ミトコンドリアのATP感受性K^+チャネルの構造と機能

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研究発表

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研究の概要と今後の展開

ATP 感受性 K^+チャネルは、細胞内の ATP 濃度の高いときに閉じ 低いときに開くチャネルである。この開閉によって細胞膜を横切る K^+イオンの流れが制御され、生じる膜電位の変化を通じて細胞膜の興奮性が調節される。これまでに我々は、膵β細胞の ATP 感受性 K^+チャネルが、ABC (ATP-binding cassette) 蛋白の仲間である SUR1 (スルフォニル尿素受容体 1) と 内向き整流 K^+チャネルの仲間の Kir6.2 から構成される事を示し、血中グルコース濃度に応じたインスリンの分泌調節にかかわることを示した。また、心臓・骨格筋では、SUR2A と Kir6.2 分子が 同様のチャネルを構成し、 虚血時や低酸素時の筋機能の保護のために働いていると考えられている。さらに血管系では、SUR2B と Kir6.1 が、類似のチャネルを構成し、エネルギー状態に応じた血管の収縮弛緩に関与することが示唆されている。

一方、ミトコンドリアにも類似の ATP 感受性 K^+チャネルが存在し、細胞内の ATP 濃度に応じたエネルギー産生の調節を行っていると言われているが、その分子的実体は、知られていない。我々は、今回の科学研究費補助金により、T 細胞白血病株 Jurkat 細胞よりミトコンドリア内膜を調製し、パッチクランプ法により、チャネル活動の記録を試みた。同ミトコンドリアに観察されるチャネルのなかに、2 mM ATP により活性が抑えられ K^+イオンに選択性が高く 単一電流のコンダクタンスが 55 pS のチャネルを見出した(p. 5)。このチャネルのキネティクスは、Kir6.1 が構成するチャネルのものに似かよっており、 今後このチャネルと Kir6.1 との関係、また SUR に相当する未知の分子についての解析を行いたい。

また、Kir6.1 の遺伝子欠損マウスを作成すると、心臓の障害と思われる症状で誕生前後に高頻度に死亡することが分かった。 しかし 心筋細胞膜では、Kir6.2 と SUR2 で構成される ATP 感受性 K^+チャネルの活動は観察されるが、Kir6.1 様チャネルの活動は観察されない。今後の Kir6.1 と、心臓の障害、さらにミトコンドリアでのチャネルの発現についての関係を究明したい。

一方 Kir6.2 と SUR1 で構成されるチャネルについて、その cAMP 依存性蛋白リン酸化酵素 (PKA) による 各サブユニットのリン酸化と機能調節についての詳細な解析を行った。その結果、Kir6.2 分子と SUR1 分子は、それぞれが PKA によるリン酸化を受け、独自の機能修飾が 行われることを解明した。

さらに、troglitazone、pioglitazone, KAD-1229 など、新しい抗糖尿病薬の、Kir6.2 と SUR1, SUR2A, SUR2B それぞれの分子で構成される ATP 感受性 K^+チャネルへの作用を検討した。これらの研究はともに、Kir6.2 によって構成されるチャネルの機能・性質の解析として重要であるばかりでなく、今後 ミトコンドリアの ATP 感受性 K^+チャネルの構造と機能の解析を進める上でも、大いに有効である。

ATP-sensitive ion channel in the inner mitochondrial membrane

Introduction

Mitochondrial ion channels are often involved in apoptosis. The permeability transition pore (PTP) is the best studied example. Our aim is to study whether mitochondrial channels contribute to neuronal apoptosis, as well. As neuronal mitochondria are difficult to patch clamp we started with T-lymphocytes, a cell type well known for a role of mitochondrial ion channels in apoptosis.

Methods

Preparation of mitoplasts: Jurkat cells were cultured in RPMI medium supplemented with 10% FCS, 50 IU/ml penicillin, 50 µM streptomycin and kept in an 5% CO_2 and H_2O-saturated atmosphere at 37 ° C. From these cells mitochondria were isolated by homogenisation in a prep. solution (250 glucose, 5 K-HEPES, 0.1 BSA, 1 K-EGTA; concentrations in mM if not otherwise stated) and several centrifugation steps at different speed. Mitochondria were kept on ice in a 150 KCl solution for up to 36 h. 10 µl samples were put into 35 mm Petri dishes filled with 2 ml of a hypotonic solution (5 K-HEPES, 1 $CaCl_2$) until the cristae of the inner membrane unfolded and the outer membrane broke. Isotonicity was restored by adding 0.5 ml of a hypertonic solution (750 KCl, 80 K-HEPES, 1 $CaCl_2$). The resulting fragile vesicles are called mitoplasts and can be recognised by a dark spot which is supposed to be the so-called cap where the outer membrane adheres to the inner membrane. Details concerning techniques were given by Borecky et al., 1997 and Siemen et al., 1999).

Patch clamp experiments: Electrodes were pulled from Clark borosilicate glass with a resistance of 10-20 MΩ. Mitoplasts were chased with these pipettes, approached from the side opposite to the cap (see inset of Fig. 1) and treated with negative pressure until seal resistances of 1.5-2.5 MΩ were reached. Current was recorded in the mitoplast-attached configuration with an EPC-7 amplifier. Solutions were applied by moving the pipettes with mitoplasts into the opening of peristaltic-pump driven capillary pipe system.

Recording of data and analysis was done by means of the pClamp6 software in combination with a TL1-DMA board (Axon Instruments, Foster City, Ca, USA). The current was low-pass filtered at 1 kHz and sampled at 5 kHz. The probability of being in the open state (P_o) was determined by all-point analysis.

Fig. 1

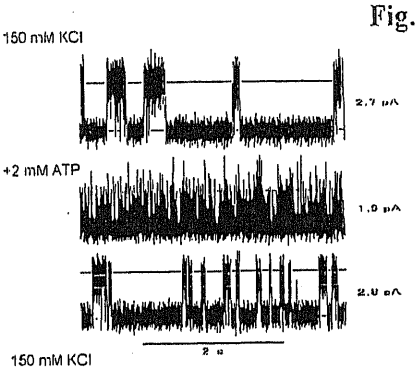


Fig. 2

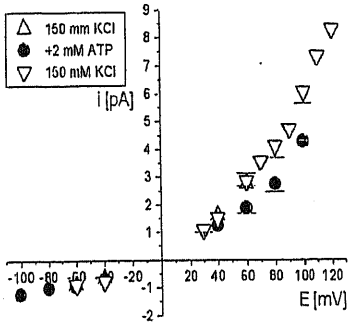


Fig. 3

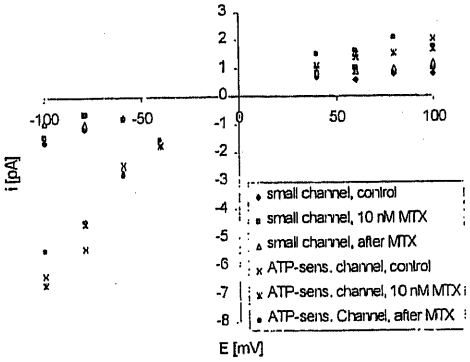
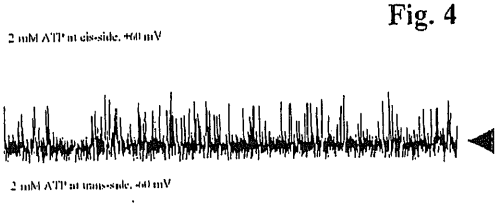
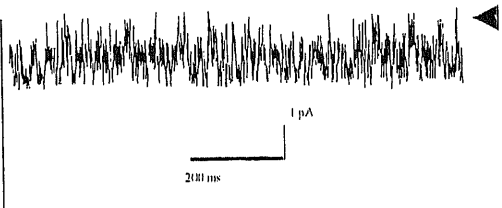


Fig. 4





Results

Fig. 1: Single-channel recordings from the inner mitochondrial membrane of Jurkat cells show a slow activity (1st trace) that can be switched to a fast flickering into the open state (2nd trace) by addition of 2 mM ATP. The effect is reversible (3rd trace). E = 60 mV.

Fig. 2: The corresponding i-E curves show activity of a rectifying channel with about 55 pS single channel conductance into the positive and about 12 pS into the opposite direction. Currents measured in 2 mM ATP (filled circles) decrease slightly in amplitude which is at least partly due to the extremely short events.

Fig. 3: In addition to the ATP-sensitive channel, a smaller ATP-insensitive channel with a conductance of 10 pS or less was found. It appears in large number with a slower kinetics than the ATP-sensitive channel. The ATP-sensitive channel is turned away from the bath with its ATP-binding site in this patch (trans-configuration) showing an i-E rectifying into the negative. Both channels are insensitive to 10 nM Margatoxin, a specific blocker of the Kv1.3 channel. The Kv1.3 was recently shown in the inner mitochondrial membrane of Jurkat cells.

Fig. 4: Current traces from two different patches recorded in the cis-configuration with the binding site exposed to ATP at +60 mV (upper trace, rectifying into the positive) and in the trans- configuration at -60 mV (lower trace, rectifying into the negative). Arrows give closed state of the channels. More than one channel present in these patches.

Fig. 5: Current knowledge of apoptosis pathways inside mitochondria. Blockade of an ATP-sensitive K-channel seems to be involved in NO-mediated activation of the PTP (Ockaili et al., 1999).

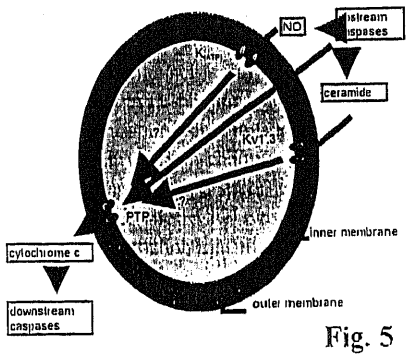


Fig. 5

Conclusions

- An ATP-sensitive ion channel can be recorded from the inner mitochondrial membrane of Jurkat cells which may be the ATP-sensitive K-channel responsible for NO-sensitive apoptosis.
- Only one side of the channel is sensitive to ATP.

References

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Ockaili, R. et al. (1999) Am. J. Physiol. 277:H2425-H2434.

Conclusions

- An ATP-sensitive ion channel can be recorded from the inner mitochondrial membrane of Jurkat cells which may be the ATP-sensitive K-channel responsible for NO-sensitive apoptosis.
- Only one side of the channel is sensitive to ATP.

PKA-mediates phosphorylation of the human K_{ATP} channel, SUR1/Kir6.2: Separate roles of phosphorylation in different subunits

Abstract

ATP-sensitive potassium (K_{ATP}) channels play important roles in many cellular functions such as hormone secretion and excitability of muscles and neurons. Classical ATP-sensitive potassium (K_{ATP}) channels are heteromultimeric membrane proteins comprising the pore-forming Kir6.2 subunits and the sulfonylurea receptor subunits (SUR1 or SUR2). The molecular mechanism by which hormones and neurotransmitters modulate K_{ATP} channels via protein kinase A (PKA) is poorly understood. We mutated the PKA consensus sequences of the human SUR1 and Kir6.2 subunits and tested their phosphorylation capacities in *Xenopus* oocyte homogenates and in intact cells. We identified the sites responsible for PKA phosphorylation in the C-terminus of Kir6.2 (S372) and SUR1 (S1571). Kir6.2 can be phosphorylated at its PKA phosphorylation site in intact cells after G-protein (Gs) coupled receptor or direct PKA stimulation. While the phosphorylation of Kir6.2 increases channel activity, the phosphorylation of SUR1 contributes to the basal channel properties by decreasing burst duration, interburst interval and open probability, and also increasing the number of functional channels at the cell surface. Moreover, the effect of PKA could be mimicked by introducing negative charges in the PKA phosphorylation sites. These data demonstrate direct phosphorylation by PKA of the K_{ATP} channel, and may explain the mechanism by which Gs protein coupled receptors stimulate channel activity. Importantly, they also describe a model of heteromultimeric ion channels in which there are functionally distinct roles of the phosphorylation of the different subunits.

Introduction

Regulation of ion channels by intracellular signals through protein phosphorylation is an important event that controls a wide variety of cellular functions (Levitan et al., 1994). Many classes of ions channels, including Ca^{2+}, Na^{+}, and K^{+} channels are regulated by protein phosphorylation (Levitan et al., 1994). The most common mechanisms of phosphorylation of ion channels are mediated by protein kinase A (PKA) and protein kinase C (PKC); the phosphorylation by PKA and PKC on serine and threonine residues alter channel properties by modifying the kinetics and/or the number of active channel present at the plasma membrane (Jonas and Kaczmarek, 1996). Despite extensive studies of the phosphorylation-function relationships of ion channels, little is known of distinct roles for the phosphorylation of the different subunits in the modulation of heteromultimeric ion channels.

K_{ATP} channels couple cell metabolism to membrane potential in many types of cell, and participate in a variety of cellular functions including hormone secretion, vascular smooth muscle tone, and excitability in neurons and muscles (Ashcroft, 1988). In pancreatic β-cells, the K_{ATP} channel is critical for glucose-and sulfonylurea-induced insulin secretion (Cook et al., 1988). Indeed, mutations of the K_{ATP} channel which result in loss of its function lead to familial persistent hyperinsulinemic hypoglycemia (Thomas et al., 1995; Kane et al., 1996). In addition, we have recently found that there is almost no insulin secretory response to either glucose or the sulfonylurea tolbutamide in K_{ATP} channel-deficient mice (Miki et al., 1998).

Classical K_{ATP} channels comprise the inward rectifier Kir6.2 subunits and sulfonylurea receptor subunits (SUR1 or SUR2), which belong to the ATP-binding cassette (ABC) superfamily with two nucleotide binding folds (NBF-1 and NBF-2) (Babenko et al., 1998; Seino, 1999). Heterologous expression studies show that differing combinations of Kir6.2 and SUR1 or SUR2 (SUR2A or SUR2B) reconstitute K_{ATP} channels with distinct nucleotide sensitivities and pharmacological properties (Inagaki et al., 1995; 1996; Isomoto et al., 1996). The pancreatic β-cell K_{ATP} channel is composed of Kir6.2 subunits and SUR1 subunits with 4:4 stoichiometry (Babenko et al., 1998; Seino, 1999). While the Kir6.2 subunits form the K^{+} ion permeable pore, and are thought to confer primarily inhibition by ATP (Tucker et al., 1997), the SUR1 subunits are thought to mediate the stimulatory effect of MgADP, and are the primary target for pharmacological agents such as sulfonylureas and K^{+} channel openers (Inagaki et al., 1995; Nichols et al., 1996; Gribble et al., 1997). Although deletion of the C-

terminus of Kir6.2 (Kir6.2 ΔC26 or ΔC36) produces K_{ATP} channel currents in the absence of SUR1 subunits (Tucker et al., 1997), both Kir6.2 subunits and SUR1 subunits are normally required for functional expression of the β-cell K_{ATP} channel (Inagaki et al., 1995).

In addition to being regulated by various nucleotides, K_{ATP} channels are modulated by hormones and neurotransmitters (De Weille et al., 1989; Wellman et al., 1998), and accumulating data indicate that intracellular signals such as G proteins (Sanchez et al., 1998) and phosphatidylinositol-4,5 phosphate (PIP_2) (Shyng and Nichols, 1998; Baukrowitz et al., 1998) modulate K_{ATP} channel activity. It has been shown that the activities of K_{ATP} channels are regulated also by protein kinase A (Ribalet et al., 1989; Quayle et al., 1994; Wellman et al., 1998). In the pancreatic β-cell lines RINm5F and HIT, for example, exogenously introduced PKA catalytic subunits activate the K_{ATP} channels (Ribalet et al., 1989). In arterial smooth muscle and in ventricular myocytes, the K_{ATP} channels are also activated by Gs-coupled receptor stimulation or by addition of exogenous protein kinase A (Quayle et al., 1994; Notsu et al., 1992). Phosphorylation by unknown kinases of the sulfonylurea receptor is also thought to modulate the effect of diazoxide on K_{ATP} channel activity (Niki et al. 1992), or to be involved in the maintenance of channel activity (Findlay and Dunne, 1986). However, the molecular basis of PKA-mediated phosphorylation of K_{ATP} channels is not known.

Due to the importance of PKA-mediated K_{ATP} channel phosphorylation in various tissues, we attempted to identify the PKA phosphorylation sites in the human pancreatic β-cell K_{ATP} channel (Kir6.2/SUR1) and have investigated the effects of such phosphorylation on its activity and kinetic properties. Our biochemical studies demonstrate directly that serine at residue 372 of Kir6.2 and serine at residue 1571 of SUR1 are both phosphorylated by PKA. While the PKA site in Kir6.2 can be phosphorylated in intact cells after G-protein (Gs) coupled receptor or direct PKA stimulation, SUR1 is already phosphorylated by PKA in the basal state. Electrophysiological analyses show that while the phosphorylation of Kir6.2 increases activity of the K_{ATP} channels, the phosphorylation of SUR1 affects channel properties by decreasing burst duration, interburst interval, and open probability. These effects were mimicked by introducing negative charges into PKA phosphorylation sites of Kir6.2 (372D) or SUR1 (1571D). The phosphorylation of SUR1 is also involved in the maintenance of functional expression of K_{ATP} channels at the cell surface.

Results

Identification of the phosphorylation site for protein kinase A in the human β-cell K_{ATP} channel (Kir6.2/SUR1)

Analysis of the amino acid sequence reveals the presence of two consensus sites (T224 and S372) for PKA phosphorylation in the cytoplasmic C-terminal region of human Kir6.2, and four sites (T949, S1446, S1500, and S1571) in human SUR1 (Fig. 1). While the putative sites for PKA phosphorylation in Kir6.2 are conserved among the different species identified to date, those in SUR1 are not conserved. S1571 is a human specific site, while the other sites are also present in rat and/or hamster. Biosynthetic labelling with ^{35}S-methionine of *Xenopus* oocytes expressing SUR1 plus wild-type (wt) Kir6.2 or SUR1 plus each mutant Kir6.2 showed that wt Kir6.2 and all the mutants are expressed at a similar level (Fig. 2A, lanes 2-5). The phosphorylation capacity by PKA stimulation was then tested in the homogenates. After immunoprecipitation of Kir6.2, an increase in the ^{32}P incorporation upon cAMP addition was detected in *Xenopus* oocytes expressing wt Kir6.2/SUR1 (Fig. 2B, compare lane 2 with 4), indicating that Kir6.2 became phosphorylated in response to PKA stimulation. Addition of the specific PKA inhibitor H-89 to the homogenate completely abolished the phosphorylation (Fig. 2B, compare lane 4 with 5), confirming the specificity of the PKA-mediated phosphorylation. Despite the similar level of expression of wt Kir6.2, the mutant S372A, and the double mutant T224A/S372A (Fig. 2A), the phosphorylation of both mutants was completely abolished (Fig. 2B, compare lane 4 with 9 and 11), while the T224A mutation did not affect the phosphorylation of Kir6.2 (Fig. 2B, compare lane 4 with 7). Abolition of the PKA-mediated phosphorylation of Kir6.2 also was obtained by mutating the serine 372 in aspartate (unpublished data). These results indicate that serine at residue 372 of Kir6.2 can be directly phosphorylated by PKA.

