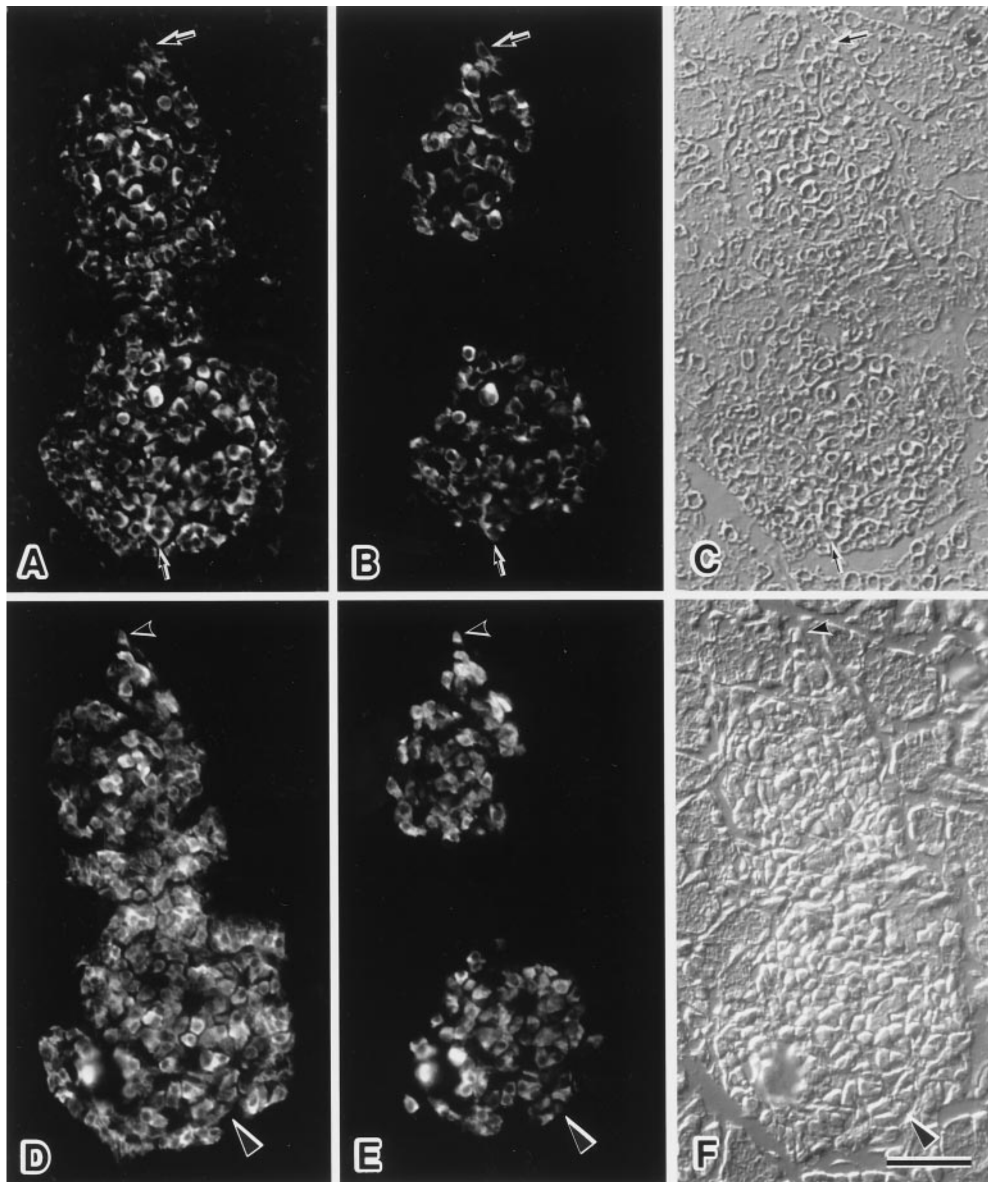

Fig. 2A–F. Indirect immunofluorescence staining against SUR1 (**A**), insulin (**B** and **E**), and Kir6.2 (**D**) in serial frozen sections (5 μm) of rat pancreas. Nomarski differential interference-contrast images (**C** and **F**) are shown as the corresponding references:'**C**' to '**A**' and '**B**'; '**F**' to '**D**' and '**E**'. A double-immunostained section (**A** and **B**) shows the colocalization of SUR1 and insulin. Further immunostained consecutive sections (**A** and **D**) show the coexistence of SUR1 and Kir6.2. Arrows and arrowheads indicate the corresponding sites, respectively. The scale bar = 20 μm

