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Receptor stimulation causes slow inhibition of IRK1 inwardly rectifying K^+ channels by direct protein kinase A-mediated phosphorylation

[inwardly rectifying K^+ channel/serotonin (5-hydroxytryptamine)/cAMP/rat basophilic leukemia cells/patch clamp]

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ABSTRACT **Strongly rectifying IRK-type inwardly rectifying K^+ channels are involved in the control of neuronal excitability in the mammalian brain. Whole-cell patch-clamp experiments show that cloned rat IRK1 (Kir 2.1) channels, when heterologously expressed in mammalian COS-7 cells, are inhibited following the activation of coexpressed serotonin (5-hydroxytryptamine) type 1A receptors by receptor agonists. Inhibition is mimicked by internal perfusion with GTP[γ-S] and elevation of internal cAMP concentrations. Addition of the catalytic subunits of protein kinase A (PKA) to the internal recording solution causes complete inhibition of wild-type IRK1 channels, but not of mutant $IRK1_{S425N}$ channels in which a C-terminal PKA phosphorylation site has been removed. Our data suggest that in the nervous system serotonin may negatively control IRK1 channel activity by direct PKA-mediated phosphorylation.**

Various cellular processes that control K^+ homoeostasis, secretion, and membrane excitability involve inwardly rectifying K^+ (Kir) channels. The recent progress in molecular cloning has led to the identification of multiple channel species that according to a recently proposed classification scheme (1) may be assigned to three major subfamilies: (*i*) Kir 1.0 channels that are ATP-sensitive and mildly rectifying, (*ii*) Kir 2.0 channels (IRK) with strong inward rectification, and (*iii*) G protein-activated Kir 3.0 channels (GIRK). Channel subtypes that belong to the GIRK subfamily have been considered to be under the obligatory control of G protein-mediated signaling pathways. Underlying the action of acetylcholine in heart atrial cells, receptor-activated $G\beta\gamma$ subunits dramatically increase the open probability of Kir 3.1 (GIRK1/KGA; refs. 2–4). Although direct demonstration is still lacking, $G\beta\gamma$ subunits are thought to directly interact with distinct N- and C-terminal parts of the channel protein (5–8). Analogously, central nervous system neurons may use this direct signaling theme by employing different combinations of several G protein-coupled receptors (9, 10) and Kir 3.0 subtypes (11–15).

In contrast to the pathways that involve GIRK and transduce signals with rate constants of 3 to 10 s^{-1} (16), slower forms of G protein-mediated control of K^+ inward rectifiers involving soluble cytoplasmic messengers may also exist. Both in excitable (17–19) and in nonexcitable cells (20, 21), Kir channels are inhibited by a process that involves the action of serine/threonine protein kinases and protein phosphatases. In addition, recent reports suggested that channel run down and loss of activity after patch-excision of recombinant Kir 1.1 and Kir 2.1 channels, respectively, were reduced in the presence of protein kinase A (PKA) (22, 23). Functional modulation of receptor/channel activity through phosphorylation/dephosphorylation processes in fact is a widespread cellular phenomenon and has been found to affect Na^+ channels (24), L-type Ca^{2+} channels (25), large-conductance Ca^{2+}-dependent K^+ channels (26), as well as receptors for GABA (A-type; ref. 27), glutamate (GluR6; refs. 28 and 29), and acetylcholine (nicotinic; ref. 30).

Although the majority of cloned Kir channels exhibit consensus sites in their primary amino acid sequences for a variety of kinases including cAMP/cGMP-dependent PKA, protein kinase C (PKC), and protein tyrosine kinases, it is still unknown as to whether the channel protein itself functions as the substrate for the kinases. In this report, we provide direct evidence that Kir 2.1 (IRK1) channels, recently cloned from a mast cell line (31), are under the inhibitory control of a receptor-stimulated PKA pathway; alteration of a single C-terminal IRK1 PKA phosphorylation site prevents this inhibition.