A similar approach was used to identify the phosphorylation site for PKA in human and rat SUR1. Metabolic labelling with ^{35}S-methionine showed that the expression levels of each SUR1 mutant in oocytes was comparable with that of wild-type (Fig. 3A, compare lane 2 with lanes 3-5). Wild-type human SUR1 became phosphorylated in response to PKA stimulation, but wild-type rat SUR1 was not phosphorylated by PKA (Fig. 3B, compare lane 3 and 4 with lane 12 and 13, respectively). The abolition of the SUR1 phosphorylation by addition of the PKA inhibitor H-89 confirmed PKA specificity of the phosphorylation (Fig. 3B, compare lane

4 with 5). Both the S1500A and S1446A mutants were phosphorylated to the same extent as the wild-type (Fig. 3B, compare lane 4 with lane 9 or 11). In contrast, the phosphorylation in the S1571A mutant in response to PKA was completely abolished (Fig. 3B, compare lane 4 with 7). PKA-mediated phosphorylation of human SUR1 was also abolished by the substitution of serine 1571 with aspartic acid (unpublished data). In all proteins that are known to be phosphorylated by PKA, two positively charged lysine and/or arginine residues are present in -2 and -3 from the phosphorylated serine or threonine (Pearson and Kemp, 1991). Neither rat nor hamster SUR1 contains a PKA consensus phosphorylation site corresponding to serine 1571 in human SUR1. Although the serine residue corresponding to 1571 in human SUR1 is present in both species, the -3 from this serine is not a positively charged amino acid, but glutamine. Accordingly, serine 1571 in human SUR1 is species specific and a unique site for PKA-mediated phosphorylation.

Since protein kinase C (PKC) has been suggested to modulate K_{ATP} channel activity (Wollheim et al., 1988), we also investigated in a similar way to find if Kir6.2 or SUR1 could be a direct substrate for PKC-mediated phosphorylation. After phorbol 12-myristate 13-acetate (PMA) stimulation (100 nM) of PKC endogenous kinases of *Xenopus* oocyte homogenates, neither Kir6.2 nor SUR1 showed an increase in phosphorylation. A similar result was also obtained in a different expression system using COS-1 cells (unpublished data). These results indicate that neither Kir6.2 nor SUR1 is directly phosphorylated by PMA-sensitive PKC in a reconstituted system.

PKA-mediated phosphorylation in the human β-cell K_{ATP} channel in intact cells

Although our data indicate clearly that phosphorylation occurs at specific sites in K_{ATP} channels, to determine if these sites are phosphorylated in intact cells, we transiently transfected COS-1 cells with either human wt Kir6.2 or mutant Kir6.2 S372A cDNA together with either human wt SUR1 or the mutant SUR1 S1571A cDNA, and tested their ability to be phosphorylated in intact cells after PKA stimulation. In untransfected COS-1 cells, neither Kir6.2 nor SUR1 could be detected after metabolic labelling with ^{35}S-methionine (Fig. 4A and B, lane 1) or ^{32}P-orthophosphate (Fig. 4C and D, lanes 1-3). In transfected cells, metabolic labelling with ^{35}S-methionine showed that wt and mutants of both subunits were expressed at similar levels (Fig. 4A and B, compare lane 2 with 3). The phosphorylation of wt Kir6.2 but not that of Kir6.2

S372A was increased by stimulation of COS-1 cells with forskolin and 3-isobutyl-1-metylxanthine (IBMX) or dibutyryl cyclic adenosine monophosphate (dbcAMP) (Fig. 4D, compare lane 4 with lane 5 or 6 and also compare lane 7 with lane 8 or 9). This indicates that serine 372 in human Kir6.2 also can be phosphorylated by PKA in intact cells. In contrast, wt SUR1 is phosphorylated under the basal condition (Fig. 4, lane 4). We could not detect any significant increase in wt SUR1 phosphorylation by stimulation with forskolin and IBMX or dbcAMP (Fig. 4C, compare lane 4 with lane 5 or 6). In addition, mutation of the PKA-site (SUR1 S1571A) did not result in any significant decrease in phosphorylation in the basal state (Fig. 4C, lane 7), and the PKA inhibitor H-89 did not reduce this basal phosphorylation (unpublished data), indicating that basal phosphorylation is due to unknown kinases rather than PKA. In contrast to Kir6.2, no direct PKA-mediated phosphorylation of human SUR1 in intact cells could be detected.

To determine if the basal phosphorylation could mask the PKA-mediated SUR1 phosphorylation, we performed a two-dimensional phosphopeptide mapping and phosphoaminoacid analysis (PAA) using the immunoprecipitated wt and mutant human SUR1 from transfected COS-1 cells with or without forskolin plus IBMX stimulation. Digestion with trypsin of immunoprecipitated wt and the mutant SUR1 S1571A showed similar maps of the phosphopeptides. However, one peptide became almost undetectable after the digestion of the mutant SUR1, indicating that the SUR1 PKA-site is located in the peptide (unpublished data). Since this phosphopeptide was detected in the non-stimulated state and could not be further increased after PKA stimulation, the SUR1 PKA-site might be already phosphorylated by PKA in the basal state in intact cells. On the other hand, phosphoaminoacid analysis identified the phosphorylation of a unique serine residue, and because the mutant SUR1 did not abolish this serine phosphorylation, the basal phosphorylation could be due primarily to unknown serine/threonine kinases (unpublished data).

To ascertain if a relatively high level of basal phosphorylation due to unknown kinases and/or PKA accounts for the failure to detect an increase in PKA-mediated SUR1 phosphorylation in intact cells, we eliminated possible unknown cytosolic kinases by preparing a crude membrane preparation from COS-1 cells transfected with the wt K_{ATP} channel (Kir6.2/SUR1) or the mutant K_{ATP} channel (Kir6.2 S372/SUR1 S1571A). Since no phosphatase inhibitors were included during the preparation, this purification step also allowed

us to obtain a completely dephosphorylated SUR1. We then carried out an "*in vitro*" phosphorylation assay using the exogenous PKA catalytic subunit. Although a slight basal phosphorylation was still present, despite the expression levels of the wt and mutant SUR1 being similar (Fig. 5A, inset 2 and 3), the addition of PKA substantially increased the phosphorylation of wt SUR1 and not that of mutant SUR1 S1571A (Fig. 5B, compare lane 3 and 4 with lane 5 and 6, respectively). This suggests that human SUR1 can be phosphorylated in COS-1 cells at serine 1571 but, in contrast to the case in *Xenopus* oocyte homogenates, such PKA-mediated phosphorylation can be detected only when the basal phosphorylation of SUR1 due to unknown kinases and PKA is low.

Receptor mediated phosphorylation of the human β-cell K_{ATP} channel in COS-1 and MIN6 cells

In order to better understand the physiological role of the PKA-mediated phosphorylation of Kir6.2, we examined to find if the activation of different G protein-coupled receptors could lead to a change in Kir6.2 phosphorylation. For this purpose, COS-1 cells were transiently transfected with wt or the mutant (Kir6.2 S372/SUR1 S1571A) K_{ATP} channel together with various G protein-coupled receptors. Metabolic labelling with ^{35}S-methionine of COS-1 cells showed that the wt and mutant Kir6.2 were expressed at a similar level (Fig. 6A, inset). We then determined whether or not stimulation with epinephrine, which activates endogenous β_2-adrenergic receptors, leads to phosphorylation of Kir6.2 at its PKA phosphorylation site. Stimulation of β_2-adrenergic receptors with epinephrine increased the phosphorylation of wt Kir6.2, but not that of the mutant (Fig. 6B, compare lane 9 and 11 with lane 10 and 12, respectively). The effect of epinephrine could be abolished by the β-antagonist alprenolol (unpublished data). These data indicate that the phosphorylation of Kir6.2 after β_2-adrenergic receptor activation is mediated by PKA. To further investigate PKA-mediated phosphorylation, Kir6.2 and SUR1 were coexpressed with other G-protein coupled receptors such as pituitary adenylate cyclase-activating polypeptide (PACAP) receptor PACAP-3 (Inagaki et al., 1994), gastrointestinal inhibitory polypeptide (GIP) receptor (Yasuda et al., 1994), or somatostatin receptor SSTR3 (Yamada et al., 1992), all of which are known to be expressed in pancreatic β-cells and to modulate insulin secretion positively or negatively. PACAP and GIP stimulation, which increase the cAMP content in cells (Inagaki et al., 1994; Yasuda et al., 1994), were able

to promote phosphorylation of wt Kir6.2 (Fig. 6, lanes 2 and 6). In contrast, the Kir6.2 S372A could not undergo PACAP- or GIP-induced phosphorylation (Fig. 6, lanes 3 and 7). As a negative control, stimulation of the somatostatin receptors, known to inhibit adenylyl cyclase activity, could not increase Kir6.2 phosphorylation (Fig. 6, lanes 13-16). These results indicate that stimulation of receptors coupled to the cAMP-dependent signalling pathway leads to the phosphorylation of Kir6.2 at serine 372.

To determine if Kir6.2 and SUR1 in native cells can be phosphorylated by direct PKA or receptor stimulation, we examined the capacity of Kir6.2 and SUR1 to be phosphorylated by forskolin and IBMX, dbcAMP, or PACAP stimulation in mouse pancreatic β-cell-derived MIN6 cells, in which both Kir6.2 and SUR1 are expressed (Inagaki et al., 1995). Similarly to human SUR1 expressed in COS-1 cells, a basal level of phosphorylation of SUR1 in MIN6 cells was observed in the absence of stimuli, and it was not increased by direct PKA or PACAP stimulation (Fig. 6C, lanes 1-4). In contrast, Kir6.2 phosphorylation was increased significantly (2-3 fold) after forskolin and IBMX stimulation (Fig. 6D, compare lane 1 with 2), but almost no increase was observed after dbcAMP or PACAP stimulation (Fig. 6D, compare lane 1 with lane 3 or 4), suggesting that due to the lower abundance of both Kir6.2 and SUR1 proteins in MIN6 cells as compared to transfected cells, only a maximal stimulation of PKA permits detection of Kir6.2 phosphorylation in these cells. These results indicate that Kir6.2 can be directly phosphorylated at S372, both after G protein-coupled stimulation in a reconstituted system and after direct PKA stimulation in native cells, suggesting a physiological significance of PKA-mediated phosphorylation of Kir6.2.

Effects of PKA-mediated phosphorylation on human β-cell K_{ATP} channel properties

To determine if PKA-mediated phosphorylation could modulate the properties of the channel, we examined the electrophysiological properties in excised inside-out membrane patch configuration of COS-1 cells expressing wt K_{ATP} channels or the PKA-site mutant K_{ATP} channels. The PKA catalytic subunit was applied to the bath solution containing 10 μM ATP to prevent rundown of the K_{ATP} channel, but this ATP concentration was sufficient for use as a PKA substrate (Ribalet et al., 1989). A progressive increase in K_{ATP} channel activity was observed from 6 to 10 min after application of the PKA catalytic subunit. As shown in Fig. 7A

and 7B, increase in activity was 2 fold for both wt Kir6.2/SUR1 (212%) and Kir6.2/SUR1 S1571A (209%). In contrast, no significant increase in activity was observed in K_{ATP} channels comprising either Kir6.2 S372A/ wt SUR1 or Kir6.2 S372A/ SUR1 S1571A (Fig. 7C and Fig. 7D). This increase in channel activity was due to neither the change in channel conductance (pS) (74.3 ± 0.7, 72.0 ± 0.8, in the absence of PKA and 72.1 ± 2.9, 74.3 ± 1.8, in the presence of PKA, for wt Kir6.2/SUR1 and Kir6.2 S372A /wt SUR1, respectively, n=5 for each), nor the change in ATP-sensitivity (K_i, μM) (27.5 ± 2.2, 26.5 ± 1.7, in the absence of PKA and 26.3 ± 2.8, 25.9 ± 2.5, in the presence of PKA, for wt Kir6.2/SUR1 and Kir6.2 S372A/wt SUR1, respectively, n=5 for each), but to an increase in open probability of single channels and/or an increase in the number of functional channels which were already present in the membrane (Fig. 7, right). These data suggest strongly that the K_{ATP} channel can be activated by direct PKA-mediated phosphorylation of S372 in Kir6.2, and that PKA phosphorylation of SUR1 is not required.

To determine if PKA-mediated phosphorylation of SUR1 might have functional significance, we analysed K_{ATP} channel kinetics from patches which had only a single channel. Interestingly, as shown in Fig.8, there is an apparent difference in the channel kinetics between the K_{ATP} channels comprising wt SUR1 and those comprising the mutant SUR1 S1571A. K_{ATP} channels have a complex kinetics, one open state (τ_o) and three closed states (τ_{c1}, τ_{c2}, τ_{c3}) (Alekseev et al., 1997). The mean open and closed times during bursts are defined by τ_o and τ_{c1}, respectively. Interburst kinetics is defined principally by longer closed times τ_{c2} and τ_{c3} ($\tau_{c3} > \tau_{c2}$). As shown in Fig.8 and Table I, the mean burst duration and the long closed time (τ_{c3}) of K_{ATP} channels comprising the mutant SUR1 S1571A were significantly (2-3 fold) and dramatically (6-9 fold) prolonged, respectively, as compared to those of K_{ATP} channels comprising wt SUR1. The cluster duration in K_{ATP} channels comprising mutant SUR1 S1571A also was greatly prolonged (Fig. 8, compare A or C with B or D, Table I) and, as a consequence, the open probability was increased (Table I). On the other hand, no significant change was observed for the other kinetic parameters, τ_o, τ_{c1} and τ_{c2}. These findings together suggest that the PKA phosphorylation site in SUR1 of K_{ATP} channels plays an important role in basal channel kinetics.

Since disruption of the SUR1 PKA phosphorylation site alone was sufficient to affect burst and interburst kinetics and open probability, we thought that the PKA site in SUR1 might

already be in the phosphorylated form in the basal state in intact cells. To evaluate these possibilities, we performed a dephosphorylation experiment in which the alkaline phosphatase was applied to the bath solution. Treatment with alkaline phosphatase showed both PKA-dependent and -independent processes: a PKA-dependent process characterized by a transient increase in channel activity (1.7 fold) of the K_{ATP} channels comprising wt SUR1 (Fig. 8A and B) that is abolished in the K_{ATP} channels comprising mutant SUR1 S1571A (Fig. 8C and D) and a PKA-independent process observed as a decrease in channel activity of both wt and mutant K_{ATP} channels after long exposure to alkaline phosphatase (Fig. 8, right). Treatment with alkaline phosphatase also prolonged significantly burst and cluster duration, and to some extent the long closed time (τ_{c3}) of the K_{ATP} channels comprising wt SUR1 (Fig. 8A and B, Table II), while no significant change was observed in the K_{ATP} channels comprising mutant SUR1 S1571A (Fig. 8C and D, Table II). These results indicate that human SUR1 is indeed already present in its PKA-phosphorylated form in intact cells and can be dephosphorylated by the addition of alkaline phosphatase. As shown in Fig. 4, SUR1 is also phosphorylated in the basal state by unknown kinases. Although alkaline phosphatase cleaves any of the phosphate groups which are phosphorylated by PKA and unknown kinases, the insensitivity of mutant SUR1 S1571A to alkaline phosphatase after short exposure indicates that the burst and cluster duration, interburst interval, and open probability effects are due to the PKA rather than unknown kinase phosphorylation. In contrast, the progressive decrease in open probability after longer exposure to alkaline phosphatase, which may be associated with "run-down", might be due to dephosphorylation of the sites phosphorylated by unknown kinases.

To confirm that negative charges in the Kir6.2 and SUR1 PKA phosphorylation sites plays a role in the modulation of channel activity, we introduced constitutively negative charges by replacing both phosphorylation sites with the acidic amino acid aspartic acid. This substitution was shown to mimic the action of kinases (Li et al., 1993). In patches which had only a single Kir6.2 S372D/wt SUR1 channel, a 2-4 fold increase in open probability was observed, compared to the wt Kir6.2/SUR1 channel (0.12 ± 0.06 and 0.37 ± 0.10, for wt Kir6.2/SUR1 and Kir6.2 S372D/wt SUR1, respectively, n=4-7). In contrast, no significant change was observed in open probability (0.12 ± 0.06 and 0.09 ± 0.03, for wt Kir6.2/SUR1 and wt Kir6.2/SUR1 S1571D, respectively, n=4-7) or channel kinetics (unpublished data) for the SUR1 S1571D mutant, as would be expected if SUR1 is already phosphorylated under

basal conditions. These results indicate that the presence of negative charges (i.e., PKA phosphorylation) in Kir6.2 and SUR1 is sufficient to activate the K_{ATP} channel and to determine the basal properties, including the decrease in open probability, respectively.

Interestingly, rubidium efflux from the K_{ATP} channels comprising SUR1 S1571A expressed in COS-1 cells decreased by 68 ± 12% (n=3), compared to that from wt K_{ATP} channels. Since this result contrasts to the increase in open probability observed by patch clamp analysis (Table I), we attempted to determine the level of functional expression at the cell surface by measuring channel density. The functional channel density is determined principally by the detectable rate of channels and the number of channels in a patch. Both the channel detectable rate and the number of channels in a patch were markedly reduced in cells transfected with mutant SUR1 S1571A (Table I). In these experiments, similar expression levels of the wild-type and mutant SUR1 were detected, as assessed by ^{35}S-methionine metabolic labelling (unpublished data). Using channel density and open probability parameters, the channel activity of K_{ATP} channel comprising SUR1 S1571A can be estimated to 30-40% of wt K_{ATP} channels. These results suggest that a reduction in both the channel detectable rate and the number of channels within a patch accounts for the decrease in rubidium flux, and that disruption of the PKA phosphorylation site in SUR1 impairs channel activity by decreasing functional expression of the K_{ATP} channels at the cell surface.

Discussion

We have shown that the human K_{ATP} channel comprising SUR1 and Kir6.2 subunits is directly phosphorylated by PKA or Gs-protein coupled receptor stimulation when reconstituted in *Xenopus* oocyte homogenates or in intact cells. We identified S372 and S1571 in Kir6.2 and SUR1, respectively, as unique targets for PKA-mediated phosphorylation in each subunit. The phosphorylation detected in MIN6 cells and the increase in channel activity in inside-out patch configuration after PKA catalytic subunit application suggest that K_{ATP} channels are positively controlled by a PKA-mediated process. The abolition of this increase in activity after disruption of serine in Kir6.2 indicates clearly that Kir6.2 is the major site of PKA phosphorylation, and that channel activity is directly modulated by such PKA-mediated protein phosphorylation. Native K_{ATP} channels in the pancreatic β-cell derived cell lines RINm5F and HIT showed a similar increase in channel amplitude (2-3 fold) after dbcAMP stimulation (Ribalet et al. 1989).

In addition, in excised inside-out patch configuration, K_{ATP} channels in RINm5F and HIT exhibit a similar increase in open probability after PKA catalytic subunit application (Ribalet et al., 1989). Rodent pancreatic β-cell K_{ATP} channels (Kir6.2/SUR1) reconstituted in COS-1 cells also require phosphorylated serine at residue 372 in Kir6.2 to significantly increase the channel activity by PKA stimulation (Nagashima et al., in preparation). Since phosphorylation of the Kir6.2 subunit is necessary for modulating both human and rodent Kir6.2/SUR1 channel activity, and, in addition, the Kir6.2 subunit couples with either SUR1, SUR2A, or SUR2B subunit (Inagaki et al., 1995; 1996; Isomoto et al., 1996), PKA-mediated phosphorylation of the Kir6.2 subunit rather than the SUR1 subunit seems to play the more general role in modulating K_{ATP} channel activity. However, the simple replacement of the PKA phosphorylation site at serine-1571 with alanine in human SUR1 prolonged the burst and cluster duration, interburst interval (τ_{c3}) and increased the open probability. The K_{ATP} channels also underwent a reduction of activity by 60-70% in intact cells, which might be explained by impaired functional expression of K_{ATP} channels at the cell surface. Moreover, alkaline phosphatase treatment clearly demonstrate that SUR1 is already phosphorylated by PKA in the basal state in intact cells. Accordingly, in contrast to the case in rodents, PKA phosphorylation of human SUR1 is responsible in part for the maintenance of functional expression of the channels at the cell surface, and seems to be a main factor in determining the kinetic properties observed in native human K_{ATP} channels. Consistently with our data, Babenko et al. (1998) et al. found that removal of only the 15 residues of SUR1 in which the PKA phosphorylation site is located eliminates almost all of the channel activity, as assessed by rubidium efflux measurements using a series of small truncations from the C-terminal end of SUR1.

