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PKA-mediated phosphorylation of the human K_{ATP} channel: separate roles of Kir6.2 and SUR1 subunit phosphorylation

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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-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.

Keywords: ATP-sensitive K^+ channel/Kir6.2/phosphorylation/PKA/SUR1

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 ion channel, 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 alters channel properties by modifying the kinetics and/or the number of active channels 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 primarily confer 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

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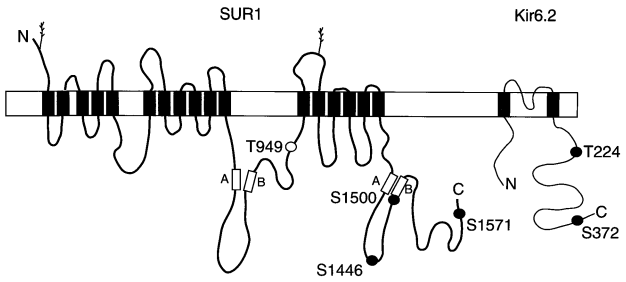

Fig. 1. Consensus phosphorylation sites for PKA in Kir6.2 and SUR1 subunits of the human K_{ATP} channel. Filled circles indicate serine and threonine residues in the consensus phosphorylation sites tested in this study. The open circle represents an additional site. Open squares A and B indicate the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

such as G proteins (Gs) (Sanchez *et al.*, 1998) and phosphatidylinositol-4,5 phosphate (PIP_2) (Baukrowitz *et al.*, 1998; Shyng and Nichols, 1998) modulate K_{ATP} channel activity. It has been shown that the activities of K_{ATP} channels are regulated also by PKA (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 PKA (Notsu *et al.*, 1992; Quayle *et al.*, 1994). Phosphorylation by unknown kinases of the sulfonylurea receptor is also thought to modulate the effect of diazoxide on K_{ATP} channel activity (Niki and Ashcroft, 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 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 (Figure 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 labeling 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 (Figure 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 (Figure 2B, compare lane 2 with lane 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 (Figure 2B, compare lane 4 with lane 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 (Figure 2A), the phosphorylation of both mutants was completely abolished (Figure 2B, compare lane 4 with lanes 9 and 11), while the T224A mutation did not affect the phosphorylation of Kir6.2 (Figure 2B, compare lane 4 with 7). Abolition of the PKA-mediated phosphorylation of Kir6.2 was also obtained by mutating the serine 372 in aspartate (unpublished data).

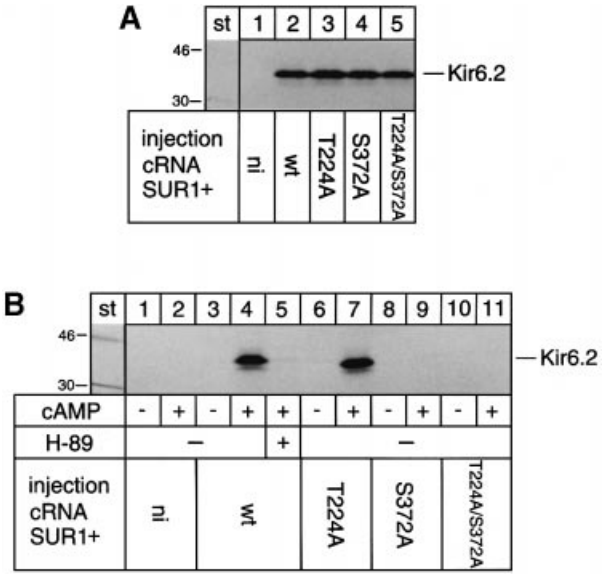

Fig. 2. Expression of human wild-type and mutant Kir6.2 in *Xenopus* oocytes and phosphorylation in homogenates upon stimulation of cellular PKA. (**A**) Cellular expression of wild-type and mutant Kir6.2 in oocytes. Control oocytes without injection (lane 1) or oocytes injected with human SUR1 cRNA together with human wt Kir6.2 (lane 2) or mutant Kir6.2 (lanes 3–5) cRNA. (**B**) Phosphorylation of wild-type and mutant Kir6.2 in homogenates. The phosphorylation reaction was done in the absence (lanes 1, 3, 6, 8 and 10) or in the presence of 50 μM cAMP (lanes 2, 4, 5, 7, 9 and 11) and in the absence (lanes 1–4 and 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.