pressed with Kir6.2, reconstituted beta-cell-type K_{ATP} channels in COS cells [4–7, 20]. Kir6.2 had been shown to be present over the whole pancreatic islet and localized in the beta, alpha, and delta cells in our previous study [11]. We showed here the occurrence of Kir6.2 in PP cells by immunofluorescence staining. Together, these results suggest that K_{ATP} channels comprising SUR1 and Kir6.2 (SUR1/Kir6.2 channels) are present in all four types of islet cells. K_{ATP} channel activity is affected by nucleotides such as ATP, GTP, ADP, and other nucleoside diphosphates (NDPs) [8, 21]. SUR1 belongs to the ATP-binding cassette (ABC) or the traffic adenosine triphosphatase (ATPase) superfamily [2] and has two putative nucleotide-binding folds (NBFs) including the Walker A and B consensus motifs. Mutations in both of its NBFs result in loss of activation of the K_{ATP} channels by MgADP [22]. Although truncated Kir6.2 channels alone are inhibited by ATP, their sensitivity is 10-fold lower than intact Kir6.2 coexpressed with SUR1 [23]. It is highly probable, therefore, that SUR1 functions as the nucleotide sensor of K_{ATP} channels in islet cells.

Electrophysiological investigation with a transfection system has shown that the pharmacological properties of K_{ATP} channels are determined by the SUR