How the PKA phosphorylation site of SUR1 is tonically phosphorylated while that of Kir6.2 is not remains unclear. There might be an unidentified molecule that modulates the phosphorylation by interacting with the SUR1 or Kir6.2 subunit. Alternatively, the affinities of the PKA phosphorylation sites of the two subunits might be different. For example, A-kinase-anchoring proteins (AKAPs) which are known to anchor PKA close to preferred substrates (Rubin et al., 1994), could be located in proximity with SUR1 but not with Kir6.2 to explain the observed difference of PKA phosphorylation in the basal state. Another interesting feature of the PKA phosphorylation sites in the different subunits is that they contribute to open probability in the K_{ATP} channels in an opposite way : SUR1 and Kir6.2 PKA phosphorylation

decreasing and increasing the channel open probability, respectively. Accordingly, both of the C-termini probably play an important role in gating the K_{ATP} channel pore by modifying the conformation of the region. Since the PKA action of each subunit can be mimicked by the Kir6.2 (S372D) or SUR1 (S1571D) mutant, the negative charges carried by both subunits seem to be good candidates for this structural change. Previous studies have also shown that the C-terminus of Kir6.2 plays a crucial role in the regulation of the K_{ATP} channel (Shyng and Nichols, 1998; Baukrowitz et al., 1998; Tucker et al., 1998). The C-terminus appears to be involved in the binding of ATP and PIP_2 (Shyng and Nichols, 1998; Baukrowitz et al., 1998; Tucker et al., 1998), to participate in the gating of the channel (Drain et al., 1998), and to be involved in the intracellular trafficking (Zerangue et al., 1999). One of the possible structural modifications by PKA phosphorylation could be interaction with cytoskeletal elements, as is known of the C-terminal region of Kir2.3, in which association or dissociation with cytoskeletal elements is under regulation by PKA-mediated phosphorylation (Cohen et al., 1996). However, the C-terminal region of Kir6.2 does not contain known motifs which could interact with cytoskeletal elements.

The PKA phosphorylation in SUR1 appears to be more complex than in Kir6.2. On one hand, the presence of the phosphate group in human SUR1 reduces open probability, which then becomes similar to rodent K_{ATP} channels (Trapp et al., 1998), suggesting that the channels in the basal state naturally tend to be in the low Po mode of gating. On the other hand, since functional expression of the channels at the cell surface is reduced in SUR1 S1571A, the PKA phosphorylation in SUR1 might also be involved in the trafficking from the intracellular vesicles to the plasma membrane, as has been described of water channel aquaporin-2 (Fushimi et al., 1997), or participate in the activation of channels already present on the plasma membrane. The C-terminus of SUR1, therefore, seems to play a critical role in the regulation of K_{ATP} channel activity.

K_{ATP} channels can be activated by Gs-coupled receptor stimulation. For example, stimulation of calcitonin gene-related peptide receptor (Wellman et al., 1998; Quayle et al., 1994), A_2 receptor (Kleppisch and Nelson, 1995), and β_1-adrenoreceptor (Narishige et al., 1994) activate K_{ATP} channels via an adenylyl cyclase/ PKA pathway in arterial smooth muscles, arterial myocytes, and coronary artery, respectively, leading to an increase in vasodilatation in these tissues. Since the present data indicate a direct phosphorylation of Kir6.2 by stimulation

of Gs-coupled receptors in intact cells, and our preliminary experiment shows that both rat SUR2A and SUR2B do not seem to be direct substrates for PKA-mediated phosphorylation (unpublished data), the mechanism by which Gs-coupled protein receptor stimulation activates K_{ATP} channels in these tissues appears to be due to a direct phosphorylation of serine 372 in Kir6.2 by PKA. In contrast, the physiological significance of PKA-mediated phosphorylation of the K_{ATP} channels in pancreatic β-cells is less certain. Activation of the K_{ATP} channels theoretically inhibits insulin secretion, while activation of the cAMP/PKA pathway normally stimulates insulin secretion (Ashcroft, 1994; Jones and Persaud, 1998). Since the basal intracellular cAMP and PKA activity in pancreatic islets are higher than in purified β-cells, and both are required to maintain secretory activity (Pipeleers et al., 1985), the K_{ATP} channel could be maximally phosphorylated in the basal state so that no more increase in channel activity could occur after the PKA stimulation. In that case, the PKA-mediated phosphorylation of the K_{ATP} channel might not participate in the stimulation process of insulin secretion. This hypothesis of tonic PKA phosphorylation of K_{ATP} channels is supported by a phosphorylation study of the glucose transporter GLUT2 which shows that GLUT2 is maximally phosphorylated by PKA in the basal state in pancreatic islets but not in purified β-cells (Thorens et al., 1996). Another possible role of PKA-mediated phosphorylation of K_{ATP} channels in β-cells could be its participation in the oscillation process comprising depolarization and hyperpolarization (Ullrich et al., 1996).

In summary, our data indicate that while the Kir6.2 subunits appear to be phosphorylated by PKA in all species identified to date, the phosphorylation of the SUR1 subunits is species specific. In addition, the role of the phosphorylation of each subunit is distinct: the PKA phosphorylation in Kir6.2 is responsible for increasing channel activity of the K_{ATP} channels and can be phosphorylated by Gs coupled receptors, while the phosphorylation in SUR1 participates in the basal state of the channel properties, including burst and cluster duration, interburst interval and open probability, and is also important for the maintenance of functional expression of the channels at the cell surface. Accordingly, the present study shows a model of modulation of heteromultimeric ion channels in which PKA-mediated phosphorylation of the different subunits has distinct roles.

Materials and methods

Site-directed mutagenesis

Point mutations were introduced into the putative PKA phosphorylation sites of human Kir6.2 and SUR1 by the polymerase chain reaction (PCR)-based method. The mutations were confirmed by DNA sequencing using automated DNA sequence analysis (ABI PRISM 310 Genetic Analyser, Perkin Elmer). Human SUR1 cloned from a human insulinoma cDNA library (Genbank accession #AF087138), human Kir6.2 (Inagaki et al., 1995), and rat SUR1 (gift from G. Bell) were subcloned into the pSD5 vector. To increase the translation of foreign cRNA in *Xenopus* oocytes, the 5'-untranslated region of human Kir6.2, human SUR1, and rat SUR1 was replaced with a modified *Xenopus* 5'-untranslated region as described previously (Béguin et al., 1996).

Expression of cRNAs in Xenopus oocytes and phosphorylation in homogenates

Stage V-VI oocytes were obtained from *Xenopus* laevis as described previously (Béguin et al., 1996). 2.5 ng of *in vitro* synthesized cRNA for human Kir6.2 and 10 ng of cRNA for human or rat SUR1 were injected into oocytes. To confirm protein expression, some of the injected oocytes were incubated in modified Barth's medium containing 0.6 mCi/ml ^{35}S-methionine for 36 h and subjected to a 12 h chase in modified Barth's medium containing 10 mM cold methionine. Even after 3 days chase, Kir6.2 and SUR1 injected alone or together were stable in the oocytes, indicating that both subunits are insensitive to the oocyte degradation pathway (unpublished data). Triton extracts were then prepared as described (Béguin et al., 1996) and Kir6.2 and SUR1 were immunoprecipitated (see below). The remaining oocytes were incubated for 2 days in modified Barth's medium and yolk-free homogenates were prepared as described previously (Béguin et al., 1996) and subjected to phosphorylation experiments.

The PKA dependent phosphorylation reaction in homogenates was done in aliquots of 8-9 oocytes in the presence of 100 μM γ-^{32}P ATP and 50 μM cAMP (Sigma) at 25°C for 30 min. In some cases, the PKA inhibitor H-89 (Calbiochem) was added 15 min prior to the start of reactions. The reactions were stopped by heating at 95°C and adding SDS (3.7% final concentration) and the samples then were subjected to immunoprecipitation (see below).

Cell culture and DNA transfection

COS-1 and MIN6 cells were grown in Dulbecco's modified Eagle's medium (high glucose) supplemented with 10 % fetal bovine serum, streptomycin (60.5 μg/ml) and penicillin (100

μg/ml). COS-1 cells were transiently transfected with wt or mutated human Kir6.2 cDNA plus human SUR1 (4 μg and 1μg of each subunit for biochimical and electrophysilogical studies, respectively), using lipofectamine and Opti-MEM I reagents (Life Technologies) or FuGENE6 (Boehringer), according to the manufacturer's instructions. In some cases, human somatostatin receptor SSTR3 cDNA (Yamada et al., 1992), GIP receptor cDNA (Yasuda et al., 1994), or PACAPR-3 cDNA (Inagaki et al., 1994) (3 μg for each) was also co-transfected into COS-1 cells. Transfected 100-mm dishes were split into three 60-mm dishes. Two days after transfection, one dish was used for biosynthetic ^{35}S-methionine labelling and the two remaining dishes were used for phosphorylation assay (see below). For electrophysiological recordings, COS-1 cells were plated on dishes containing glass coverslips and either wild-type or mutated Kir6.2 cDNA and SUR1 cDNA were cotransfected with the green fluorescent protein cDNA (pSR α-GFP, 0.5 μg) as a reporter gene.

Metabolic labelling and " in vitro" and "in vivo" phosphorylation in COS-1 and MIN6 cells

COS-1 cells were metabolically labelled by equilibrated cells in Dulbecco's modified Eagle's medium without methionine for 1 h and then incubated in the same medium containing ^{35}S-methionine (0.2 mCi/ml) (Amersham) for 18h at 37°C. The phosphorylation experiments were performed as follows: COS-1 or MIN6 cells were equilibrated in phosphate-free Dulbecco's modified Eagle's medium for 2h and then incubated in the same medium containing carrier-free ^{32}P (0.2mCi/ml; Amersham) for two additional hours at 37°C. PKA-mediated phosphorylation after direct PKA stimulation was tested by adding 100 μM forskolin (Sigma) in the presence of 1 mM (IBMX) (Sigma) or by adding 1 mM dibutyryl cAMP (dbcAMP) (Sigma) for 20 min. PKA-mediated phosphorylation after G protein-coupled receptor stimulation was tested by adding agonists for endogenous or transfected receptors during 20 min as indicated in Fig. 5. Microsomes were prepared as described (Borghini et al., 1994) and Kir6.2 or SUR1 were immunoprecipitated (see below).

The membrane preparation for the "*in vitro*" phosphorylation from COS-1 cells was done as described above, except that the lysis buffer did not contain β-mercaptoethanol or phosphatase inhibitors and the final pellet was resuspended in a buffer containing 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 5 μg/ml each of leupeptin, pepsatin, and antipain. The PKA dependent phosphorylation was performed under the

μg/ml). COS-1 cells were transiently transfected with wt or mutated human Kir6.2 cDNA plus human SUR1 (4 μg and 1μg of each subunit for biochimical and electrophysilogical studies, respectively), using lipofectamine and Opti-MEM I reagents (Life Technologies) or FuGENE6 (Boehringer), according to the manufacturer's instructions. In some cases, human somatostatin receptor SSTR3 cDNA (Yamada et al., 1992), GIP receptor cDNA (Yasuda et al., 1994), or PACAPR-3 cDNA (Inagaki et al., 1994) (3 μg for each) was also co-transfected into COS-1 cells. Transfected 100-mm dishes were split into three 60-mm dishes. Two days after transfection, one dish was used for biosynthetic ^{35}S-methionine labelling and the two remaining dishes were used for phosphorylation assay (see below). For electrophysiological recordings, COS-1 cells were plated on dishes containing glass coverslips and either wild-type or mutated Kir6.2 cDNA and SUR1 cDNA were cotransfected with the green fluorescent protein cDNA (pSR α-GFP, 0.5 μg) as a reporter gene.

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The membrane preparation for the "*in vitro*" phosphorylation from COS-1 cells was done as described above, except that the lysis buffer did not contain β-mercaptoethanol or phosphatase inhibitors and the final pellet was resuspended in a buffer containing 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 5 μg/ml each of leupeptin, pepsatin, and antipain. The PKA dependent phosphorylation was performed under the

following conditions: 85 µg of resuspended microsome preparation was incubated at 30°C for 30 min in the presence of 100 µM γ^{32}P ATP and 20 U of DTT-reconstituted bovine heart PKA catalytic subunits (Sigma). The reactions were stopped by heating at 95°C and adding SDS (3.7% final concentration) and then the samples were subjected to immunoprecipitation (see below).

Immunoprecipitation

Aliquots of phosphorylated or ^{35}S-methionine-labelled oocyte homogenates and COS-1 or MIN6 microsomes were immunoprecipitated by specific antibodies prepared against unconjugated 24 mer peptide (amino acids 364-387) of the C-terminal region of mouse Kir6.2 or against KL-hemocyanin-conjugated 21 mer peptide (amino acids 1563-1583) of the C-terminal region of rat SUR1. Immunoprecipitations were performed under denaturing conditions, resolved by SDS-PAGE and revealed by fluography as described previously (Béguin et al., 1996).

Rubidium efflux

Rubidium efflux in COS-1 cells expressing the wild-type and mutant K_{ATP} channels was performed as described (Inagaki et al., 1995). To confirm a similar protein expression of the different transfected subunits, ^{35}S-metabolic labelling was performed as described above.

Electrophysiology

Recordings were made 24-72 hours after transfection. Single-channel recordings were made with a patch-clamp amplifier EPC-7 (List Electronics) as described (Inagaki et al.,1995). The bath solution contained 110 mM potassium aspartate, 30 mM KCl, 2 mM $MgSO_4$, 1 mM EGTA, 0.084 mM $CaCl_2$ and 10 mM MOPS (pH 7.2) at a concentration of free Mg^{2+} of 0.8 mM. The pipette solution contained 140 mM KCl, 2 mM $CaCl_2$ and 5 mM MOPS (pH 7.4). Experiments were performed at room temperature (24-26°C). The holding potential was -70mV. Before experiments, the cells were perifused in the bath solution for at least 30 min. In inside-out patch configuration, the patches were first recorded in a bath solution containing 10 µM ATP for at least 2 min and then exposed to the test solution. PKA catalytic subunit (Sigma) and alkaline phosphatase (Boehringer, Mannheim) were used in a final concentration of 100 U/ml and 10 U/ml, respectively. Before and after PKA stimulation, ATP-sensitivity was determined at 1, 10, 100 and 1000 µM ATP as described (Inagaki et al., 1995). Channel kinetics after AP treatment were obtained by analysing the data between 0 and 2 min. To

measure channel density at the cell surface, single channel recordings were performed between 48 and 72 hours after transfection. Detectable rate of channels was determined by the ratio between patches expressing K_{ATP} channels and the total number of examined patches (n=80). Three independent experiments were performed. The number of channels in a patch was estimated by dividing the maximum current amplitude by the K_{ATP} channel unitary current.

Data collection

Single channel currents were continuously stored on VCR tape with a digital data recorder for later analysis. Data were analysed by a combination of pCLAMP (ver6.0, Axon Instruments, CA,USA) and in-house software. For evaluating the effects of the PKA catalytic subunit and alkaline phosphatase on the channel activity (NPo) before and after applying the enzymes, the currents were measured for 2 min and averaged. The burst and cluster duration, intraburst and interburst kinetics were measured form patches possessing only one functional channel. For burst analysis, based on the preliminary experiment, a burst in channel activity was defined as a set of openings and closures terminated by a close event with a duration that exceeded 2.5 ms (Alekseev et al., 1997; Sanchez et al., 1998). A group of bursts which are separated by closed time less than 20 ms are considered as one cluster of bursts. Closed time distributions in the total record were well fitted by a sum of three exponents (τ_{c1},τ_{c2}, τ_{c3}) (Alekseev et al., 1997). Statistical significances were tested using unpaired and paired Student's t-tests and results are expressed as mean ± S.E.

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Figure legends

Fig. 1. Consensus phosphorylation sites for PKA in Kir6.2 and SUR1 subunits of the human K_{ATP} channel. Filled circles indicate serine and threonine residues in the consensus phosphorylation sites tested in this study. Open circle represents an additional site. Open square A and B indicate the position of Walker A and B motifs in nucleotide binding fold (NBF).

Fig. 2. Expression of human wild type (wt) and mutant Kir6.2 in *Xenopus* oocytes and phosphorylation in homogenates upon stimulation of cellular PKA. (A) Cellular expression of wt and mutant Kir6.2 in oocytes. Control oocytes without injection (lane 1) or oocytes injected with human SUR1 cRNA together with human wt Kir6.2 (lane 2) or mutant Kir6.2 (lanes 3-5) cRNA. (B) Phosphorylation of wt and mutant Kir6.2 in homogenates. The phosphorylation reaction was done in the absence (lanes 1, 3, 6, 8, 10) or in the presence of 50 μM cAMP (lanes 2, 4, 5, 7, 9, 11) and in the absence (lanes 1-4, 6-11) or in the presence of 2 μM PKA inhibitor H-89 (lane 5). Autoradiographs of immunoprecipitation of human Kir6.2 are shown. A representative example from three similar results is shown. st, proteins of known molecular mass (97.4, 66, 46, 30, 14.3 kDa). ni, non-injected oocytes.

Fig. 3. Expression of human wild type (wt), rat wt, and human mutant SUR1 in *Xenopus* oocytes and phosphorylation in homogenates upon stimulation of cellular PKA. (A) Cellular expression of wt and mutant SUR1 in oocytes. Control oocytes without injection (lane 1) or injected with human Kir6.2 cRNA together with human wt (lane 2), rat wt (lane 6), or human mutant (lanes 3-5) SUR1 cRNA. (B) Phosphorylation of wt and mutant SUR1 in homogenates. The phosphorylation reaction was done in the absence (lanes 1, 3, 6, 8, 10, 12) or in the presence of 50 μM cAMP (lanes 2, 4, 5, 7, 9, 11, 13, 14) and in the absence (lanes 1-4, 6-13) or in the presence of 2 μM PKA inhibitor H-89 (lanes 5, 14). Autoradiographs of immunoprecipitation of human and rat SUR1 are shown. A representative example from three similar results is shown. st, proteins of known molecular mass (97.4, 66, 46, 30, 14.3 kDa). ni, non-injected oocytes.

Fig. 4. Expression and phosphorylation of human wt and mutant K_{ATP} channels in intact cells upon PKA stimulation. (A, B) Microsomal expression of wt Kir6.2/SUR1 and mutant Kir6.2

S372A/ SUR1 S1571A K_{ATP} channels in COS-1 cells. Untransfected control COS-1 cells (lane 1) or COS-1 cells transfected with wt Kir6.2 and wt SUR1 (lane 2) or mutant Kir6.2 S372A and SUR1 S1571 (lane 3) cDNA. (C, D) Phosphorylation of wt and mutant K_{ATP} channels in COS-1 cells. The phosphorylation assay was done in the absence of PKA stimulators (lanes 1, 4, 7) or in the presence of 100 μM forskolin and 1 mM IBMX (lanes 2, 5, 8) or in the presence of 1 mM dbcAMP (lanes 3, 6, 9). Autoradiographs of immunoprecipitation of human Kir6.2 and SUR1 are shown. A representative example from three similar results is shown. st, proteins of known molecular mass (97.4, 66, 46, 30, 14.3 kDa). Non-specific bands (*) were observed after immunoprecipitation of the phosphorylated samples from untransfected and transfected COS-1 cells.

Fig. 5. Expression and phosphorylation of microsomal human wt and mutant SUR1 with PKA. (A) Microsomal expression of wild type (wt) and mutant SUR1. Untransfected control COS-1 cells (lanes 1, 2) or COS-1 cells transfected with wt Kir6.2 and wt SUR1 cDNAs (lanes 3, 4) or the mutant Kir6.2 S372A and the mutant SUR1 S1571A cDNAs (lanes 5, 6). (B) Phosphorylation of wt and mutant SUR1 in COS-1 cells. The "*in vitro*" phosphorylation assay was done in the absence (lanes 1, 3, 5) or in the presence (lanes 2, 4, 6) of the PKA catalytic subunit (20 U/reaction). Autoradiographs of immunoprecipitation of human SUR1 are shown. A representative example from three similar results is shown. st, proteins of known molecular mass (97.4, 66, 46, 30, 14.3 kDa). Non-specific bands (*) were observed after immunoprecipitation of the phosphorylated samples from untransfected and transfected COS-1 cells.