MATERIALS AND METHODS

Molecular Biology and Cell Culture. A rat ortholog of IRK1 (Kir 2.1) with an open reading frame of 1284 bp that showed 94% base pair identity to the mouse IRK1 (32) has been cloned by reverse transcriptase-PCR from rat basophilic leukemia type 1 cells (31). For phosphorylation experiments, a synthetic oligonucleotide primer was used to PCR-construct a mutant $IRK1_{S425N}$ in which the serine at amino acid position 425 was replaced by an asparagine residue. The mutation was verified by sequencing on both strands using the prism Sequenase dye terminator kit on an automatic sequencer (Perkin–Elmer). cDNAs encoding IRK1, $IRK1_{S425N}$, as well as the human serotonin (5-hydroxytryptamine; 5-HT) type 1A receptor ($5\text{-}HT_{1A}R$; ref. 33) were subcloned into the eukaryotic expression vector pSVSport 1 (Life Technologies). Simian virus 40 transformed COS-7 kidney cells (ATCC #CRL1650) were cultured at 37°C and 5% CO_2/95% air in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 0.1 mg/ml streptomycin, and 100 units per ml penicillin, and plated on poly-L-lysine-coated glass coverslips. Expression plasmids were transfected into the cells grown at confluency using Lipofectamine and Opti-MEM I (Life Technologies) following the manufacturer's protocol. cDNA amounts used for transfections were optimized for the electrophysiological assay to 0.8 μg/ml medium (IRK1s) and 0.4 μg/ml medium ($5\text{-}HT_{1A}R$).

Electrophysiology. Whole-cell and single channel recordings in the cell-attached and excised patch configuration (34)

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Abbreviations: 5-HT, serotonin (5-hydroxytryptamine); $5\text{-}HT_{1A}R$, 5-HT type 1A receptor; 8-OH DPAT, 8-hydroxy-2-(di-*n*-propylamino)tetralin; H7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazinedihydrochloride; Kir, inwardly rectifying K^+ channel; PKA, protein kinase A; PKC, protein kinase C.

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were performed at room temperature 48–72 h posttransfection in a bath solution consisting of 135 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 5 mM Hepes (pH 7.4). Patch pipettes were pulled from Kimax-51 borosilicate glass capillary tubes (Kimble, Sussex, England), coated with Sylgard (Dow-Corning), and heat-polished to give input resistances of 3–6 MΩ (whole-cell) and 7–9 MΩ (single-channel). The intracellular recording solution contained 140 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 5 mM Hepes, 1 mM Na_2ATP, 100 μM cyclic AMP, and 100 μM GTP (optional) (pH 7.3). A motor-driven fast microperfusion system was used to perform local solution changes (in < 30 ms) near the measured cells. *N*-(heptyl)-5-chloro-1-naphthalenesulfonamide (SC-10) and bisindolylmaleimide were from Calbiochem, and α-methyl-5-HT was from Research Biochemicals (Natick, MA); all other drugs and chemicals were provided by Sigma. Bovine heart PKA catalytic subunits (P2645, Sigma) were reconstituted in water freshly for each experiment using dithiothreitol.

Currents were recorded with an EPC9 (Heka Electronics, Lamprecht/Pfalz, Germany) patch clamp amplifier and low pass-filtered at 1–2 kHz. Capacitive currents were canceled using the automatic compensation of the EPC9. Stimulation and data acquisition were controlled by the PULSE/PULSEFIT software package (Heka Electronics) on a Macintosh computer, and data analysis was performed with TAC (Heka Electronics) and IGOR software (WaveMetrics, Lake Oswego, OR). Data are presented as mean ± SD (number of cells).

RESULTS

Coexpression of IRK1 Channels and 5-HT_{1A}R in COS-7 Cells. An IRK1 (Kir 2.1) ortholog cloned from rat basophilic leukemia type 1 cells (31), when transfected into COS-7 cells, exhibited steeply "voltage-dependent" K^+ currents with strong rectification in the presence of 1 mM internal Mg^{2+}. Current amplitudes averaged to 6.64 ± 3.68 nA (n = 18) at a holding potential of V_h = −120 mV ($[K^+]_e$ = 25 mM). With respect to gating kinetics, activation potential, K^+ permeability, and block by external Cs^+, Ba^{2+}, and Na^+, both whole-cell and single channel data indicated that IRK1 in COS-7 cells exhibited functional features typical for strongly rectifying "classic" IRK1- (Kir2.1-) K^+ inward rectifiers (data not shown).