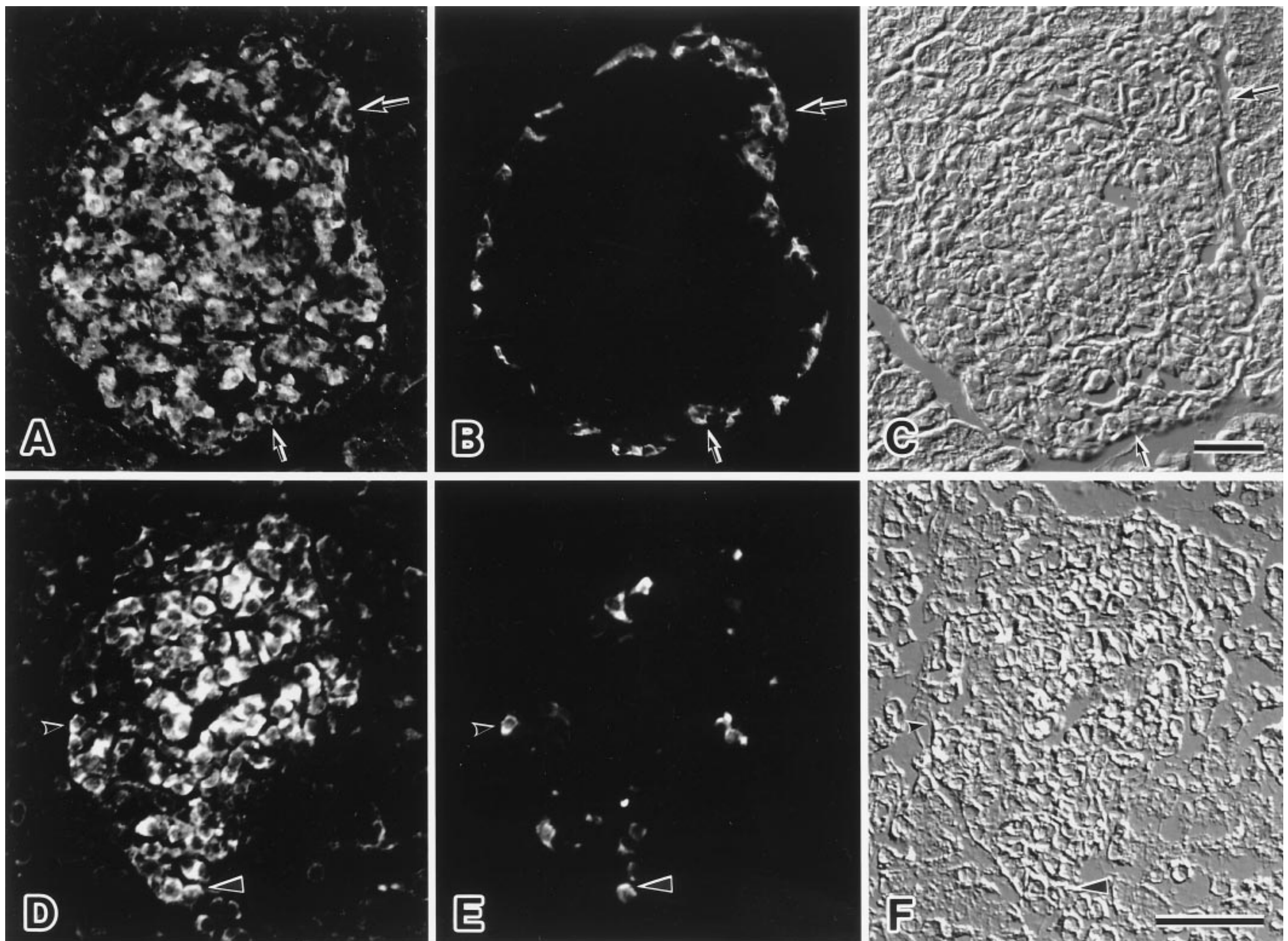

Fig. 3A–F. Double-immunofluorescence staining against SUR1 (**A**) and glucagon (**B**), and against SUR1 (**D**) and somatostatin (**E**) in frozen sections (5 μm) of rat pancreas. Nomarski differential interference-contrast images (**C** and **F**) are shown as the corresponding references:'**C**' to '**A**' and '**B**'; '**F**' to '**D**' and '**E**'. Both glucagon and somatostatin colocalize with SUR1. The scale bar = 20 μm

subunits [4, 6, 8]. As to the inhibitory effect by glibenclamide, half-maximal values of $^{86}Rb^{+}$ efflux were approximately 8.6 nmol/l and 350 nmol/l for the Kir6.2/SUR1 and Kir6.2/SUR2A channels, respectively, reflecting the affinity of SUR for glibenclamide [6]. The Kir6.2/SUR1 channel is activated by a beta-cell K_{ATP} channel opener, diazoxide, but not by cardiac K_{ATP} channel openers which stimulate Kir6.2/SUR2A channel activity. There are accumulating reports on stimulators and inhibitors of insulin secretion which modify K_{ATP} channel activity. Our study suggests that these chemicals, including the sulphonylureas, could have a direct effect not only on the beta cells but also on the alpha, delta, and PP cells. It is, therefore, important to consider such paracrine effects on insulin secretion in the investigation of pharmaceutical therapies targeting beta-cell-type K_{ATP} channels.

Patch clamp studies had failed to detect K_{ATP} channel activities in alpha cells which were purified by an autofluorescence-activated cell sorting system [24, 25]. By whole-cell current monitoring, however, other research [26] showed the ATP- and tolbutamide-sensitive K^{+} outflow in alpha-TC glucagon-secreting cells, the magnitude of which is similar to that in beta-TC3 insulinoma cells. Both tolbutamide and diazoxide affect the glucagon-releasing activity of alpha-TC cells. In addition, the expression of both SUR1 and Kir6.2 mRNAs in alpha-TC-6 cells has been detected by Northern blot analysis [2, 6]. We have histologically localized both SUR1 and Kir6.2 in alpha-cells in situ, suggesting the occurrence of Kir6.2/SUR1 channels. The role of the Kir6.2/SUR1 channel in the alpha cells, however, remains to be determined, since experimental results are quite different in the effects of sulphonylureas on glucagon release from alpha-cells [26–32].