Fig. 6. Expression and phosphorylation of human wt and mutant K_{ATP} channels in COS-1 cells and the K_{ATP} channels in MIN6 cells upon G protein-coupled receptor and PKA stimulation. (A) Microsomal expression of wt Kir6.2 and mutant Kir6.2 S372A in COS-1 cells. COS-1 cells expressing endogenous β_2-adrenergic receptor (lanes 9-12) or expressing various G-coupled receptors: PACAP receptor (lanes 1-4), GIP receptor (lanes 5-8), or somatostatin receptor (lanes 13-16) cDNA was co-transfected with wt Kir6.2 and wt SUR1 (lanes 1, 2, 5, 6, 9, 10, 13, 14) or co-transfected with the mutant Kir6.2 S372A and SUR1 S1571A cDNAs (lanes 3, 4, 7, 8, 11, 12). (B) Phosphorylation of wt and the mutant Kir6.2 in

COS-1 cells. The phosphorylation assay was done in the absence of agonist (lanes 1, 3, 5, 7, 9, 11, 13, 15) or in the presence of 8 nM PACAP-38 (lanes 2, 4), 1 μM GIP (lanes 6, 8), 10 μM epinephrine (lanes 10, 12) or 50 nM somatostatin (lanes 14, 16). (C, D) Phosphorylation of Kir6.2 and SUR1 in mouse pancreatic β-cell-derived MIN6 cells. The phosphorylation assay was performed in the absence of stimulator (lane 1) or in the presence of 100 μM forskolin and 1 mM IBMX (lane 2), 1 mM dbcAMP (lane 3), or 8 nM PACAP-38 (lane 4). Autoradiographs of immunoprecipitation of human Kir6.2 (A, B) from transfected COS-1 cells or mouse SUR1 (C) and Kir6.2 (D) from MIN6 cells are shown. A representative example from three similar results is shown. st, proteins of known molecular mass (97.4, 66, 46, 30, 14.3 kDa). Non-specific bands (*) were observed after immunoprecipitation of the phosphorylated samples from transfected COS-1 cells and MIN6 cells.

Fig. 7. Changes in activities of wt and various mutant K_{ATP} channels before and after application of the PKA catalytic subunit. Current recordings were obtained in inside-out patches excised from COS-1 cells expressing wt Kir6.2 and wt SUR1 (A), wt Kir6.2 and the mutant SUR1 S1571A (B), the mutant Kir6.2 S372A and wt SUR1 (C), and the mutant Kir6.2 S372A and mutant SUR1 S1571A (D). The effect of PKA on channel activity was examined in the same patch for each K_{ATP} channel. Holding membrane potential was -70 mV. Representative recordings before and at 8-10 min after PKA application are shown. Right, relative open probability (NPo) recorded at 8-10 min, in the absence (hatched columns) or presence (filled columns) of PKA catalytic subunit in the bath solution. n=9-10 patches for (A)-(D).

Fig. 8. Changes in burst and cluster duration, interburst interval and open probability before and after application of alkaline phosphatase (10U/ml). Single channels were obtained in inside-out patches excised from COS-1 cells expressing wt Kir6.2 and wt SUR1 (A), wt Kir6.2 and the mutant SUR1 S1571A (B), the mutant Kir6.2 S372A and wt SUR1 (C), the mutant Kir6.2 S372A and mutant SUR1 S1571A (D). Holding membrane potential was -70 mV. Representative recordings before and at 2 min after alkaline phosphatase application are shown. Right, time course of the relative channel activiy (NPo) recorded in the absence (open circles) or presence (filled circles) of alkaline phosphatase in the bath solution. n=7-11 patches in (A)-(D).

Table I. Properties of wt and various mutant K_{ATP} channels

K_{ATP} channel	Open probability (Po)	Burst duration (ms)	Cluster duration (ms)	Intraburst kinetics (ms)		Interburst kinetics (ms)		Channel density	
				Open time (τ_o)	Short closed time (τ_{c1})	Long closed time (τ_{c2})	(τ_{c3})	Detectable rate (%)	Channel number in a patch
Kir6.2 wt / SUR1 wt	0.12 ± 0.06	25 ± 11	75 ± 18	1.94 ± 0.19	0.49 ± 0.03	10.1 ± 2.9	131 ± 66	66.3	26.4 ± 6.5
Kir6.2 wt / SUR1 S1571A	0.44 ± 0.06 *	73 ± 17*	394 ± 113*	2.21 ± 0.13	0.44 ± 0.03	12.9 ± 4.5	1120 ± 71 *	40.0	4.8 ± 0.8 *
Kir6.2 S372A / SUR1 wt	0.13 ± 0.03	35 ± 12	75 ± 12	1.92 ± 0.10	0.49 ± 0.02	8.0 ± 2.6	102 ± 36	65.0	19.2 ± 3.4
Kir6.2 S372A / SUR1 S1571A	0.62 ± 0.08 *	74 ± 20 *	274 ± 37 *	2.22 ± 0.30	0.49 ± 0.04	10.5 ± 1.0	863 ± 49 *	35.0	3.2 ± 0.4 *

Recordings were made at -70 mV in ATP-free conditions [n= 5-7 in all cases, except for the channel density determination (n=60-80)].

Data marked with * are significantly different from those for wt K_{ATP} channels ($P \leq 0.05$).

Table II. Properties of wt and various mutant K_{ATP} channels after AP treatment

K_{ATP} channel	Burst duration (ms)	Cluster duration (ms)	Long closed time (τ_{c3})
Before alkaline phosphatase			
Kir6.2 wt / SUR1 wt	15.7 ± 2.4	40 ± 13	186 ± 53
Kir6.2 wt / SUR1 S1571A	26.6 ± 7.6	152 ± 35	1318 ± 208
Kir6.2 S372A / SUR1 wt	18.7 ± 3.0	59 ± 5	197 ± 57
Kir6.2 S372A / SUR1 S1571A	53.6 ± 3.7	169 ± 19	1654 ± 594
After alkaline phosphatase			
Kir6.2 wt / SUR1 wt	50.3 ± 9.2 *	155 ± 32 *	402 ± 60
Kir6.2 wt / SUR1 S1571A	36.9 ± 11.1	169 ± 41	1236 ± 279
Kir6.2 S372A / SUR1 wt	41.1 ± 3.2 *	103 ± 15 *	576 ± 162
Kir6.2 S372A / SUR1 S1571A	52.6 ± 6.1	178 ± 37	1743 ± 485

Recordings were made at -70 mV in 10μM ATP conditions (n= 3-6 in all cases).
Data marked with * are significantly different after AP treatment ($P<0.05$).

Fig. 1

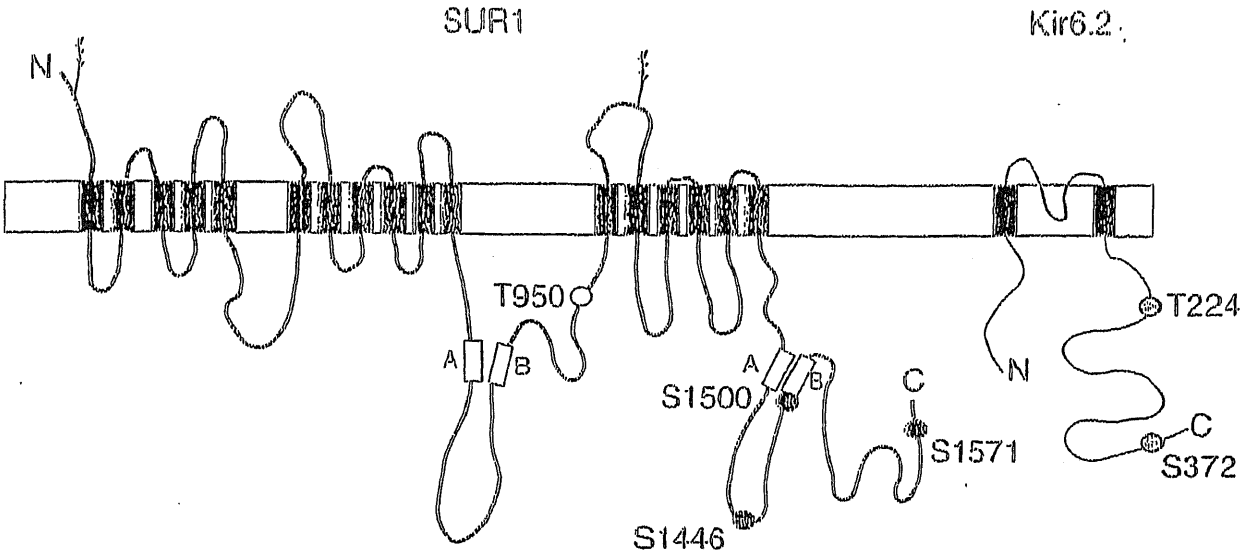
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Fig. 2

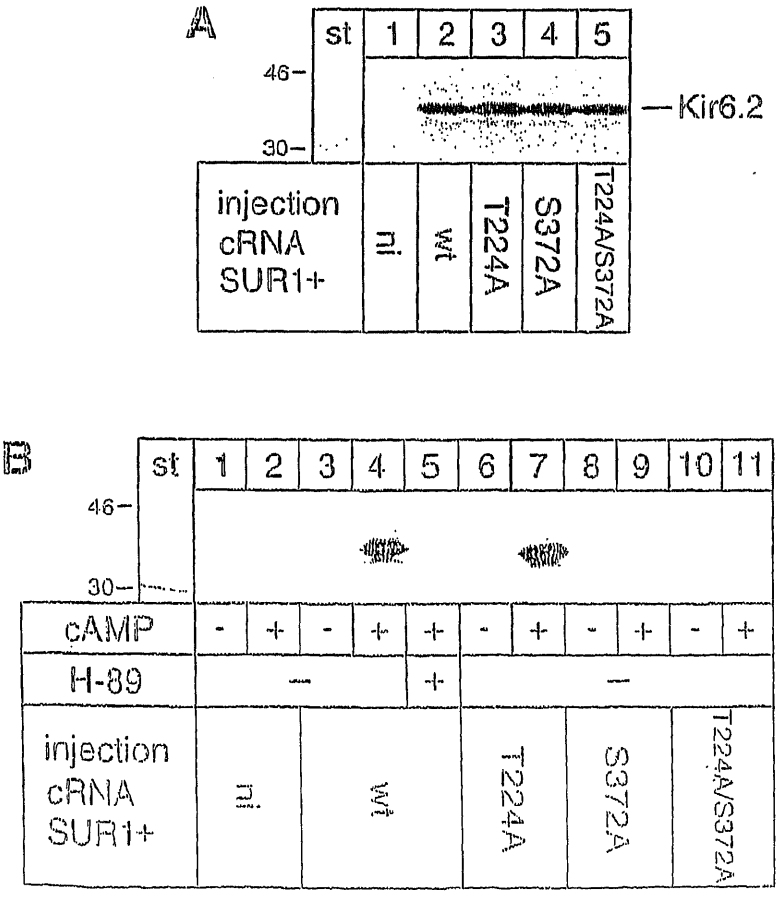
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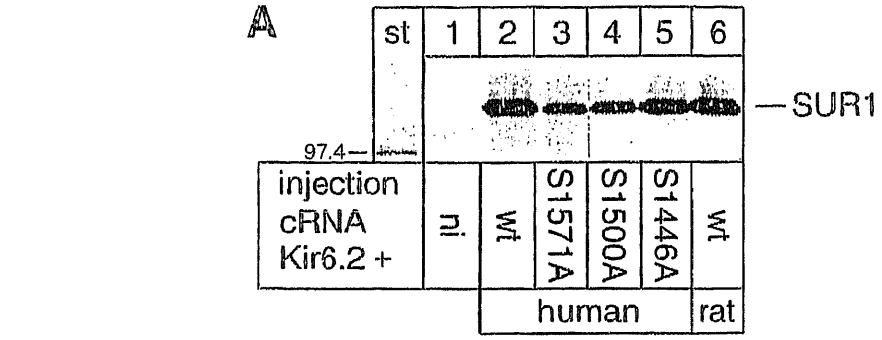
Fig. 3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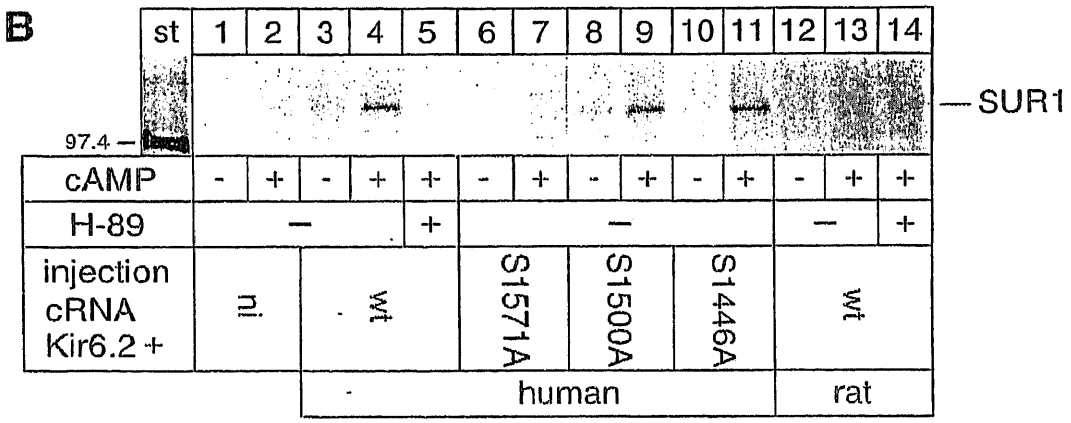
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Fig. 4

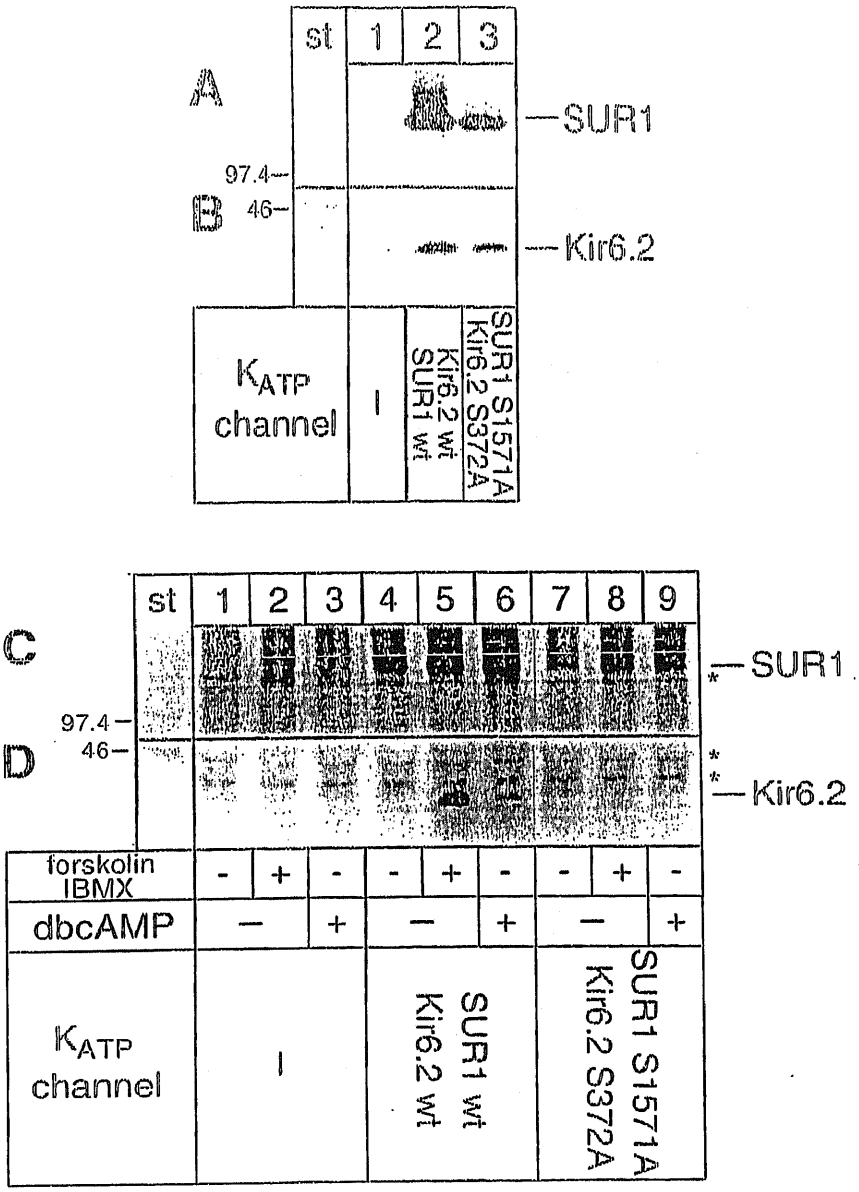
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Fig. 5