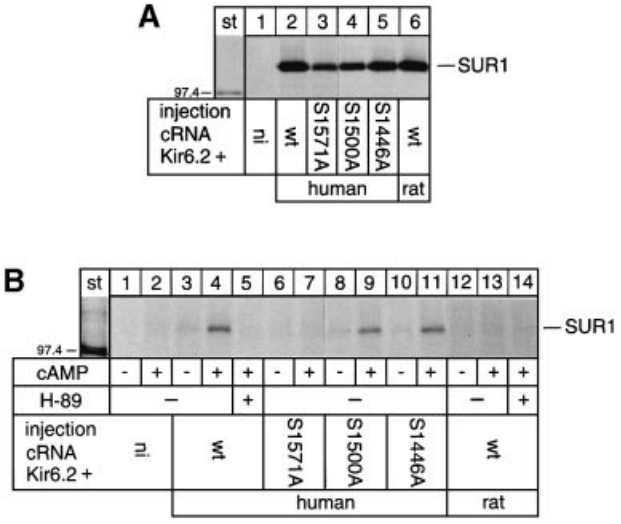

Fig. 3. Expression of human wild-type, rat wild-type and human mutant SUR1 in *Xenopus* oocytes and phosphorylation in homogenates upon stimulation of cellular PKA. (**A**) Cellular expression of wild-type and mutant SUR1 in oocytes. Control oocytes without injection (lane 1) or injected with human Kir6.2 cRNA together with human wild-type (lane 2), rat wild-type (lane 6) or human mutant (lanes 3–5) SUR1 cRNA. (**B**) Phosphorylation of wild-type and mutant SUR1 in homogenates. The phosphorylation reaction was done in the absence (lanes 1, 3, 6, 8, 10 and 12) or in the presence of 50 μM cAMP (lanes 2, 4, 5, 7, 9, 11, 13 and 14) and in the absence (lanes 1–4 and 6–13) or in the presence of 2 μM PKA inhibitor H-89 (lanes 5 and 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.

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 labeling with [^{35}S]methionine showed that the expression levels of each SUR1 mutant in oocytes was comparable with that of wild type (Figure 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 (Figure 3B, compare lanes 3 and 4 with lanes 12 and 13, respectively). The abolition of the SUR1 phosphorylation by addition of the PKA inhibitor H-89 confirmed PKA specificity of the phosphorylation (Figure 3B, compare lane 4 with 5). Both the S1500A and S1446A mutants were phosphorylated to the same extent as the wild type (Figure 3B, compare lane 4 with lane 9 or 11). In contrast, the phosphorylation in the S1571A mutant in response to PKA was completely abolished (Figure 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 at –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 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 labeling with [^{35}S]methionine (Figure 4A and B, lane 1) or [^{32}P]orthophosphate (Figure 4C and D, lanes 1–3). In transfected cells, metabolic labeling with [^{35}S]methionine showed that wild-type and mutants of both subunits were expressed at similar levels (Figure 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-methylxanthine (IBMX) or dibutyryl cyclic adenosine monophosphate (dbcAMP) (Figure 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 can also be phosphorylated by PKA in intact cells. In contrast, wt SUR1 is phosphorylated under the basal condition (Figure 4C, lane 4). We could not detect any significant increase in wt SUR1 phosphorylation by stimulation with forskolin and IBMX or dbcAMP (Figure 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 (Figure 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 phosphoamino acid analysis (PAA) using the immunoprecipitated wild-type and mutant human SUR1 from transfected COS-1 cells with or without forskolin plus IBMX stimulation. Digestion with trypsin of immunoprecipitated wild-type 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