Kir channel current, I_{Kir}, in native rat basophilic leukemia cells is under the inhibitory control of an as yet undetermined signaling pathway that involves putatively pertussis-toxin-insensitive G proteins (31, 35). In an attempt to reconstitute receptor-mediated Kir modulation in COS cells, we cotransfected rat IRK1 together with G protein-coupled human 5-HT_{1A}Rs. When cotransfected COS cells were recorded in the whole-cell configuration, 22 of 46 cells tested (and expressing IRK1) were clearly affected by bath application of the agonist 5-HT (10–100 μM). COS cell responses to hyperpolarizing voltage steps revealed that 5-HT inhibits inward currents through IRK1 channels (Fig. 1). Current inhibition by 100 μM 5-HT averaged to 42 ± 20% (n = 16). The block was voltage-independent and rapidly developing (τ_{ON} = 4277 ± 1267 ms; n = 5) and reversible within seconds (τ_{OFF} = 3025 ± 1515 ms; n = 5) as determined from continuous recordings of cell responses to 5-HT applied through a fast microperfusion system (V_h = −120 mV; Fig. 1*B*).

Of the more specific receptor agonists tested, the 5-HT type 2 (5-HT_2) receptor agonist α-methyl-5-HT (n = 13) did not significantly affect I_{Kir} (Fig. 2). 5-HT inhibition was only exceeded by applying the selective 5-HT_{1A}R agonist 8-hydroxy-2-(di-*n*-propyl-amino)tetralin (8-OH-DPAT). Current block by 100 μM 8-OH-DPAT averaged to 72.7 ± 4% (n = 6; Fig. 2) and was otherwise indistinguishable from the 5-HT effect. When coapplied with the agonist, the broad 5-HT_1 receptor antagonist pindolol (50 μM) diminished the inhibitory effect of 5-HT (Fig. 2). These results provide strong evidence that inhibition in COS cells of I_{KIR} by 5-HT may indeed be mediated by 5-HT_{1A}Rs (see *Discussion*).

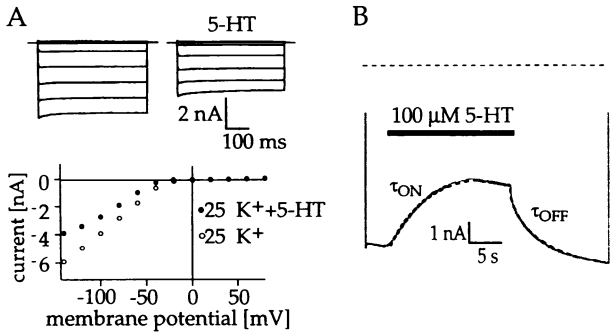

FIG. 1. 5-HT inhibition of IRK1 through cotransfected 5-HT_{1A}Rs in COS-7 cells. (*A*) Whole-cell voltage clamp responses of IRK1/5-HT_{1A}R-cotransfected COS-7 cells to step potentials between +80 mV and −120 mV from a holding potential of −60 mV in the absence (*Left*) and presence (*Right*) of 100 μM 5-HT. Steady state currents plotted against voltage below reveal a ≈40% reduction in current amplitude induced by 5-HT. (*B*) Kinetics of 5-HT-induced inhibition of IRK1 currents in a cell clamped at V_h = −120 mV. The on and off time course of inhibition is fitted by single exponentials with constants of τ_{ON} = 5.52 s and τ_{OFF} = 4.65 s, respectively. $[K^+]_e$ = 25 mM.