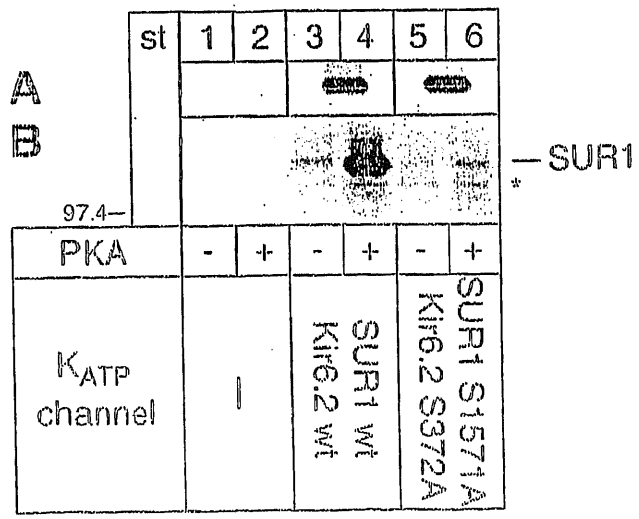
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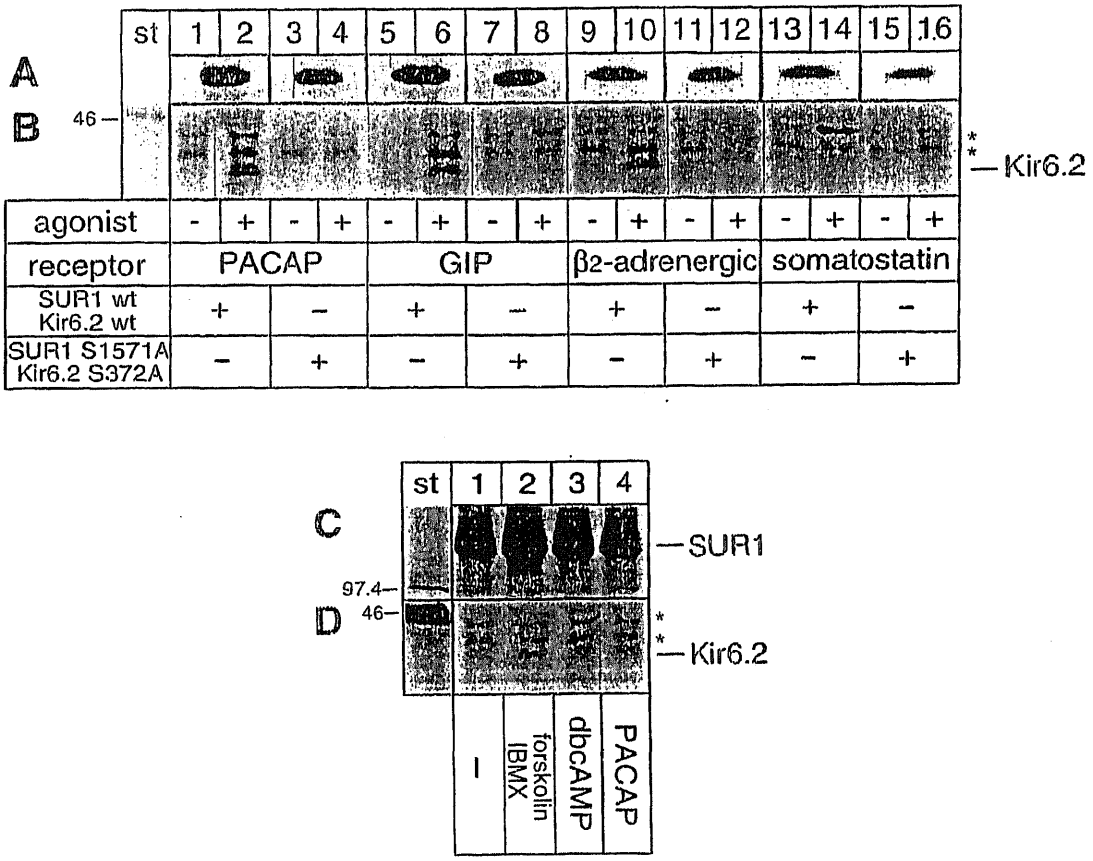
Fig. 6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4
5
6
7
8
9
10
11
12
13
14
15
16
A
B
46
**
Kir6.2
agonist
receptor
PACAP
GIP
β2-adrenergic
somatostatin
SUR1 wt
Kir6.2 wt
SUR1 S1571A
Kir6.2 S372A

st
1
2
3
4
C
D
97.4
46
SUR1
**
Kir6.2
–
forskolin IBMX
dbcAMP
PACAP

Fig. 7

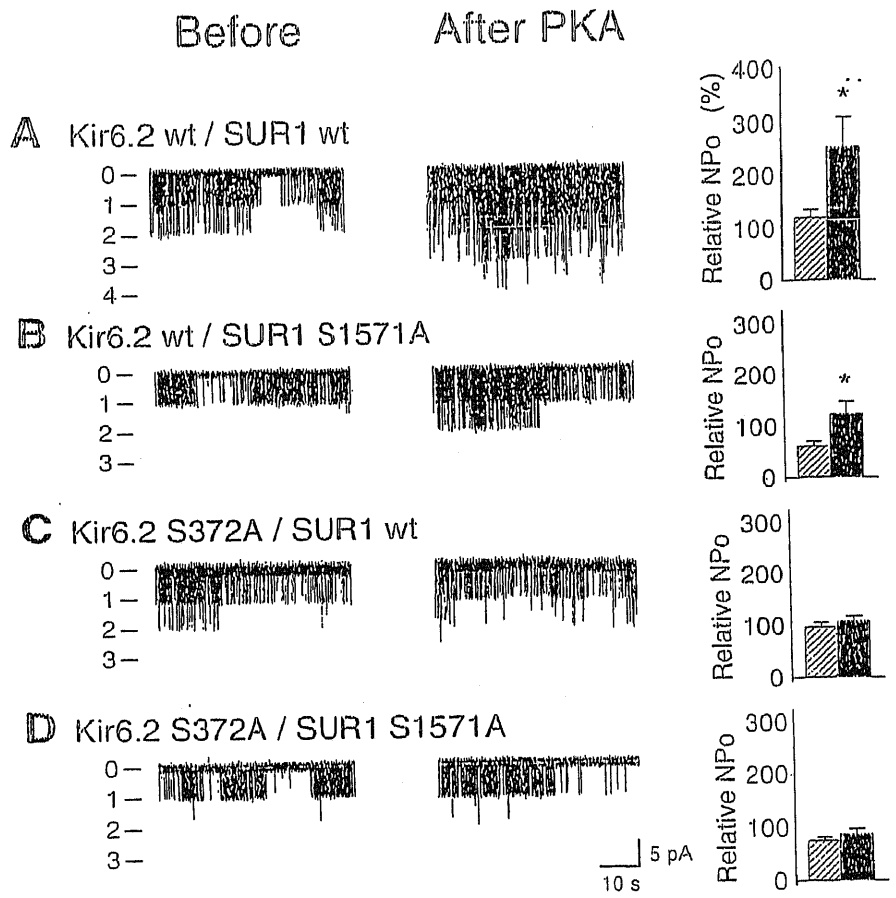
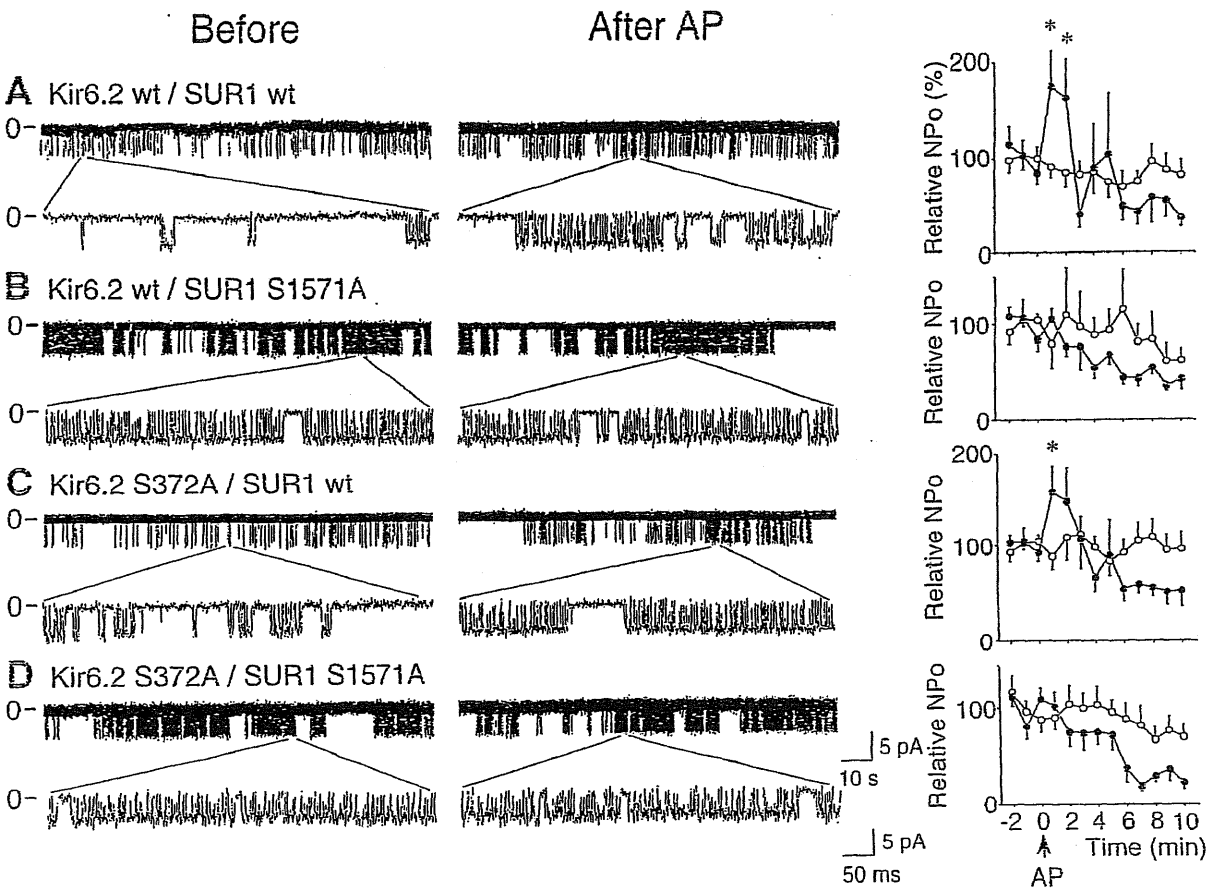

Before
After PKA
A Kir6.2 wt / SUR1 wt
B Kir6.2 wt / SUR1 S1571A
C Kir6.2 S372A / SUR1 wt
D Kir6.2 S372A / SUR1 S1571A
0
1
2
3
4
5 pA
10 s
Relative NPo (%)
Relative NPo
400
300
200
100
0
*

Fig. 8


Before
After AP
A Kir6.2 wt / SUR1 wt
B Kir6.2 wt / SUR1 S1571A
C Kir6.2 S372A / SUR1 wt
D Kir6.2 S372A / SUR1 S1571A
0
0
5 pA
10 s
5 pA
50 ms
Relative NPo (%)
Relative NPo
Relative NPo
Relative NPo
200
100
0
-2 0 2 4 6 8 10
Time (min)
AP

Inhibition of ATP-sensitive K^+ channel activity with troglitazone, but not with pioglitazone: Studies on the reconstituted K_{ATP} channels, Kir6.2/SUR1 and Kir6.2/SUR2A

Abstract

We compared the effects of the two thiazolidinedione derivatives, troglitazone and pioglitazone, on ATP-sensitive K^+ (K_{ATP}) channel activities. Pancreatic β-cell type and cardiac type K_{ATP} channels were reconstituted in COS-1 cells (SV 40-transformed African green monkey kidney (AGMK) cells) by heterologously expressing sulfonylurea receptor 1 (SUR1) plus Kir6.2 and sulfonylurea receptor 2A (SUR2A) plus Kir6.2, respectively.

Troglitazone inhibited [$^{86}Rb^+$] efflux in both K_{ATP} channel types in the presence of metabolic inhibitors, which was confirmed by electrophysiological techniques. The [$^{86}Rb^+$] efflux increased by the channel openers diazoxide and pinacidil was abolished by troglitazone. In contrast, pioglitazone did not affect these channel activities in either type K_{ATP} channel. These results suggest that troglitazone modulates the various cellular functions including insulin secretion by inhibiting the K_{ATP} channels, while pioglitazone has no effect on K_{ATP} channel activity.

1. Introduction

ATP-sensitive K^+ channels (K_{ATP} channels) are characterized by an inhibition of channel opening when the ATP/ADP ratio at the cytoplasmic cell surface is increased (Noma, 1983). K_{ATP} channels play an important role in various cellular responses such as secretion and muscle contraction, by linking the metabolic status of the cell to its membrane potential (Ashcroft, 1988);they have been found in various tissues including pancreatic β-cells, skeletal muscle, brain, and vascular and nonvascular smooth muscle (Ashford et al., 1988; Cook and Hales, 1984; Spruce et al., 1985; Standen et al., 1989). Since the discovery of K_{ATP} channels in pancreatic b-cells, the sulfonylureas, insulin secretagogues widely used as oral hypoglycemic agents in the treatment of diabetes mellitus, have been shown to inhibit the activity of these K_{ATP} channels (Sturgess et al., 1985; Trube et al., 1986). Molecular cloning of the high affinity sulfonylurea receptor (SUR) revealed it to be a member of the ATP-binding cassette (ABC) superfamily (Aguilar Bryan et al., 1995). It has been shown that classical K_{ATP} channels are complexes of two subunits, Kir6.2 subunits, which form the K^+-selective ion channel pore, and SUR subunits, receptors for sulfonylureas (Seino, 1999); pancreatic β-cell type and cardiac type K_{ATP} channels comprise Kir6.2 and SUR1 subunits (Inagaki et al., 1995; Sakura et al., 1995) and Kir6.2 and SUR2A (Inagaki et al., 1996) subunits, respectively. The thiazolidinedione derivatives troglitazone and pioglitazone are recently developed orally active hypoglycemic compounds that improve insulin resistance in diabetic rodents and in patients with diabetes mellitus (Ciaraldi et al., 1990; Fujiwara et al., 1988; Fujiwara et al., 1991; Hofmann et al., 1992; Kemnitz et al., 1994; Kobayashi et al., 1982). We have previously shown that troglitazone is capable of directly stimulating insulin secretion from pancreatic β-cells, although it is not apparent until after a few minutes (Masuda et al., 1995). In this study, we compare the effects of trogliatizone and piogliatazone on reconstituted pancreatic β-cell type and cardiac type K_{ATP}

channels, and find that while troglitazone has an effect, pioglitazone has none, suggesting the different effects of these two anti-diabetic agents.

2. Materials and Methods

2.1. Cell culture and transfection

COS-1 cells (SV 40-transformed African green monkey kidney (AGMK) cells) were plated at a density of 2 x 10^5 cells per dish (35mm in diameter) for single channel analysis or 3 x 10^5 per well (30 mm 6-well dish) for [$^{86}Rb^+$] efflux measurements, and cultured in Dulbecco's modified Eagles medium (DMEM, 4500 mg/L glucose) supplemented with 10 % fetal calf serum. For single channel analysis, cytomegalovirus-promoter-driven hamster SUR1-expression plasmid, pCMVhaSUR1 (1.5 μg), or rat SUR2A-expression plasmid, pCMVrSUR2A (1.5 μg) and mouse Kir6.2-expression plasmid, pCMVmKir6.2 (1.5 μg), with the expression plasmid for green fluorescence protein (pSRαGFP, 0.05 μg) or pS65T-C1 (Clontech, CA) as a reporter gene (Marshall et al., 1995), were transfected into COS-1 cells with Lipofectoamine and OPTI-MEM I reagents (Life Technologies, Inc.) and pAdVantage (0.5 μg, Promega), according to the instructions of the manufacturer. For [$^{86}Rb^+$] efflux measurements, pCMVhaSUR1 (1.0 μg) or pCMVrSUR2A (1.0 mg) and pCMVmKir6.2 (1.0 μg) were transfected into COS-1 cells with Lipofectoamine and OPTI-MEM I reagents.

2.2. [$^{86}Rb^+$] efflux measurements

Two days after transfection, [^{86}Rb]Cl (1 mCi/ml, Amersham Pharmacia Biotech, UK) was added in fresh DMEM containing 10 % fetal calf serum and incubated for 12-24 h. The cells were further incubated for 30 min at 37 °C in Krebs-Ringer solution (118 mM NaCl, 5.0 mM $NaHCO_3$, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 20 mM HEPES, pH=7.4) containing 1 mCi/ml [^{86}Rb]Cl with or without 2.5 mg/ml of oligomycin and 1 mM of 2-deoxy-D-glucose. After washing the cells once in [$^{86}Rb^+$]-free Krebs-Ringer solution, with or without added metabolic inhibitors and thiazolidinedione derivatives, [$^{86}Rb^+$] efflux was

measured at 37 °C as previously described (Inagaki et al., 1995): briefly, the medium was removed at each time point and replaced with fresh medium containing the indicated concentrations of troglitazone or pioglitazone, with or without metabolic inhibitors. The medium at each time point was counted, and the values were summed to determine flux. The data are presented as the percentage of total cellular [$^{86}Rb^{+}$]. All of the curves are the average of three or more independent experiments. Troglitazone and pioglitazone were dissolved in dimetylsulfoxide (DMSO) at a concentration of 100 mM

2.3. Electrophysiology

After transfection, the cells were cultured for 48 to 92 h before recordings. The transfected cells were selected by green fluorescence under a microscope (Marshall et al., 1995). Single channel recordings were made in the excised inside-out patch configurations as described (Hamill and Sakmann, 1981; Inagaki et al., 1995). The bath solution contained 110 mM potassium aspartate, 30 mM KCl, 2 mM $MgSO_4$, 1 mM EGTA, 0.084 mM $CaCl_2$ and 10 mM MOPS (pH7.2). Dipotassium ATP (0.001 mM) was added to the bath solution unless otherwise noted. The pipette solution contained 140 mM KCl, 2 mM $CaCl_2$ and 5 mM MOPS (pH 7.4). Troglitazone and pioglitazone were dissolved in DMSO at a concentration of 300 mM, and then suspended in the bath solution before use. Recordings were made at 20-22 °C

3. Results

The [$^{86}Rb^{+}$] efflux from COS-1 cells cotransfected with SUR1 and Kir6.2 is greater than the efflux from those transfected with vector alone in the absence or presence of metabolic inhibitors, indicating that the efflux represents the activity of the K_{ATP} channels (Fig.1). Troglitazone had an inhibitory effect on [$^{86}Rb^{+}$] efflux from COS-1 cells cotransfected with SUR1 and Kir6.2. K_{ATP} channel activity was decreased by 30 μM and 100 μM (data not shown) troglitazone, in the absence (Fig.1A) or presence (Fig.1B) of metabolic inhibitors.

The [$^{86}Rb^{+}$] efflux from COS-1 cells cotransfected with SUR2A and Kir6.2 is greater than that from sham transfected COS-1 cells only in the presence of metabolic inhibitors, showing that SUR2A/Kir6.2 channels are closed in their absence in COS-1 cells. The effect of troglitazone was, therefore, examined on [$^{86}Rb^{+}$] efflux from COS-1 cells cotransfected with SUR2A and Kir6.2 in the presence of metabolic inhibitors. [$^{86}Rb^{+}$] efflux through the reconstituted SUR2A/Kir6.2 channels was inhibited by as little as 3 μM of troglitazone (Fig. 2).

Pioglitazone did not affect [$^{86}Rb^{+}$] efflux through K_{ATP} channels of either the pancreatic β-cell type or the cardiac type (Fig. 3).

Pancreatic β-cell type and cardiac type K_{ATP} channel activities are inhibited by glibenclamide. 10 μM or 30 μM troglitazone augmented the submaximal inhibitory effects of 3 nM or 1 μM glibenclamide on pancreatic β-cell type and cardiac type K_{ATP} channels, respectively (Fig. 4). In addition, troglitazone (10 μM or 30 μM) abolished the stimulatory effects of diazoxide (200 μM) and pinacidil (200 μM) on pancreatic β-cell type and cardiac type K_{ATP} channels, respectively (Fig. 5).

Troglitazone inhibited activity of K_{ATP} channels reconstituted from SUR1 and Kir6.2 at concentrations of 30 μM, 100 μM (data not shown), and 300 μM (Fig. 6A). Single channel conductance was not affected by the troglitazone application (Fig. 6A). The inhibitory effect of 30

μM troglitazone was abolished soon after washout of the drug (Fig. 6A-a). Extensive wash out of the drug was required for full recovery of channel activity after application of 300 μM troglitazone (Fig. 6A-b). Similarly, troglitazone inhibited activity of K_{ATP} channels reconstituted from SUR2A and Kir6.2 at concentrations higher than 100 μM (Fig. 6B). In contrast to the effect of troglitazone, pioglitazone did not inhibit K_{ATP} channel activity at concentrations up to 300 μM in COS-1 cells transfected with either SUR1 plus Kir6.2 (Fig. 6C) or SUR2A plus Kir6.2 (Fig. 6D)

4. Discussion

We have previously reported that troglitazone stimulates insulin secretion from pancreatic islet (Masuda et al., 1995). In this study we show that the insulinotropic effect of troglitazone, at least in part, may be inhibition of pancreatic β-cell type K_{ATP} channels. [$^{86}Rb^{+}$] efflux and electrophysiological characterization using COS-1 cells heterologously expressing SUR1 plus Kir6.2 and SUR2A plus Kir6.2 shows that troglitazone but not pioglitazone inhibits the activity of both types of K_{ATP} channel. These results are consistent with reports that troglitazone inhibits channel activity in Cambrige rat insulinoma-G1 (CRI-G1) insulin-secreting cells (Lee et al., 1997) and in neurons in the ventromedial hypothalamus (Lee and Boden, 1997). The initial examination of pancreatic β-cell type K_{ATP} channel activity in the presence of troglitazone failed to show its inhibitory effect, alhough troglitazone has a putative non-competitive binding site at the sulfonylurea receptor (Masuda et al., 1995).

The thiazolidinedione derivatives have been shown to bind at the ligand-binding domain of the peroxisomal proliferator-activated receptor-gamma (PPARγ). (Berger et al., 1996; Forman et al., 1995), so it seems unlikely that activation of PPARγ should be followed by inhibition of K_{ATP} channel activity, since pioglitazone, another thiazolidinedione derivative also activates PPARγ. Recently, Ohtani et al. reported that pioglitazone stimulates insulin secretion in hamster β-cell line (HIT-T15) by inducing Ca^{2+} influx (Ohtani et al., 1996). Taken together, these data suggest that the thiazolidinedione derivatives could have several target proteins including the K_{ATP} channels in pancreatic β-cells.