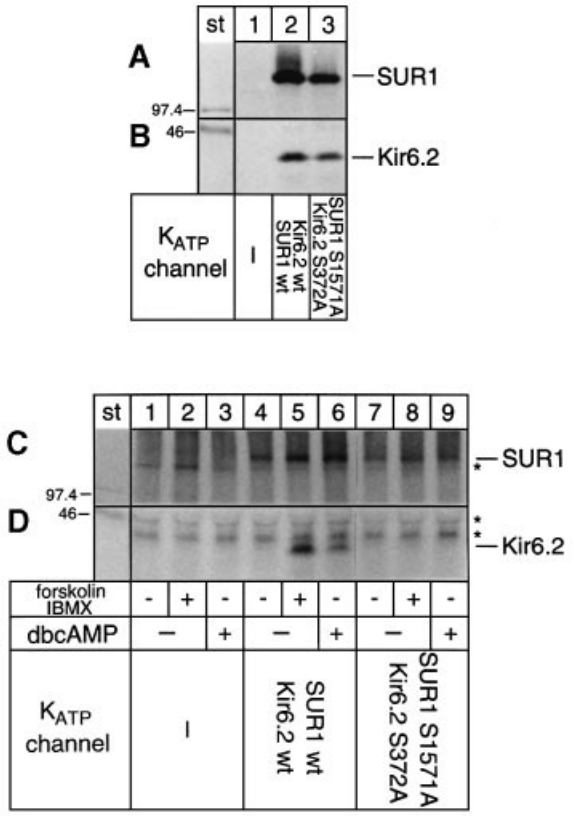

Fig. 4. Expression and phosphorylation of human wild-type and mutant K_{ATP} channels in intact cells upon PKA stimulation. (**A** and **B**) Microsomal expression of wt Kir6.2/SUR1 and mutant Kir6.2 S372A/ SUR1 S1571A K_{ATP} channels in COS-1 cells. Untransfected control COS-1 cells (lane 1) or COS-1 cells transfected with wt Kir6.2 and wt SUR1 (lane 2) or mutant Kir6.2 S372A and SUR1 S1571A (lane 3) cDNA. (**C** and **D**) Phosphorylation of wild-type and mutant K_{ATP} channels in COS-1 cells. The phosphorylation assay was done in the absence of PKA stimulators (lanes 1, 4 and 7) or in the presence of 100 μM forskolin and 1 mM IBMX (lanes 2, 5 and 8) or in the presence of 1 mM dbcAMP (lanes 3, 6 and 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.

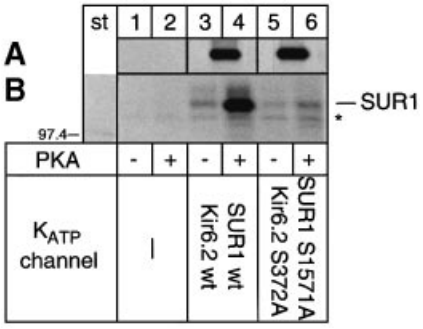

Fig. 5. Expression and phosphorylation of microsomal human wild-type and mutant SUR1 with PKA. (**A**) Microsomal expression of wild-type and mutant SUR1. Untransfected control COS-1 cells (lanes 1 and 2) or COS-1 cells transfected with wt Kir6.2 and wt SUR1 cDNAs (lanes 3 and 4) or the mutant Kir6.2 S372A and the mutant SUR1 S1571A cDNAs (lanes 5 and 6). (**B**) Phosphorylation of wild-type and mutant SUR1 in COS-1 cells. The *in vitro* phosphorylation assay was done in the absence (lanes 1, 3 and 5) or in the presence (lanes 2, 4 and 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.