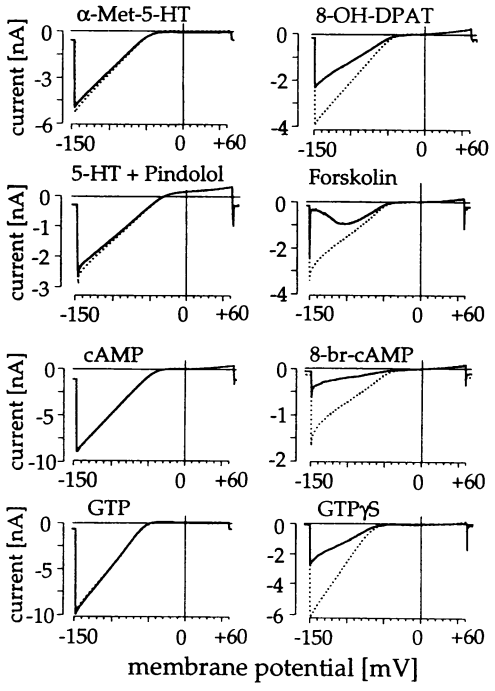

FIG. 2. Regulation of IRK1 in transfected COS-7 cells. Whole cell responses of IRK1/5-HT_{1A}R-cotransfected COS-7 cells to 500 ms voltage ramps between −150 mV and +60 mV in the presence and absence of modulatory agents. Neither the extracellularly applied 5-HT_2 receptor agonist α-methyl-5-HT (50 μM) nor cAMP (100 μM), but the selective 5-HT_{1A}R agonist 8-OH-DPAT (100 μM) and the membrane-permeable cAMP analogue, 8-bromo-cAMP (100 μM), significantly inhibit I_{IRK1}. Block induced by receptor agonist (50 μM 5-HT) is antagonized by the 5-HT_1 receptor antagonist pindolol (50 μM). Bath application of 100 μM forskolin induces a voltage-dependent block of I_{IRK1}. Dotted lines depict cell response before administration of drug. Addition of 200 μM GTP to the internal solution did not, but the same concentration of GTP[γ-S] did reduce I_{IRK1} amplitude over time. Dotted lines depict current responses at the time of break-in, solid lines after 10 min of whole-cell recording. A <10% degree of inhibition is allowed to account for "undetermined" current run-down during 5 min of in the whole-cell recording. $[K^+]_e$ = 25 mM.

To further delineate components involved in the 5-HT_{1A}R-mediated inhibition of I_{Kir}, we investigated signaling through the second messenger cAMP. First, in 8 out of 12 cells expressing IRK, addition of GTP[γ-S]; 100–400 μM) to the internal solution resulted in a 74 ± 8% blockade of I_{Kir} during the first 10 min after obtaining the whole-cell configuration. In comparison, I_{Kir} remained basically constant during that time period in the presence of internal GTP. When applied to the bath, the adenylate cyclase activator forskolin (100 μM) was also found to inhibit I_{Kir} in a time- and voltage-dependent manner (Fig. 2). Forskolin block was observed in 10 of 16 cells and characteristically proceeded with both a fast time constant (τ_{ON} = 285 ± 884 ms; n = 6; probably due to direct, voltage-dependent interaction with the channel; ref. 36) and a slow time constant (τ_{ON} = 1.97 ± 0.45 s; n = 3; probably due to elevation of internal cAMP concentration) of both onset of inhibition and recovery from inhibition (Fig. 3). As expected, the second messenger cAMP was ineffective in regulating I_{Kir} when applied to the outside of the cells (n = 8). In contrast, microperfusion of transfected COS cells with the membrane-permeable analogue 8-bromo-cAMP (50–100 μM) induced a pronounced (>10%) and reversible block of I_{Kir} (80 ± 9%) in 8 out of 31 cells tested (Fig. 2). Interestingly, coapplication of 5-HT and 8-bromo-cAMP induced a block that was comparable in amplitude (88 ± 11%; n = 9) and occurred more frequently (9 from 12 cells) than the responses to both substances individually. Fig. 3 compares the kinetics of the inhibitory effect by 5-HT, 8-bromo-cAMP, and both applied together; the average time constant for the onset of block was τ_{ON} = 3320 ± 1495 ms (n = 8) for the receptor agonists (5-HT and 8-OH-DPAT), and this was ≈3 times slower than inhibition induced by 8-bromo-cAMP (τ_{ON} = 1144 ± 759 ms; n = 4) or 5-HT together with 8-bromo-cAMP (τ_{ON} = 958 ± 678 ms; n = 5). These kinetic data may both serve as reference for the action/regulation of other putative signaling components (e.g., adenylate cyclase stimulated by forskolin) and suggest a function of cAMP downstream of 5-HT_{1A}Rs in the regulation of I_{Kir}.

PKA-Mediated Phosphorylation of IRK1 and Mutant $IRK1_{S425N}$ Channels. Both the potent nonspecific protein kinase inhibitor 1-(5-isoquinolinesulfonyl)-2-methylpiperazinedihydrochloride (H7) as well as PKA catalytic subunits were used to test whether cAMP-dependent protein kinase may be involved in the inhibition of I_{Kir}. Fig. 4*A* demonstrates that H7 (100 μM), when added to the bath, antagonized the I_{Kir} inhibition induced by 50 μM 8-bromo-cAMP (32 ± 15%; n = 3), suggesting that I_{Kir} inhibition may occur through the action of a cAMP-dependent protein kinase. To verify this, 20 units per ml of the catalytic subunit of PKA were added to the internal recording solution and voltage step/ramp responses of transfected COS cells documented from entering the whole-cell mode. Comparison with untreated control cells (rundown <10% in 3 min) demonstrated that internally applied catalytic PKA subunits, after a delay of ≈100 s of whole-cell recording, drastically reduced the amplitude of I_{Kir} by 87 ± 8% (n = 8) within 5 min (Fig. 4*B*). In contrast, under identical conditions, preincubation of cotransfected COS cells with 100 μM H7 for 10 min prevented PKA subunit-induced inhibition of I_{Kir} in all cells tested (n = 8).