In heart and skeletal muscle, the opening of the K_{ATP} channels has been implicated in the shortening of the action potential duration and the cellular loss of K^{+} during ischemia, hypoxia, and other metabolic insults, and leads to cytoprotection and vascular dilatation (Terzic et al., 1995). The inhibitory effects of troglitazone on K_{ATP} channel activity, therefore, could adversely

affect patients during cardiac ischemia or exercise which causes a reduction of ATP in cardiac and skeletal muscles.

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Figure Legends

Figure 1. The effect of troglitazone on [$^{86}Rb^{+}$] efflux from COS-1 cells coexpressing SUR1 and Kir6.2. Basal efflux from COS-1 cells transfected pCMV6c alone (crosses) and SUR1 plus Kir 6.2 (open circles) in the absence (A) or presence (B) of metabolic inhibitors. The cells expressing K_{ATP} channels were incubated with 1 μM (closed circles), 3 μM (open squares), 10 μM(closed squares), 30 μM (open triangles), and 100 μM (closed triangles). Since the data points are tightly clustered, the symbols have been offset ± 1 or 2 minutes for clarity.

Figure 2. The effect of troglitazone on [$^{86}Rb^{+}$] efflux from COS-1 cells coexpressing SUR2A and Kir6.2. Basal efflux from COS-1 cells transfected pCMV6c alone (crosses) and SUR2A plus Kir6.2 (open circles) in the presence of metabolic inhibitors. The cells expressing K_{ATP} channels were incubated with 1 μM (closed circles), 3 μM (open squares), 10 μM (closed squares), 30 μM (open triangles), and 100 μM (closed triangles). Since the data points are tightly clustered, the symbols have been offset ± 1 or 2 minutes for clarity.

Figure 3. The effects of troglitazone and pioglitazone on K_{ATP} channels reconstituted from SUR1 plus Kir6.2 or SUR2A plus Kir6.2. (A, B) Relative values of [$^{86}Rb^{+}$] efflux for 40 min from COS-1 cells coexpressing SUR1 plus Kir6.2 (A) and SUR2A plus Kir6.2 (B) with the indicated concentrations of troglitazone (open columns) or pioglitazone (closed columns) in the presence of metabolic inhibitors. Values are expressed as means ± S.E.M, relative to the [$^{86}Rb^{+}$] efflux from COS-1 cells without troglitazone or pioglitazone.

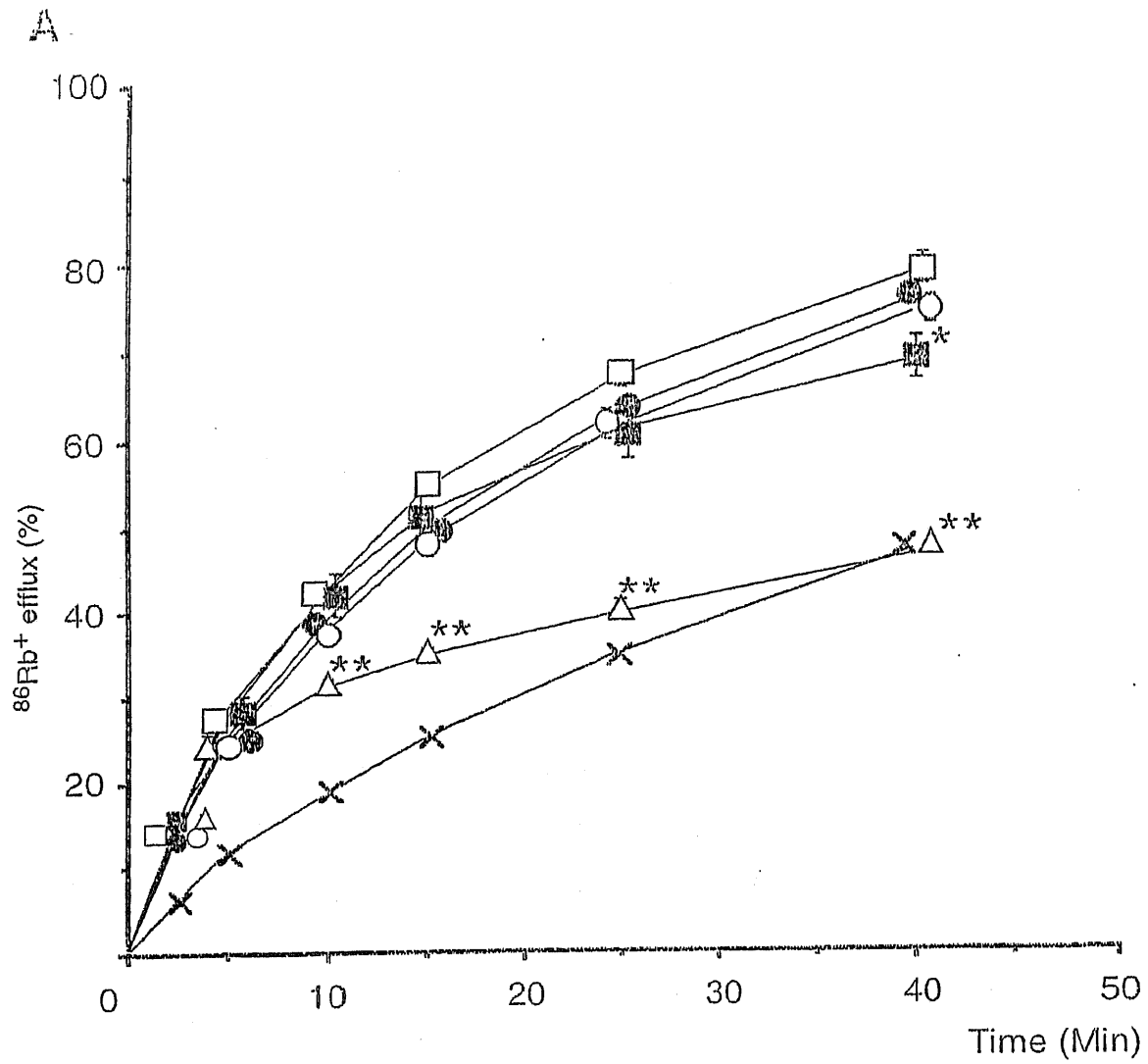
Figure 4. The effect of troglitazone on K_{ATP} channels partially inhibited by glibenclamide. (A, B) Relative values of [$^{86}Rb^{+}$] efflux for 40 min from COS-1 cells coexpressing SUR1 plus Kir6.2

(A) and SUR2A plus Kir6.2 (B) with or without the indicated concentrations of troglitazone (A, 30 μM; B, 10 μM) and glibenclamide (A, 3 nM; B, 1 μM) in the presence of metabolic inhibitors. Data are given as means ± S.E.M., relative to the [$^{86}Rb^{+}$] efflux from COS-1 cells without troglitazone and glibenclamide.

Figure 5. The effect of troglitazone on K_{ATP} channels activated by K_{ATP} channel openers. (A, B) Relative values of [$^{86}Rb^{+}$] efflux for 40 min from COS-1 cells coexpressing SUR1 plus Kir6.2 (A) and SUR2A plus Kir6.2 (B) with or without the indicated concentrations of troglitazone (A, 30 μM; B, 10 μM) and the K_{ATP} channel opener diazoxide (A, 200 μM) or pinacidil (B, 200 μM) in the absence of metabolic inhibitors. Data are given as means ± S.E.M., relative to the [$^{86}Rb^{+}$] efflux from COS-1 cells without troglitazone and K_{ATP} channel openers.

Figure 6. Electrophysiological recordings from COS-1 cells expressing reconstituted K_{ATP} channels. (A, B) The effects of troglitazone on K_{ATP} channel currents in COS-1 cells expressing SUR1 plus Kir6.2 (A) or SUR2A plus Kir6.2 (B). Troglitazone inhibits SUR1/Kir6.2 channel currents reversibly at a concentration of 30 μM (a) or 300 μM (b). (B) Troglitazone inhibits SUR2A/Kir6.2 channel currents at concentrations of 100 μM and 300 μM. (C and D) (C, D) The effect of pioglitazone on K_{ATP} channel currents in COS-1 cells expressing SUR1 plus Kir6.2 (C) or SUR2A plus Kir6.2 (D). Pioglitazone shows no apparent inhibitory effect on these channels. The recordings were made in the inside-out configuration of patch-clamp technique. The horizontal bars and numbers indicate application periods of ATP, troglitazone and pioglitazone. Calibrations are shown in each panel. The state in which all the channels are closed is represented by the symbol C.

Fig. 1A



*p<0.05 vs 0 mM troglitazone,**p<0.005 vs 0 mM troglitazone

Fig. 1B

B

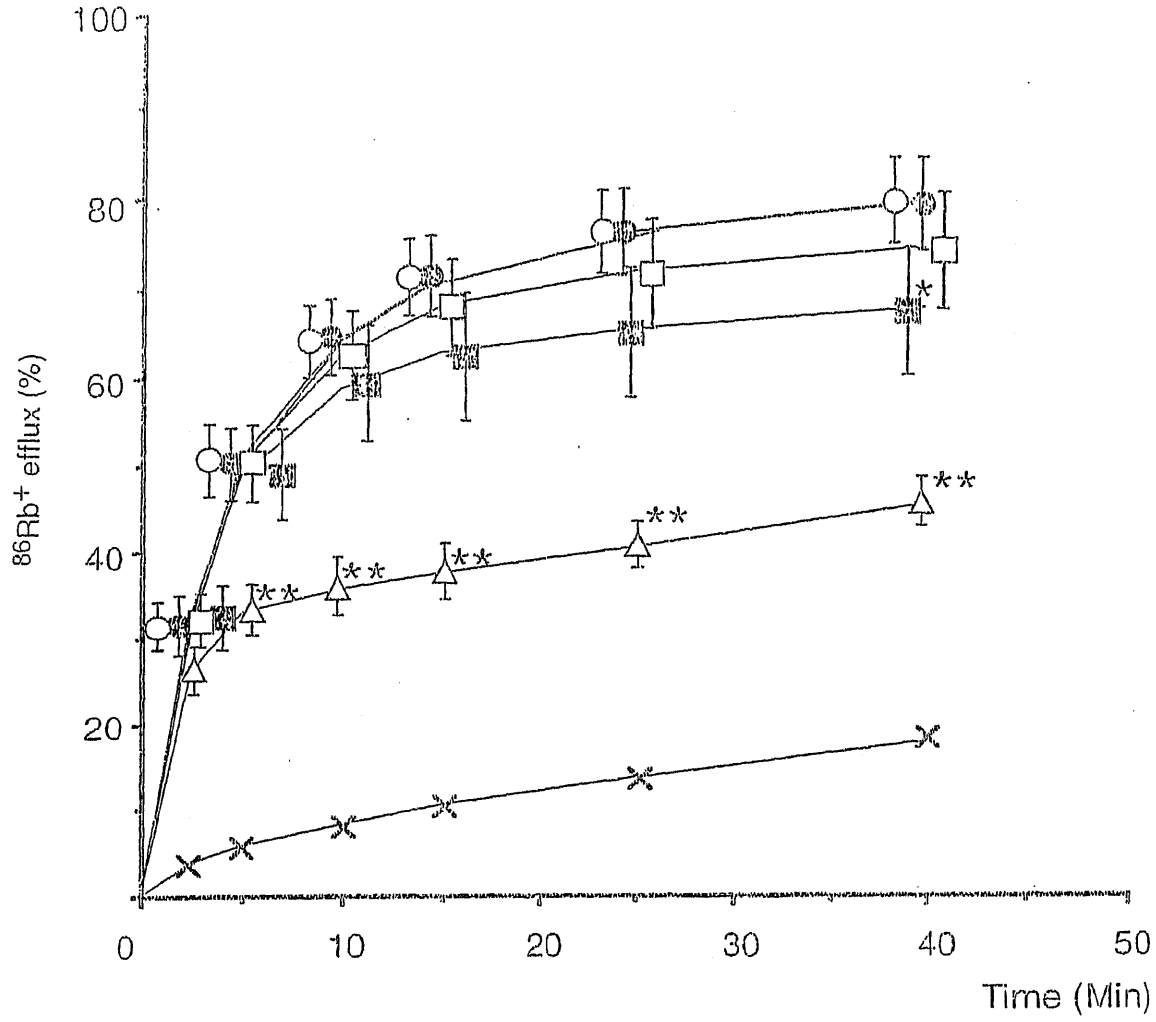


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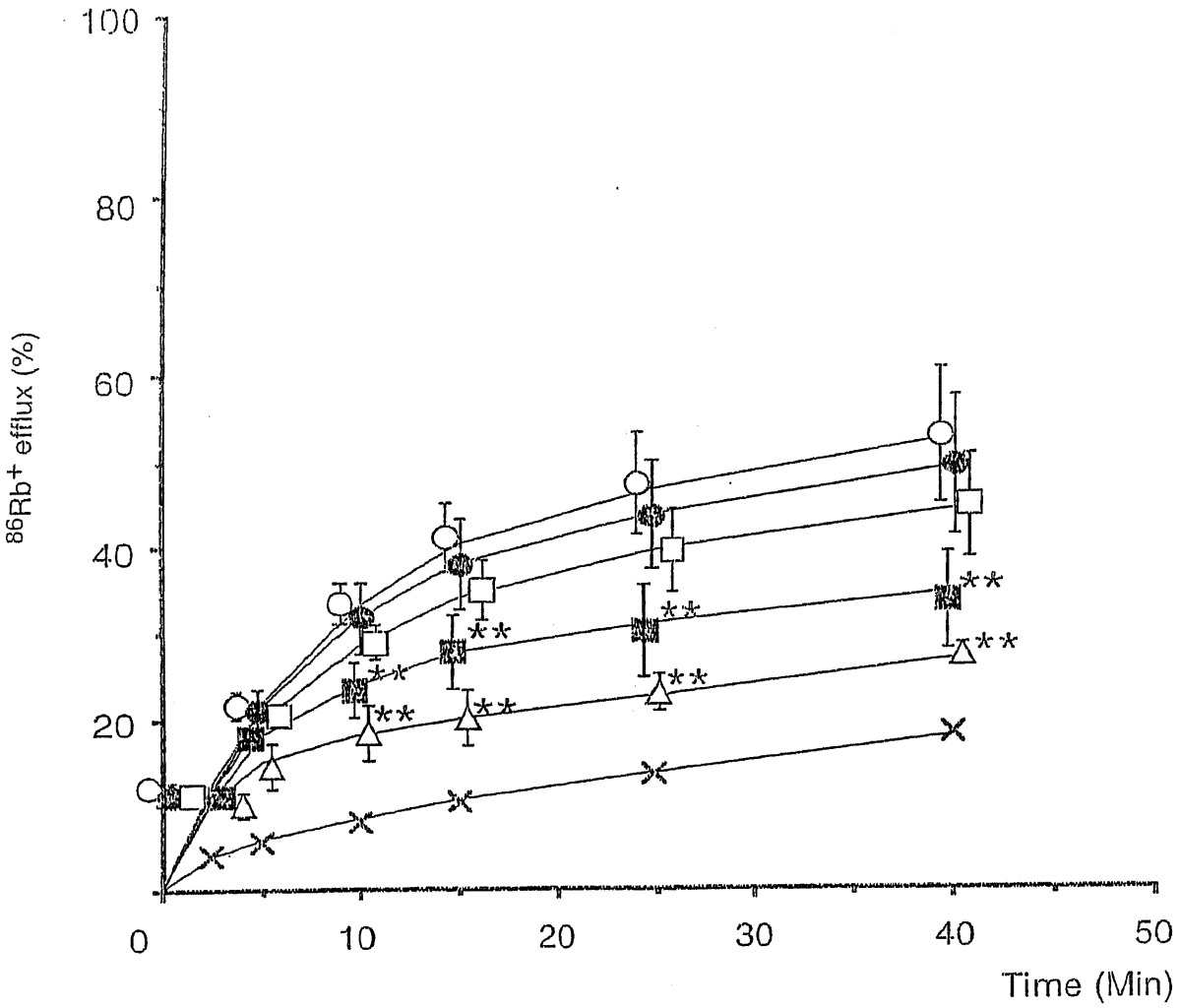
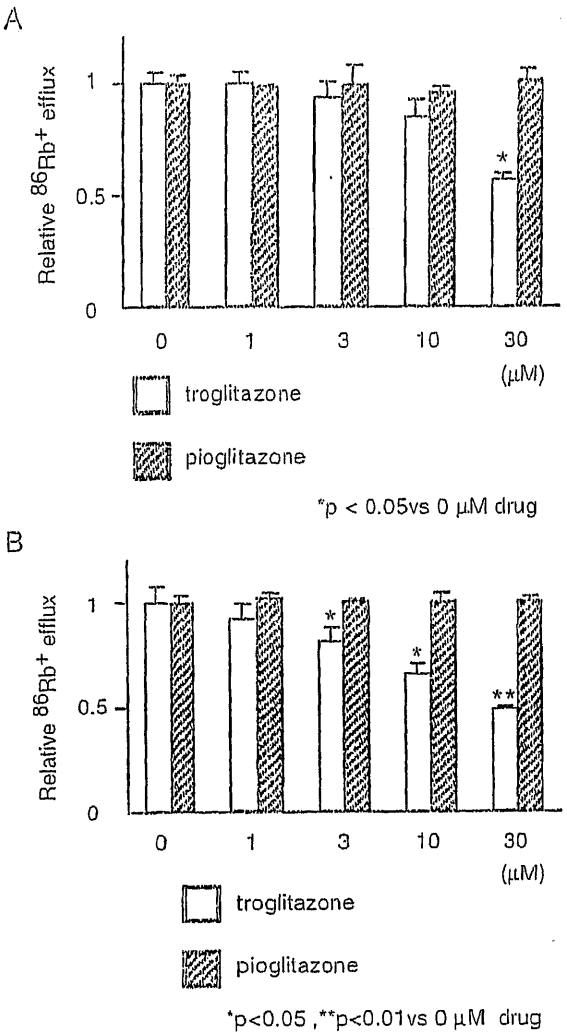
Fig. 2



*p<0.05 vs 0 mM troglitazone,**p<0.005 vs 0 mM troglitazone

Fig. 3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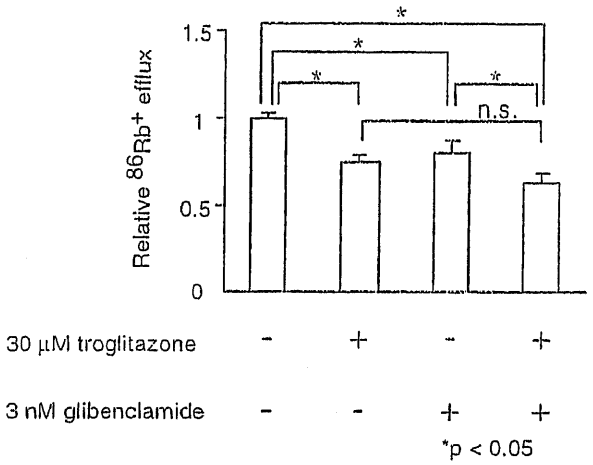
0.5
0
0
1
3
10
30
(μM)
troglitazone
pioglitazone
*p < 0.05vs 0 μM drug
B
Relative $^{86}Rb^+$ efflux
1
0.5
0
0
1
3
10
30
(μM)
troglitazone
pioglitazone
*p<0.05 ,**p<0.01vs 0 μM drug

Fig. 4

A


Relative $^{86}Rb^+$ efflux
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1
0.5
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*
*
*
n.s.
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*p < 0.05