was detected in the non-stimulated state and could not be further increased after PKA stimulation, the SUR1 PKA site might already be phosphorylated by PKA in the basal state in intact cells. On the other hand, phosphoamino acid 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 wild-type and mutant SUR1 being similar (Figure 5A, insets 2 and 3), the addition of PKA substantially increased the phosphorylation of wt SUR1 and not that of mutant SUR1 S1571A (Figure 5B, compare lanes 3 and 4 with lanes 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 understand better the physiological role of the PKA-mediated phosphorylation of Kir6.2, we investigated whether the activation of different Gs-coupled receptors could lead to a change in Kir6.2 phosphorylation. For this purpose, COS-1 cells were transiently transfected with wild-type or the mutant (Kir6.2 S372A/SUR1 S1571A) K_{ATP} channel together with various Gs-coupled receptors. Metabolic labeling with [^{35}S]methionine of COS-1 cells showed that the wild-type and mutant Kir6.2 were expressed at a similar level (Figure 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 (Figure 6B, compare lanes 9 and 11 with lanes 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 investigate PKA-

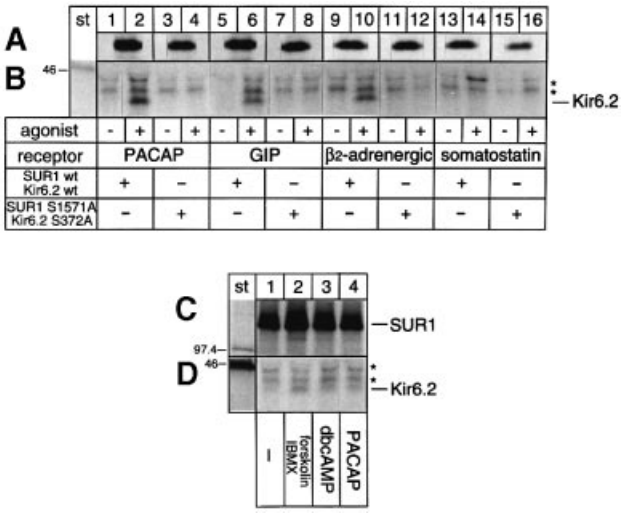

Fig. 6. Expression and phosphorylation of human wild-type and mutant K_{ATP} channels in COS-1 cells and the K_{ATP} channels in MIN6 cells upon G-coupled receptor and PKA stimulation. (**A**) Microsomal expression of wt Kir6.2 and mutant Kir6.2 S372A in COS-1 cells. COS-1 cells expressing endogenous 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 and 14) or co-transfected with the mutant Kir6.2 S372A and SUR1 S1571A cDNAs (lanes 3, 4, 7, 8, 11, 12, 15 and 16). (**B**) Phosphorylation of wild-type 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 and 15) or in the presence of 8 nM PACAP-38 (lanes 2 and 4), 1 μM GIP (lanes 6 and 8), 10 μM epinephrine (lanes 10 and 12) or 50 nM somatostatin (lanes 14 and 16). (**C** and **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 and 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.

mediated phosphorylation further, Kir6.2 and SUR1 were coexpressed with other G-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 (Figure 6B, lanes 2 and 6). In contrast, the Kir6.2 S372A could not undergo PACAP- or GIP-induced phosphorylation (Figure 6B, lanes 4 and 8). As a negative control, stimulation of the somatostatin receptors, known to inhibit adenylyl cyclase activity, could not increase Kir6.2 phosphorylation (Figure 6B, lanes 13–16). These results indicate that stimulation of receptors coupled to the cAMP-dependent signaling 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 (Figure 6C, lanes 1–4). In contrast, Kir6.2 phosphorylation was increased significantly (2- to 3-fold) after forskolin and IBMX stimulation (Figure 6D, compare lane 1 with 2), but almost no increase was observed after dbcAMP or PACAP stimulation (Figure 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 with 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-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 Figure 7A and B, 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 (Figure 7C and D). This increase in channel activity was due to neither the change in channel conductance (p*S*) (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 (Figure 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 analyzed K_{ATP} channel kinetics from patches which had only a single channel. Interestingly, as shown in Figure 8, there is an apparent difference in the channel kinetics between