Our results suggest that the Kir6.2/SUR1 channel is present also in delta and PP cells. Using perfused rat pancreas, it was shown [33] that tolbutamide dose-dependently stimulated somatostatin release

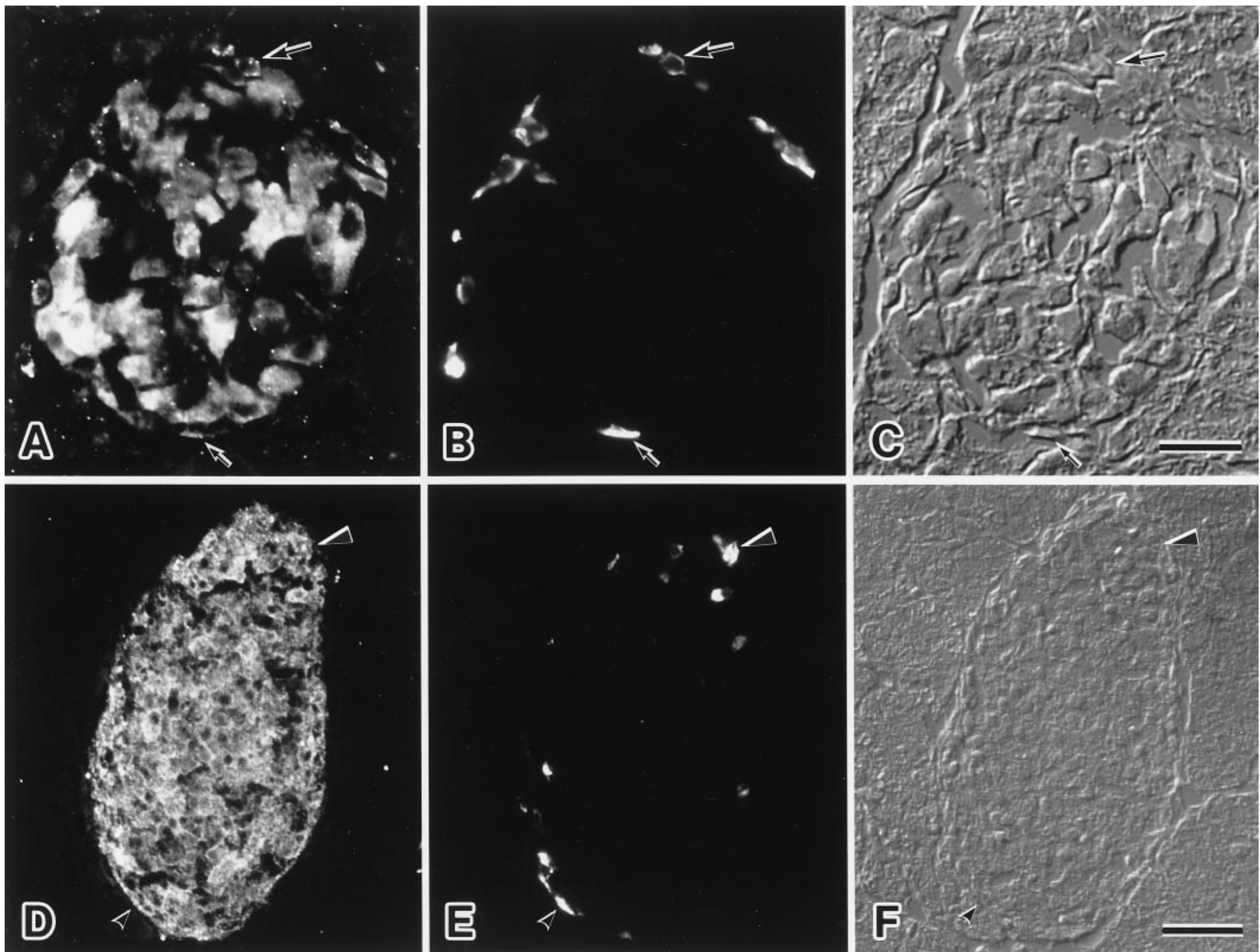

Fig. 4A–F. Double-immunofluorescence staining against SUR1 (**A**) and pancreatic polypeptides (PP) (**B**), and against Kir6.2 (**D**) and PP (**E**) in frozen sections (5 μm) of rat pancreas. Nomarski differential interference-contrast images (**C** and **F**) are shown as the corresponding references: '**C**' to '**A**' and '**B**'; '**F**' to '**D**' and '**E**'. Both SUR1 and Kir6.2 are detected in the PP-positive cells. The scale bar **A**, **B**, and **C** = 20 μm; the bar to **D**, **E**, and **F** = 40 μm

and that the A-4166-induced somatostatin release was inhibited by diazoxide. Although paracrine effects of insulin, glucagon, or PP could be involved in this response of delta cells, our combined results suggest that these chemicals might act on the delta cells directly. This is the first report of the occurrence of Kir6.2/SUR1 channels in PP cells and nothing is known of the effect of sulphonylureas and K_{ATP} channel openers on these cells. Our results suggest, however, that these compounds could have a direct influence on PP cells.