B

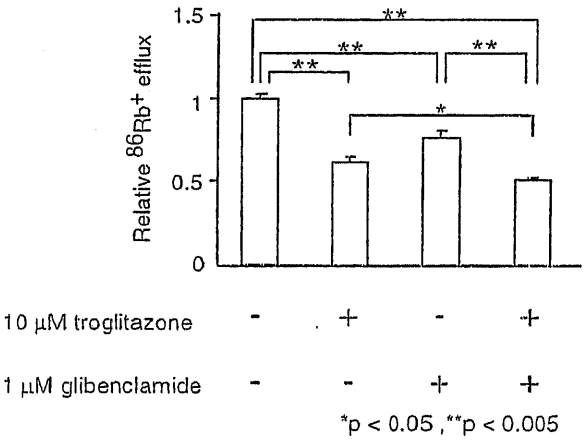
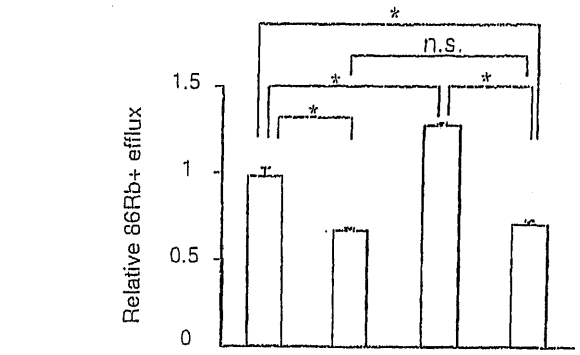

Relative $^{86}Rb^+$ efflux
1.5
1
0.5
0
**
**
**
*
10 μM troglitazone - + - +
1 μM glibenclamide - - + +
*p < 0.05 ,**p < 0.005

Fig. 5

A

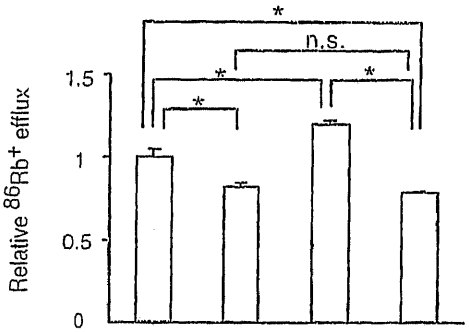


30 μM troglitazone - + - +

200 μM Diazoxide - - + +

*p < 0.05

B

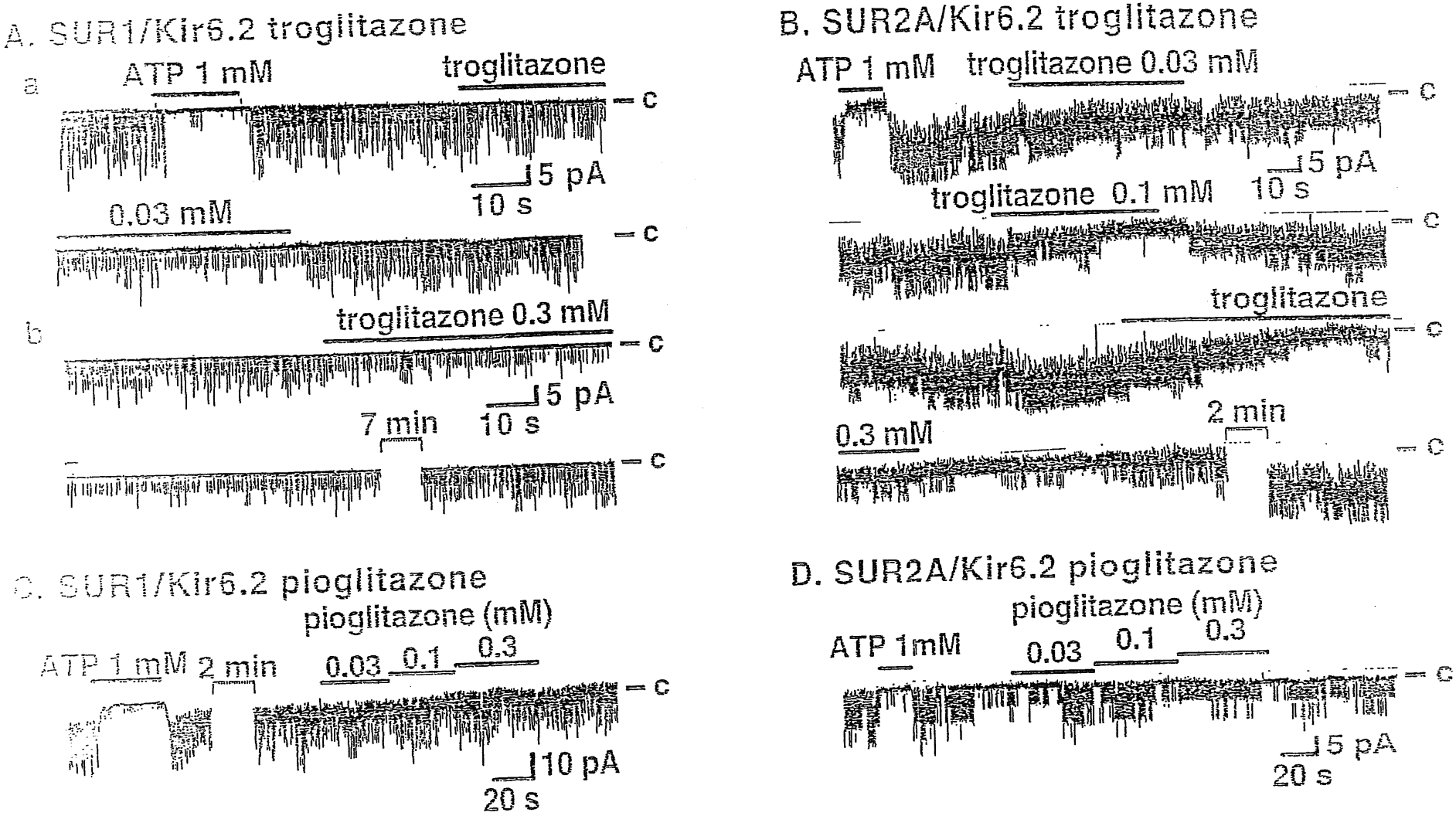


10 μM troglitazone - + - +

200 μM Pinacidil - - + +

*p < 0.05

Fig.6


A. SUR1/Kir6.2 troglitazone
a
ATP 1 mM
troglitazone
c
5 pA
10 s
0.03 mM
c
b
troglitazone 0.3 mM
c
5 pA
7 min
10 s
c
B. SUR2A/Kir6.2 troglitazone
ATP 1 mM
troglitazone 0.03 mM
c
5 pA
troglitazone 0.1 mM
10 s
c
troglitazone
c
2 min
0.3 mM
c
C. SUR1/Kir6.2 pioglitazone
pioglitazone (mM)
ATP 1 mM
2 min
0.03
0.1
0.3
c
10 pA
20 s
D. SUR2A/Kir6.2 pioglitazone
pioglitazone (mM)
ATP 1mM
0.03
0.1
0.3
c
5 pA
20 s

The effects of KAD-1229, a new anti-diabetic drug, on ATP-sensitive K^+ channels comprising Kir6.2 and SUR1, SUR2A or SUR2B

Abstract

KAD-1229, a new anti-diabetic drug, is thought to stimulate insulin secretion by closing the ATP-sensitive K^+ (K_{ATP}) channels in pancreatic β-cells. However, its selectivity for the various K_{ATP} channels is not known. In this study, the effects of KAD-1229 on the various cloned K_{ATP} channels (Kir6.2/SUR1, Kir6.2/SUR2A, and Kir6.2/SUR2B) reconstituted in COS-1 cells were examined. Single channel analysis showed that KAD-1229 inhibits Kir6.2/SUR1 channel currents in a dose-dependent manner (IC_{50} = 7.1 nM), but does not inhibit significantly either the Kir6.2/SUR2A or Kir6.2/SUR2B channel currents even at high doses (more than 10 μM). Binding experiments of SUR1 expressed in COS-1 cells revealed that KAD-1229 inhibits the binding of [^{3}H]glibenclamide to SUR1 (IC_{50} value, 280 nM), suggesting that KAD-1229 shares a binding site with glibenclamide. Insulin responses to glucose, KAD-1229, tolbutamide, and glibenclamide in MIN6 cells after chronic KAD-1229 treatment were similar to those after tolbutamide treatment. These results indicate that, similarly to tolbutamide, KAD-1229 is highly specific to the Kir6.2/SUR1 complex, i.e., the pancreatic β-cell K_{ATP} channel , and suggest that KAD-1229 is a clinically useful anti-diabetic drug.

Key Words: K_{ATP} channel; sulfonylurea receptor (SUR); KAD-1229; insulin secretion; secondary failure; desensitization

1. Introduction

ATP-sensitive K^+ (K_{ATP}) channels occur in many tissues, including pancreatic β-cells (Cook and Hales, 1984), heart (Noma, 1983), vascular smooth muscle cells (Standen et al., 1989), and other tissues (Ashford et al., 1988; Spruce et al., 1985). K_{ATP} channels play an important role in various cellular responses such as secretion and muscle contraction by linking cell metabolism to membrane potential (Ashcroft, 1988). Classical K_{ATP} channels are now known to comprise two subunit molecules: the Kir6.2 subunits that form the K^+-selective ion channel pore, and the SUR (SUR1 or SUR2) subunits, the receptor of sulfonylureas (Aguilar-Bryan et al., 1998; Ashcroft and Gribble, 1998; Seino, 1999). Co-expression of Kir6.2 and SUR1 in heterologous expression systems forms K_{ATP} channels with properties similar to those in native pancreatic β-cells (Inagaki et al., 1995; Sakura et al., 1995). Various forms of SUR2 have been identified (Chutkow et al., 1996, 1999; Inagaki et al., 1996; Isomoto et al., 1996). It is generally accepted that Kir6.2/SUR2A channels form cardiac and presumably skeletal muscle K_{ATP} channels (Aguilar-Bryan et al., 1998; Ashcroft and Gribble, 1998; Seino, 1999). Although it was initially proposed that Kir6.2/SUR2B channels form smooth muscle K_{ATP} channels (Isomoto et al., 1996), SUR2B coupled to Kir6.1, were later suggested to be the constituents of the vascular smooth muscle K_{ATP} channel (Yamada et al., 1997).

The K_{ATP} channels in pancreatic β-cells are critical in the regulation of both glucose-

induced and sulfonylurea-induced insulin secretions (Miki et al., 1998). The binding of sulfonylureas to SUR1 probably inhibits the cooperativity of the nucleotide biding folds (NBF) -1 and -2 of SUR1, which induces the closed state of the K_{ATP} channels (Ueda et al., 1999). Recently, the non-sulfonylurea insulinotropic agent metiglinide also has been shown to inhibit the β-cell K_{ATP} channel by binding to SUR1 (Gribble et al., 1998). KAD-1229 (mitiglinide), a new anti-diabetic drug with a chemical structure different from sulfonylureas has been developed in the United States, Europe, and Japan. Preclinical studies have shown the drug to have a short-lasting and immediate effect on hypoglycemic action and to increase insulin release from both pancreatic β-cells and HIT-T15 cells (Ohnoda et al., 1994, 1995). KAD-1229 has been shown to displace the bound [^{3}H]glibenclamide in the mouse insulin-secreting cell line MIN6 and to inhibit the K_{ATP} channel currents in these cells (Mogami et al., 1994), suggesting that it stimulates insulin secretion by closing the K_{ATP} channels by binding to SUR.

Evaluation of the tissue selectivity of the anti-diabetic drugs that act through SUR is crucial in predicting side effects since SURs are distributed in many tissues. While tissue specificity has been reported for some sulfonylureas (Gribble et al., 1998), that for KAD-1229 has not previously been examined. In the present study, we evaluated the selectivity of KAD-1229 to various K_{ATP} channels, using COS-1 cells co-expressing cloned Kir6.2 and SUR1, SUR2A, or SUR2B. Because it is known that chronic treatment with sulfonylureas sometimes leads to impairment of sulfonylurea-induced

insulin secretion *in vivo* and *in vitro* (Karam et al., 1986; Davalli et al., 1992; Rabuazzo et al., 1992), we have also investigated the effect of chronic KAD-1229 treatment on insulin secretion.

2. Materials and Methods

2.1. Materials

The KAD-1229 was obtained from Kissei Pharmaceutical Co. Ltd. (Nagano, Japan). Glibenclamide and tolbutamide were purchased from RBI (Natick, MA, USA) and Nacalai Tesque (Kyoto, Japan), respectively. [^{3}H]-labeled glibenclamide was from NEN Life Science Products (Boston, MA, USA).

2.2. Cell culture and Transfection

COS-1 cells were plated at a density of 2×10^6 cells per dish (10 cm in diameter) for displacement assay of [^{3}H]glibenclamide binding and at 2×10^5 cells per dish (3.5 cm in diameter) for patch clamp channel recordings. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. The full-length cDNAs encoding Kir6.2 (human or mouse) and a SUR isoform (human SUR1, hamster SUR1, rat SUR2A or rat SUR2B) were subcloned into a mammalian expression vector, pCMV6b or 6c. The human or mouse Kir6.2 expression vector and human SUR1, hamster SUR1, rat SUR2A, or rat SUR2B expression vector were co-transfected with pEGFP (Clontech, Palo Alto, USA), a transfection marker, into COS-1 cells, using lipofectamine and Opti-MEM I reagents (Life Technologies, Rockville, MD, USA).

For chronic treatment of MIN6 cells with the insulinotropic agents, the cells were

plated at a density of 1×10^5 cells per dish (10 cm in diameter) and cultured in DMEM containing 25 mM glucose, 10% fetal bovine serum, and 63 μM 2-mercaptoethanol. One day after plating, KAD-1229 (100 nM) or tolbutamide (100 μM) was added to the culture medium. The cells were cultured with the medium containing one of these drugs for 14 days, and were then plated onto a 48-well plate at a density of 1×10^5 to measure insulin secretion in response to various stimuli.

2.3. Electrophysiological analysis of K_{ATP} channels

The transfected COS-1 cells were cultured for 48-92 hr before recordings and were selected by green fluorescence under a microscope (Inagaki et al., 1996). Recordings were made in the excised inside-out patch clamp mode, as previously described (Inagaki et al., 1995). The intracellular solution contained 140 mM KCl, 2 mM EGTA, 2 mM $MgCl_2$, and 10 mM HEPES (pH=7.3). The pipette solution contained 140 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES (pH=7.4). Recordings were made at 20-22°C.

2.4. Inhibition of [^{3}H]glibenclamide binding to human SUR1 by various anti-diabetic drugs

COS-1 cells transfected with human SUR1 were washed twice with KRH buffer containing 119 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM

$MgSO_4$, 5 mM $NaHCO_3$ and 20 mM HEPES (pH=7.4), and then resuspended in the same buffer. Cells at a density of 5×10^5/tube were incubated with 2 nM [^{3}H]-labeled glibenclamide in the absence or presence of various concentrations of unlabeled KAD-1229, glibenclamide, or tolbutamide at room temperature for 2 h. The incubation was terminated by rapid filtration through GF/C filters (Whatman International, Maidstone, U.K.). The filters were washed twice with ice-cold KRH buffer and radioactivities were measured using a liquid scintillation counter. [^{3}H]glibenclamide binding is expressed as percentage of the binding of [^{3}H]glibenclamide without nonlabeled compounds.

2.5. Measurements of insulin secretion

The cells were pre-incubated for 30 min in KRB buffer containing 154 mM NaCl, 6.2 mM KCl, 3.3 mM $CaCl_2$, 1.5 mM KH_2PO_4, 1.6 mM $MgSO_4$, 12.4 mM $NaHCO_3$, 20mM HEPES (pH=7.4), and 0.2% bovine serum albumin (BSA-KRB) containing 3 mM glucose. The cells were then stimulated by adding 500 μl of BSA-KRB buffer containing 25 mM glucose or 100 nM KAD-1229, 100 μM tolbutamide, or 100 nM glibenclamide in the presence of 3 mM glucose. Insulin secreted into the medium was measured by radioimmunoassay (Eiken Chemical, Tokyo, Japan), as previously described (Kawaki et al, 1999).

3. Results

Representative recordings obtained from excised inside-out patches in COS-1cells expressing K_{ATP} channels are shown in Figure 1. ATP almost completely inhibited the three types of K_{ATP} channel currents at a concentration of 1 mM (Fig. 1A-C). KAD-1229 at a concentration of 100 nM inhibited the Kir6.2/SUR1 channel currents (Fig. 1A). In contrast, no significant inhibition of the Kir6.2/SUR2A channel currents with KAD-1229 was observed even at the highest concentration tested (100 μM) (Fig. 1B). The Kir6.2/SUR2B channel currents also were not affected by KAD-1229 (Fig. 1C). The dose-dependent effects of KAD-1229 on K_{ATP} channel currents are shown in Fig. 2. KAD-1229 inhibited both human Kir6.2/human SUR1 K_{ATP} channel currents and mouse Kir6.2/hamster SUR1 K_{ATP} channel currents in a dose-dependent manner (IC_{50} value: 100 nM for human Kir6.2/human SUR1 channel; 22 nM for mouse Kir6.2/hamster SUR1 channel). In contrast, KAD-1229 even at high concentrations (1 μM or more) showed no significant inhibitory effect on either mouse Kir6.2/rat SUR2A or mouse Kir6.2/rat SUR2B channels. The inhibitory effect of KAD-1229 (10 nM) on the Kir6.2/SUR1 channel is reversible (Fig.1A). The current amplitude recovered to 50% of the value obtained before application of the drug in less than 2 min (115 ± 18 s) after starting washout. In contrast, the effect of glibenclamide was irreversible, and the current did not recover significantly even after continuous washing for more than 1h

(n=5) (Fig. 1D).

Specific binding of [^{3}H]-labeled glibenclamide to human SUR1 expressed in COS-1 cells was inhibited by unlabeled glibenclamide, KAD-1229, and tolbutamide in a concentration-dependent manner (Fig. 3). The IC_{50} values for glibenclamide, KAD-1229, and tolbutamide were 7.1 nM, 280 nM, and 140 μM, respectively.

Acute insulin secretory responses to various stimuli in MIN6 cells treated chronically with KAD-1229 or tolbutamide are shown in Fig. 4 (A-C). As control, the cells treated with vehicle alone were used. Basal insulin secretion in the presence of 3 mM glucose was significantly lower in MIN6 cells treated with KAD-1229 and tolbutamide for 14 days than in control. Following chronic exposure to KAD-1229 at a concentration of 100 nM, basal secretion and secretion induced by the various stimuli were decreased, compared to control. The inhibitory effects of chronic KAD-1229 treatment on insulin secretion were similar to those with tolbutamide treatment. There was no significant difference in insulin content in MIN6 cells treated with KAD-1229 or tolbutamide (Fig.4 D).

4. Discussion

We have shown that KAD-1229 inhibits Kir6.2/SUR1 (pancreatic β-cell type) K_{ATP} channels in a dose dependent-manner but has no significant inhibitory effect on either Kir6.2/SUR2A (cardiac type) K_{ATP} channels or Kir6.2/SUR2B K_{ATP} channels even at high concentrations (100 μM and more). In radioligand binding assay, KAD-1229 inhibited the binding of [^{3}H]glibenclamide to SUR1 expressed in COS-1 cells. The affinity of human SUR1 for KAD-1229 was about 500 fold that for tolbutamide, but 40 fold lower than that for glibenclamide. The IC_{50} for the effect of KAD-1229 on Kir6.2/SUR1 channel current was consistent with that by the [^{3}H]glibenclamide displacement assay of KAD-1229 to human SUR1. These results demonstrate that KAD-1229 inhibits human pancreatic β-cell type K_{ATP} channels by binding to SUR1.