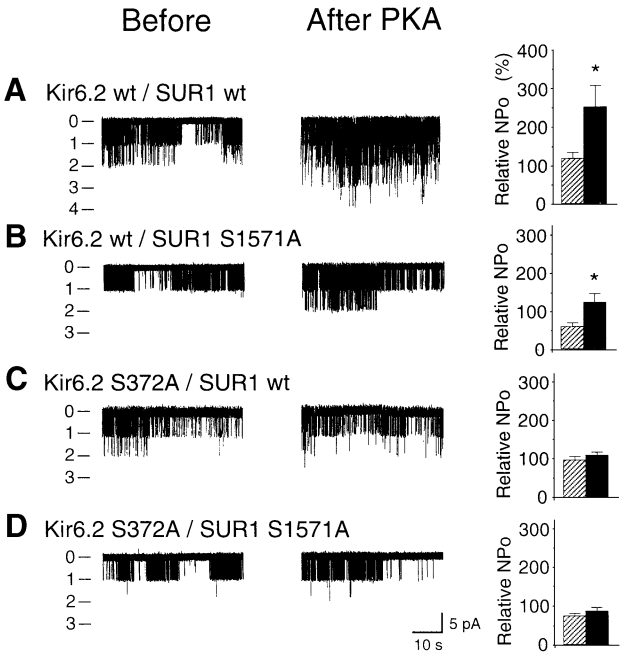

Fig. 7. Changes in activities of wild-type and various mutant K_{ATP} channels before and after application of the PKA catalytic subunit. Current recordings were obtained in inside-out patches excised from COS-1 cells expressing wt Kir6.2 and wt SUR1 (**A**), wt Kir6.2 and the mutant SUR1 S1571A (**B**), 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).

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 are defined principally by longer closed times τ_{c2} and τ_{c3} ($\tau_{c3} > \tau_{c2}$). As shown in Figure 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- to 3-fold) and dramatically (6- to 9-fold) prolonged, respectively, as compared with those of K_{ATP} channels comprising wt SUR1. The cluster duration in K_{ATP} channels comprising mutant SUR1 S1571A also was greatly prolonged (Figure 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 (Figure 8A and C) that is abolished in the K_{ATP} channels comprising mutant SUR1 S1571A (Figure 8C and D) and a PKA-independent process observed as a decrease in channel activity of both wild-type and mutant K_{ATP} channels after long exposure to alkaline phosphatase (Figure 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 (Figure 8A and C; Table II), while no significant change was observed in the K_{ATP} channels comprising mutant SUR1 S1571A (Figure 8B 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 Figure 4, SUR1 is also phosphorylated in the basal state by unknown kinases. Although alkaline phosphatase cleaves any of the phosphate groups that 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 play 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- to 4-fold increase in open probability was observed, compared with the wt Kir6.2/SUR1 channel (0.12 $\pm$ 0.06 and 0.37 $\pm$ 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 $\pm$ 0.06 and 0.09 $\pm$ 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, respectively.

Interestingly, rubidium efflux from the K_{ATP} channels comprising SUR1 S1571A expressed in COS-1 cells decreased by 68 $\pm$ 12% (n = 3), compared with that from wt K_{ATP} channels. Since this result contrasts with 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