Genetic studies suggest that mutations in the SUR1 gene cause familial hyperinsulinaemia, or familial persistent hyperinsulinaemic hypoglycaemia of infancy, in several families [34]. Various mutations are found in the SUR1 gene of affected subjects, e.g., a 109-base pair deletion within the NBF-2 region, a G to A point mutation at the 3' end of the skipped exon χ [35], and an in-frame deletion of three nucleotides in exon 34 (ΔF1388) [36]. $^{86}Rb^{+}$ efflux assays further indicate that the Kir6.2/ΔF1388 SUR1 channel becomes insensitive to intracellular metabolism and diazoxide [36]. Our study suggests that in some patients with familial hyperinsulinism, K_{ATP} channel activity might be abolished in all types of islet cells, including alpha, delta, and PP cells as well as beta cells. Glucagon deficiency has been considered to be the cause for persistent hypoglycaemia in some patients after partial pancreatectomy for hyperinsulinism [37, 38]. Plasma glucagon concentrations in some cases are considerably lower than those of normal subjects [39, 40]. Therefore, it is possible that mutated K_{ATP} channels might impair the secretory activity of glucagon in alpha cells. Abnormality of circulating glucagon associated with PHHI supports that hyperinsulinism is possibly not the sole hormonal imbalance, but rather that this disease is one of generalized disturbance of islet cell function [40, 41]. A re-

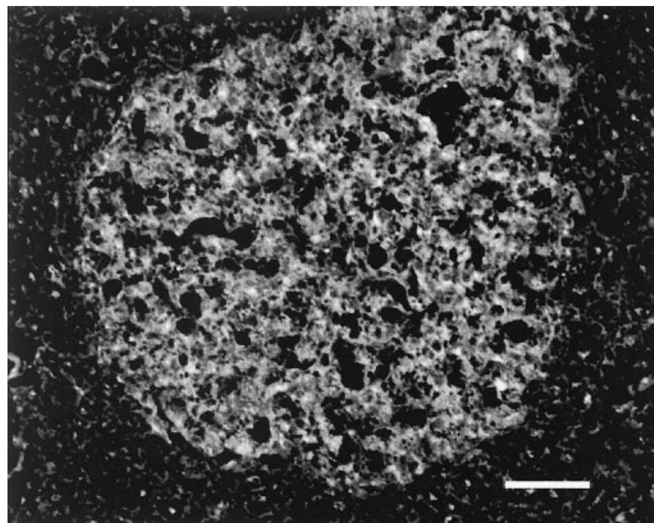

Fig. 5. Immunofluorescence staining of frozen sections (5 μm) of rat pancreas using a second antibody against a different portion of SUR1. Immuno-positive labels are observed all over the islet. Some islet cells are off in clusters, showing black patches. The scale bar = 50 μm

cent study found no insulin response to either glucose or tolbutamide stimulation in mice deficient of K_{ATP} channel [42]; it should be interesting to find if these mice show impaired secretory responses of glucagon, somatostatin, and PP to the glucose concentrations.

The immunofluorescence staining against SUR1 and Kir6.2 appeared to cause weak labels in pancreatic exocrine cells. Western blot analysis using anti-SUR1 antibodies also showed faint signals at 140 kDa, close to the molecular weight predicted from the deduced amino acid sequence of SUR1, when the immuno-blotted membranes were exposed longer. By extending exposure time, however, the band of GLUT2 was also shown in the homogenate of exocrine cells. Hence, the exocrine cells might have been contaminated with dispersed islet cells in preparation, causing the false signals in the immuno-blot analysis. In addition, immunohistological labelling in exocrine cells was not strong enough to judge whether these cells were positive for SUR1.

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