The tissue selectivity of some anti-diabetic drugs has been demonstrated by Gribble et al. (1998): (i) tolbutamide inhibits Kir6.2/SUR1 channels but not Kir6.2/SUR2A channels, (ii) glibenclamide blocks both Kir6.2/SUR1 (Ki = 4 nM) and Kir6.2/SUR2B (Ki = 27 nM) channels; and (iii) meglitinide, a non-sulfonylurea hypoglycemic agent with a benzamide moiety, inhibited Kir6.2/SUR1 and Kir6.2/SUR2A channels at similar concentrations (Ki = 0.3 and 0.5 μM, respectively). They proposed that SUR1 possesses two drug-binding sites (a sulfonylurea–binding site and a benzamide-binding site), while SUR2A has only a benzamide-binding site. They also suggested that tolbutamide binds to the sulfonylurea-binding site and glibenclamide binds to both sites,

whereas meglitinide binds only to the benzamide–binding site, and, therefore, that glibenclamide and meglitinide both bind to SUR1 and SUR2, while tolbutamide binds only to SUR1. In the present study, we demonstrate that KAD-1229 has a high selectivity in its inhibition for cloned Kir6.2/SUR1 channels, i.e., the pancreatic β-cell type K_{ATP} channels. A high tissue selectivity of KAD-1229 has also been found in native K_{ATP} channels (ID_{50} in MIN6 cells is 20 nM) (Mogami et al., 1994). KAD-1229, therefore, resembles tolbutamide in terms of its tissue selectivity. In addition, KAD-1229 inhibits the binding of [^{3}H]glibenclamide to human SUR1. These results suggest that the KAD-1229 binds to the sulfonylurea-binding site in SUR1 but does not bind to the benzamide-binding site SUR2A.

Electrophysiological study also shows that KAD-1229 has no significant inhibitory effect on either Kir6.2/SUR2A (cardiac type K_{ATP} channel) or Kir6.2/SUR2B channel currents. The K_{ATP} channels in heart play an important role in protecting against myocardial damage during ischaemia and reperfusion (Coles et al., 1991) and recovery of cardiac contractility after ischaemia and reperfusion is known to be more deteriorated in the presence of glibenclamide than in its absence (Shigematsu et al., 1995). Sulfonylureas have been used in the treatment of type 2 diabetes mellitus for a many years, but several reports have shown that chronic sulfonylurea treatment causes unresponsiveness to subsequent stimulation both *in vitro* and *in vivo* (Karam et al., 1986; Davalli et al., 1992; Rabuazzo et al., 1992). In the present study, we found that

insulin secretion in response to various stimuli, including KAD-1229 and tolbutamide, after chronic exposure in MIN6 cells is impaired, but is retained to some extent. In a previous study, we found that insulin secretion in acute response to glibenclamide in MIN6 cells was severely impaired after chronic exposure to glibenclamide (Kawaki et al., 1999). These findings indicate that the chronic effect of KAD-1229 on pancreatic β-cells is similar to that of tolbutamide, but different from that of glibenclamide.

In conclusion, we provide direct evidence that KAD-1229 binds to human SUR1 and exhibits a high degree of the specificity to the pancreatic β-cell K_{ATP} channel. Because the effects of KAD-1229 on the pancreatic β-cell type K_{ATP} channel are reversible and the insulin secretory responses to various stimuli after chronic exposure to the drug are similar to those of tolbutamide, KAD-1229 would be a clinically useful anti-diabetic drug in treatment of type 2 diabetes.

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Figure legends

Fig. 1 **Effects of KAD-1229 and glibenclamide on K_{ATP} channel currents**

Representative traces of channel currents recorded from COS-1 cells co-expressing mouse Kir6.2 and hamster SUR1 (A and D), mouse Kir6.2 and rat SUR2A (B), or mouse Kir6.2 and rat SUR2B (C) are shown. The effects of KAD-1229 (A-C) and glibenclamide (D) on the channel currents were examined. The recordings were made using the excised inside-out patch technique. The horizontal bars and the numbers above them indicate application period and concentrations of test agents, respectively. Calibration bars are shown in each panel. "zero" current levels are indicated by horizontal lines marked "0 pA". In D, ATP (1 mM) was applied as indicated by bar.

Fig. 2 **Effects of KAD-1229 on K_{ATP} channel currents**

COS-1 cells were transfected with mouse Kir6.2 and hamster SUR1 (●), human Kir6.2 and human SUR1 (▲), mouse Kir6.2 and rat SUR2A (◆), or mouse Kir6.2 and rat SUR2B (■). The channel currents were normalized to the amplitude before applying KAD-1229. Data are presented as the means ± S.E. of 5-12 experiments.

Fig. 3 **Inhibition of [^{3}H]glibenclamide binding to human SUR1 by KAD-1229, glibenclamide, and tolbutamide**

[^{3}H]glibenclamide binding to human SUR1 was displaced by unlabeled KAD-1229 (▲),

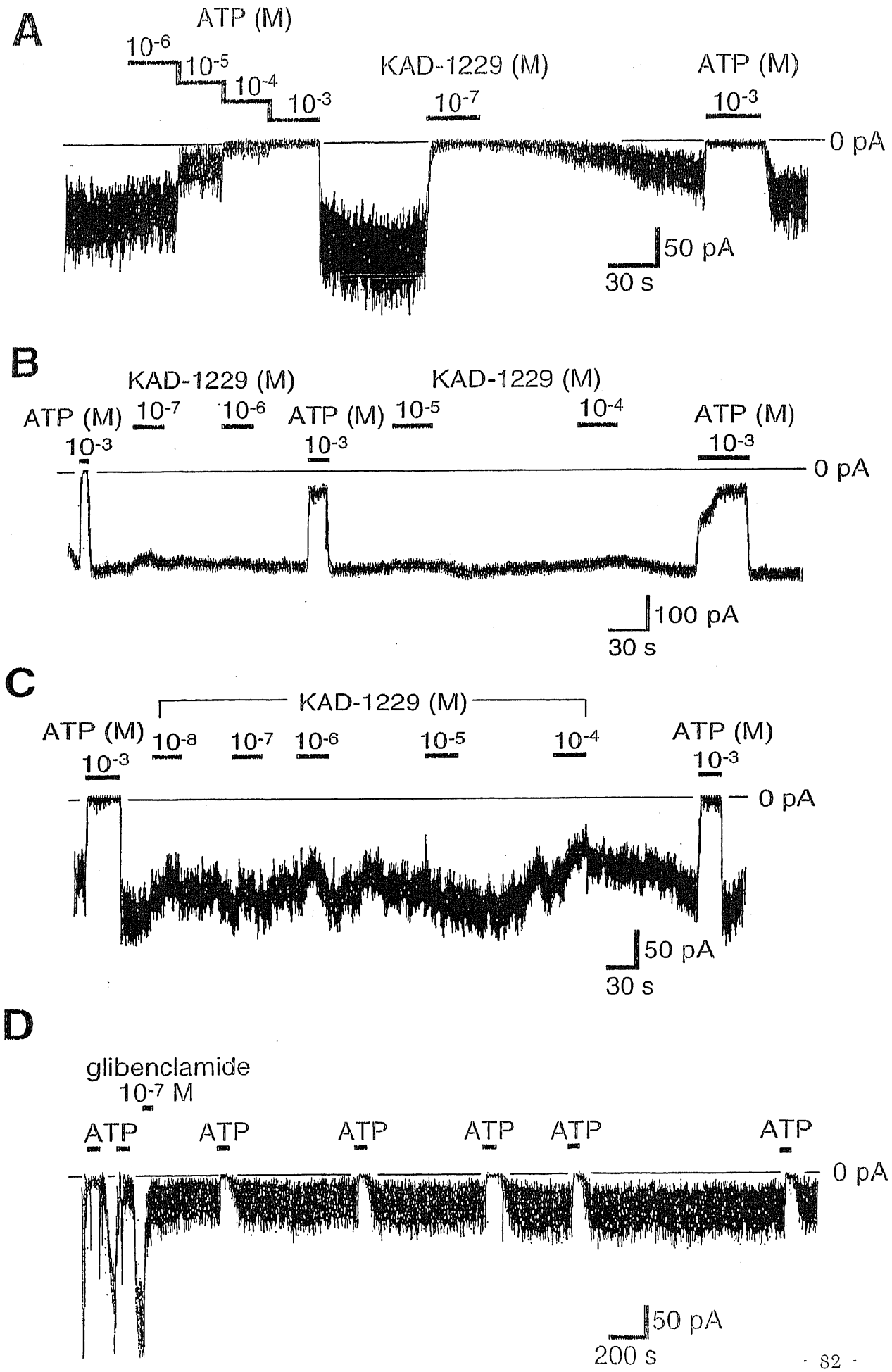
glibenclamide (●), and tolbutamide (■). Values are the means of three independent experiments.

Fig. 4 **Insulin secretion and insulin content in MIN6 cells after chronic treatment with KAD-1229 or tolbutamide.**

A, Insulin secretion to various stimuli in MIN6 cells treated with vehicle (0.1% DMSO)(control). B, Insulin secretion to various stimuli in MIN6 cells treated with 100 nM KAD-1229. C, Insulin secretion to various stimuli in MIN6 cells treated with 100 μM tolbutamide. Basal insulin secretion (in the presence of 3 mM glucose) in MIN6 cells treated with KAD-1229 or tolbutamide is significantly lower than that in control. Insulin responses to high glucose (25 mM), KAD-1229, tolbutamide or glibenclamide in MIN6 cells treated with KAD-1229 or tolbutamide are significantly lower than those in control. There were no differences in insulin responses to various stimuli between KAD-1229 and tolbutamide treatment. D, Insulin content of MIN6 cells after treatment with vehicle (control), 100 nM KAD-1229, or 100 μM tolbutamide.

*$p<0.01$ vs 3 mM glucose

Fig. 1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ATP (M)
10-6
10-5
10-4
10-3
KAD-1229 (M)
10-7
ATP (M)
10-3
0 pA
50 pA
30 s
B
KAD-1229 (M)
KAD-1229 (M)
ATP (M)
10-3
10-7
10-6
ATP (M)
10-3
10-5
10-4
ATP (M)
10-3
0 pA
100 pA
30 s
C
KAD-1229 (M)
ATP (M)
10-3
10-8
10-7
10-6
10-5
10-4
ATP (M)
10-3
0 pA
50 pA
30 s
D
glibenclamide
10-7 M
ATP
ATP
ATP
ATP
ATP
ATP
0 pA
50 pA
200 s

Fig. 2

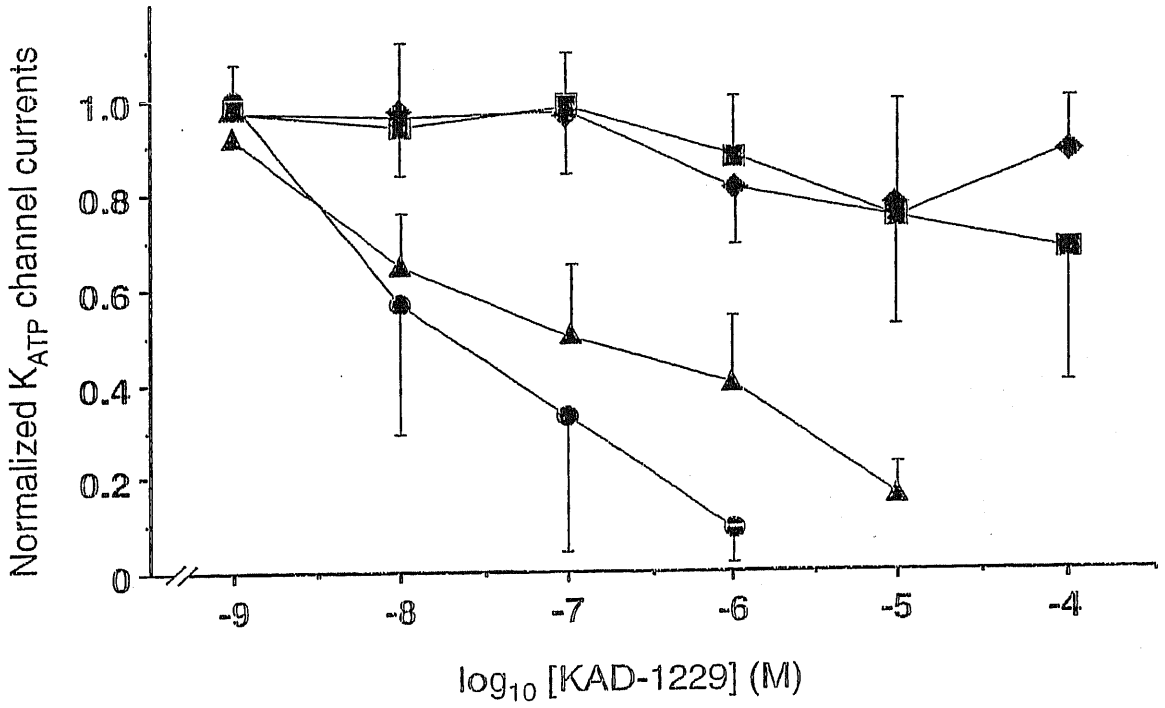

Normalized K_{ATP} channel currents
1.0
0.8
0.6
0.4
0.2
0
-9
-8
-7
-6
-5
-4
$\log_{10}$ [KAD-1229] (M)

Fig. 3

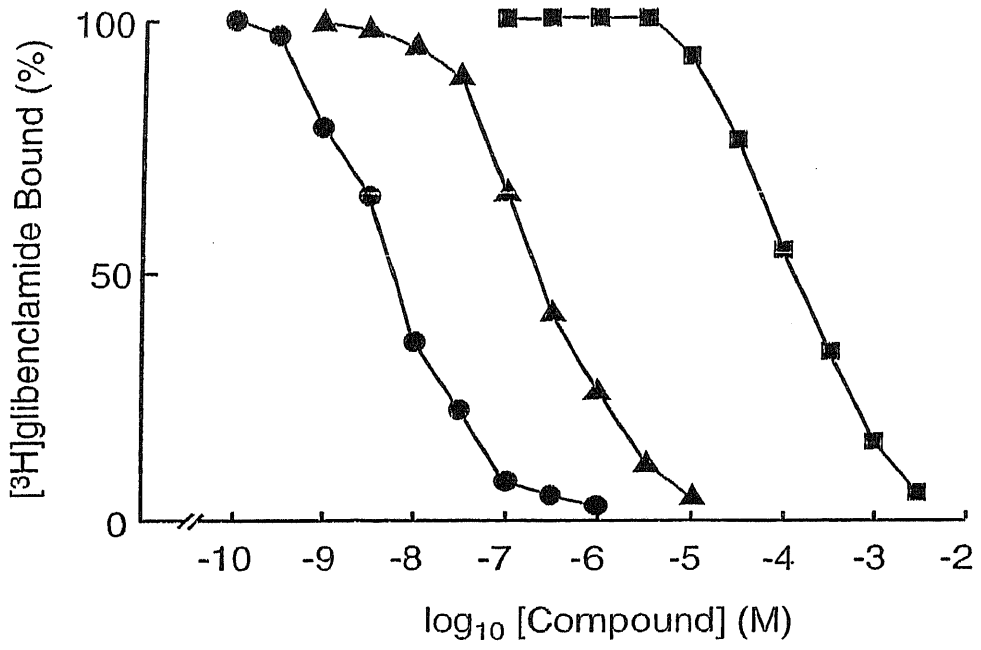
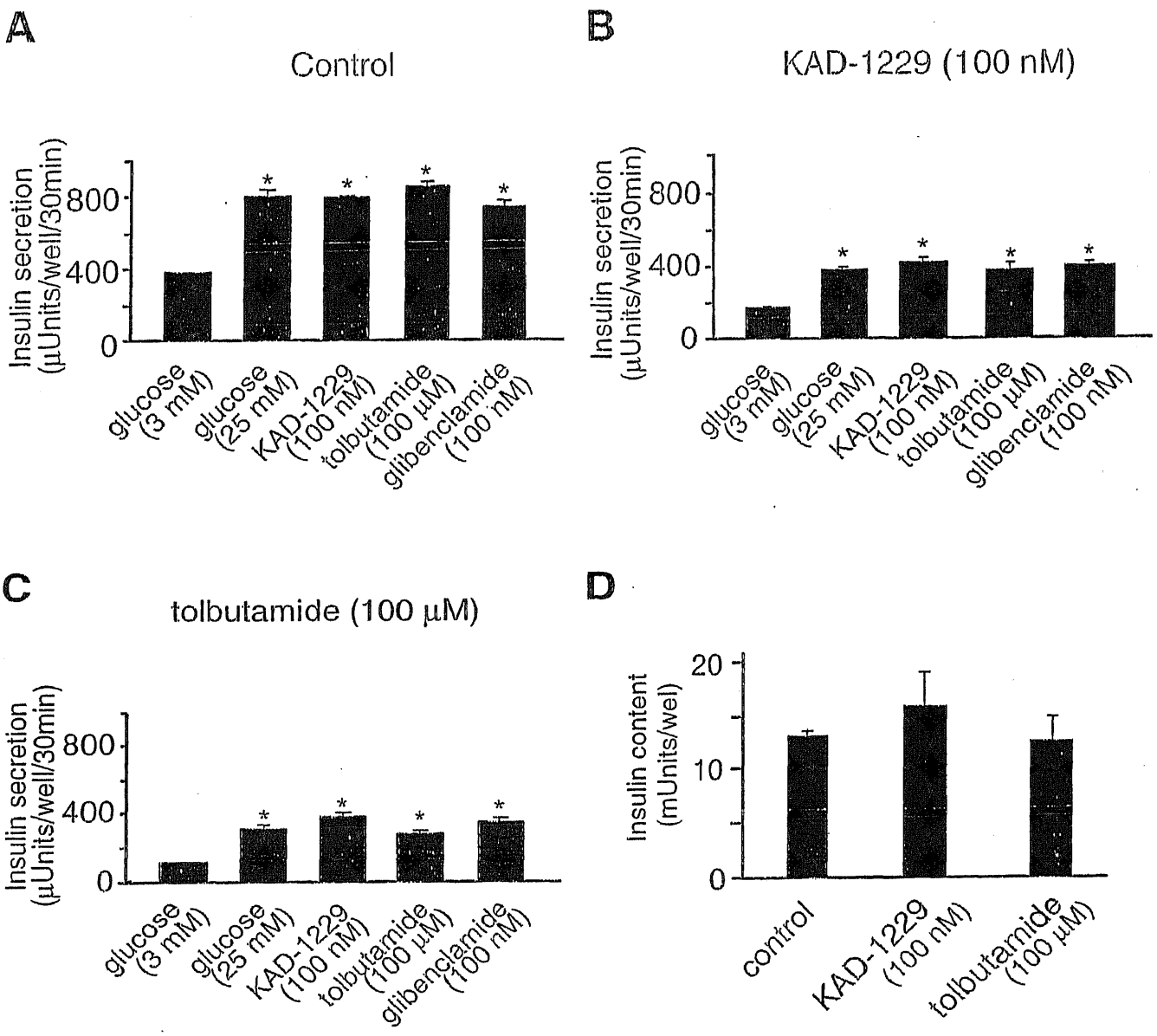

100
50
0
[3H]glibenclamide Bound (%)
-10
-9
-8
-7
-6
-5
-4
-3
-2
log10 [Compound] (M)

Fig. 4


A
Control
Insulin secretion (μUnits/well/30min)
800
400
0
glucose (3 mM)
glucose (25 mM)
KAD-1229 (100 nM)
tolbutamide (100 μM)
glibenclamide (100 nM)
B
KAD-1229 (100 nM)
Insulin secretion (μUnits/well/30min)
800
400
0
glucose (3 mM)
glucose (25 mM)
KAD-1229 (100 nM)
tolbutamide (100 μM)
glibenclamide (100 nM)
C
tolbutamide (100 μM)
Insulin secretion (μUnits/well/30min)
800
400
0
glucose (3 mM)
glucose (25 mM)
KAD-1229 (100 nM)
tolbutamide (100 μM)
glibenclamide (100 nM)
D
Insulin content (mUnits/wel)
20
10
0
control
KAD-1229 (100 nM)
tolbutamide (100 μM)