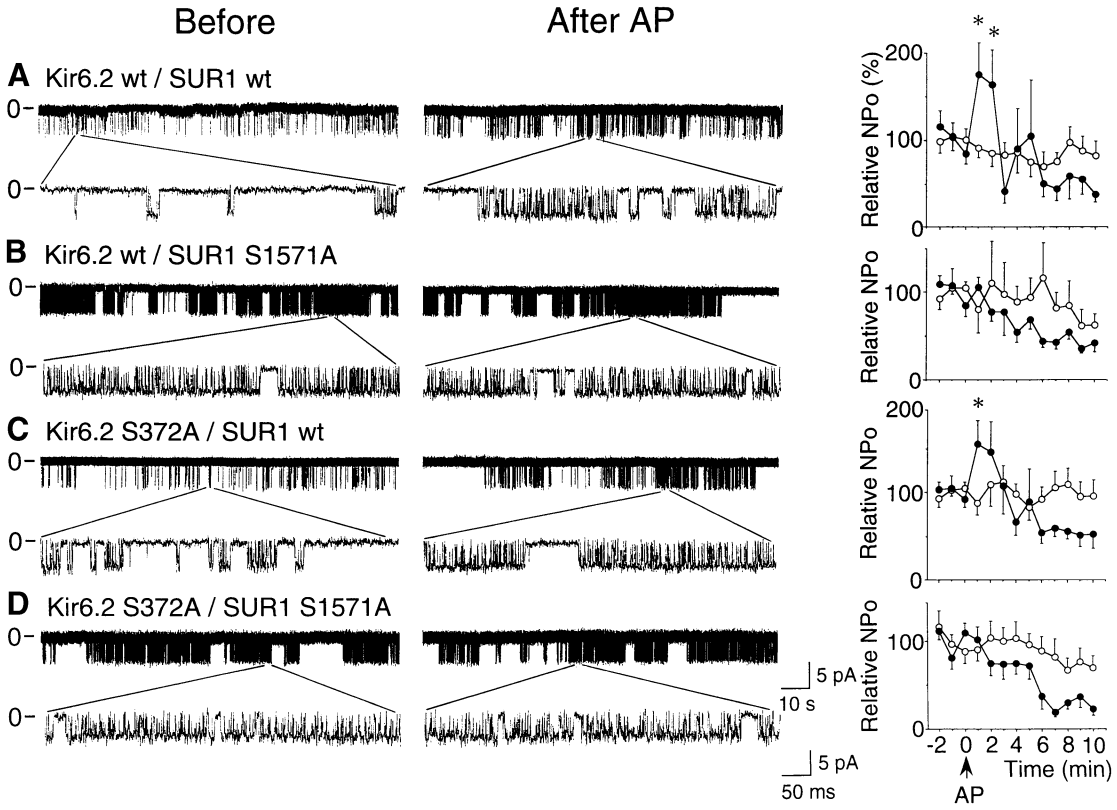

Fig. 8. Changes in burst and cluster duration, interburst interval and open probability before and after application of alkaline phosphatase (10 U/ml). Single channels were obtained in inside-out patches excised from COS-1 cells expressing wt Kir6.2 and wt SUR1 (**A**), wt Kir6.2 and the mutant SUR1 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 after (2 min) 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).

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 labeling (unpublished data). Using channel density and open probability parameters, the channel activity of K_{ATP} channel comprising SUR1 S1571A can be estimated as 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 efflux, 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-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- to 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 increase the channel activity by PKA stimulation significantly (K.Nagashima, P.Béguin, N.Inagaki and S.Seino, 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

Table I. Properties of wild-type 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 ± 0.02[a]	73 ± 17[a]	394 ± 113[a]	2.21 ± 0.13	0.44 ± 0.03	12.9 ± 4.5	1120 ± 71[a]	40.0	4.8 ± 0.8[a]
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[a]	74 ± 20[a]	274 ± 37[a]	2.22 ± 0.30	0.49 ± 0.04	10.5 ± 1.0	863 ± 49[a]	35.0	3.2 ± 0.4[a]

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)].
[a]Values significantly different from those for wild-type K_{ATP} channels ($P < 0.05$).

expression of K_{ATP} channels at the cell surface. Moreover, alkaline phosphatase treatment clearly demonstrates 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. Consistent with our data, Babenko *et al.* (1998) 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, 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 (Baukrowitz *et al.*, 1998; Shyng and Nichols, 1998; Tucker *et al.*, 1998). The C-terminus appears to be involved in the binding of ATP and PIP_2 (Baukrowitz *et al.*, 1998; Shyng and Nichols, 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

Table II. Properties of wild-type 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[a]	155 ± 32[a]	402 ± 60
Kir6.2 wt/SUR1 S1571A	36.9 ± 11.1	169 ± 41	1236 ± 279
Kir6.2 S372A/SUR1 wt	41.1 ± 3.2[a]	103 ± 15[a]	576 ± 162
Kir6.2 S372A/SUR1 S1571A	52.6 ± 6.1	178 ± 37	1743 ± 485

Recordings were made at –70 mV in 10 μM ATP conditions (n = 3–6 in all cases).
[a]Values significantly different after AP treatment ($P < 0.05$).

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 (Quayle *et al.*, 1994; Wellman *et al.*, 1998), 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 vasodilation 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 (DDBJ/EMBL/GenBank accession No. 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). *In vitro* synthesized cRNA (2.5 ng) 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 eight or nine 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 before the start of reactions. The reactions were stopped by heating at 95°C

and adding SDS (3.7% final concentration) and the samples 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 (DMEM; 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 wild-type or mutated human Kir6.2 cDNA plus human SUR1 (4 and 1 μg of each subunit for biochemical and electrophysiological studies, respectively), using lipofectamine and Opti-MEM I reagents (Life Technologies) or FuGENE6 (Boehringer Mannheim), 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 labeling and the two remaining dishes were used for phosphorylation assay (see below). For electrophysiological recordings, COS-1 cells were plated on dishes (35-mm) 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 labeling, and in vitro and in vivo phosphorylation in COS-1 and MIN6 cells

COS-1 cells were metabolically labeled by equilibrated cells in DMEM without methionine for 1 h and then incubated in the same medium containing [^{35}S]methionine (0.2 mCi/ml; Amersham) for 18 h at 37°C. The phosphorylation experiments were performed as follows: COS-1 or MIN6 cells were equilibrated in phosphate-free DMEM for 2 h and then incubated in the same medium containing carrier-free ^{32}P (0.2 mCi/ml; Amersham) for an additional 2 h 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 dbcAMP (Sigma) for 20 min. PKA-mediated phosphorylation after Gs-coupled receptor stimulation was tested by adding agonists for endogenous or transfected receptors during 20 min as indicated in Figure 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 dithiothreitol and 5 μg/ml each of leupeptin, pepstatin and antipain. 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 the samples were then subjected to immunoprecipitation (see below).

Immunoprecipitation

Aliquots of phosphorylated or [^{35}S]methionine-labeled oocyte homogenates and COS-1 or MIN6 microsomes were immunoprecipitated by specific antibodies prepared against unconjugated 24mer peptide (amino acids 364–387) of the C-terminal region of mouse Kir6.2 or against KL-hemocyanin-conjugated 21mer 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 fluorography 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 labeling was performed as described above.

Electrophysiology

Recordings were made 24–72 h 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 –70 mV. Before experiments, the cells were perfused 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 at a final concentration of 100 and 10 U/ml, respectively. Before and after PKA stimulation, ATP sensivity was determined at 1, 10, 100 and 1000 μM ATP as described (Inagaki *et al.*, 1995). Channel kinetics after AP treatment were obtained by analyzing the data between 0 and 2 min. To measure channel density at the cell surface, single channel recordings were performed between 48 and 72 h 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 analyzed by a combination of pCLAMP (ver6.0, Axon Instruments, Foster City, CA) 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 from 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 >2.5 ms (Alekseev *et al.*, 1997; Sanchez *et al.*, 1998). A group of bursts which are separated by closed time <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 ± SE.

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