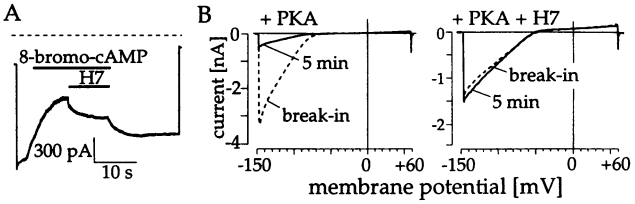

FIG. 4. Inhibition of IRK1 involves a cAMP-dependent protein kinase. (*A*) Inhibition of I_{IRK1} induced by 50 μM 8-bromo-cAMP in IRK1/5-HT_{1A}R-cotransfected COS-7 cells (V_h = −120 mV) is antagonized by coapplication of the nonselective protein kinase inhibitor H7 (100 μM). (*B*) Supplementation of catalytic PKA subunits (20 units per ml) to the pipette solution results in a >80% decrease in I_{IRK1} after 5 min of cell perfusion; in contrast, preincubation for > 5 min with 100 μM H7 prevents I_{IRK1} inhibition by PKA catalytic subunits. Recordings show cell responses to 500 ms voltage ramps between −150 mV and +60 mV.

To demonstrate that PKA-mediated inhibition occurs by direct IRK1 phosphorylation, we engineered a mutant $IRK1_{S425N}$ channel in which a putative serine site for PKA-mediated serine/threonine phosphorylation had been conservatively replaced by an asparagine residue. When transfected into COS cells, $IRK1_{S425N}$ displayed identical features as the native channel with respect to efficiency of transfection, expression level, $[K^+]_e$-dependence of activation potential and slope conductance, "voltage-dependent" gating, and gating kinetics (n = 24; data not shown). However, mutant channels were always found to be unresponsive to 5-HT/8-OH-DPAT/8-bromo-cAMP stimulation in cells both with and without coexpression of 5-HT_{1A}Rs (n = 15; data not shown). More importantly, the response to the infusion of catalytic PKA subunits through the patch pipette was dramatically altered.

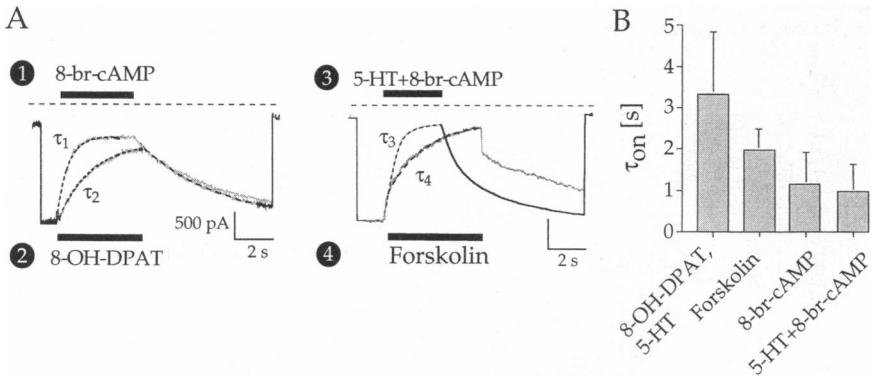

FIG. 3. Kinetics of I_{IRK1} block induced by receptor activation, 8-bromo-cAMP, and forskolin (*A*) Whole cell voltage-clamp of IRK1/5-HT_{1A}R cotransfected COS-7 cells (V_h = −120 mV) reveals different kinetics for extracellularly applied 8-bromo-cAMP (100 μM) and receptor ligand 8-OH-DPAT (100 μM) in the same cell. (*Left*) The onset of I_{IRK1} inhibition is fitted to single exponentials with time constants of τ_1= 0.71 s for 8-bromo-cAMP and τ_2= 2.13 s for 8-OH-DPAT. Time constants for relaxation from inhibition is ≈5 s for both agents. The effect of 8-bromo-cAMP when administered simultaneously with receptor agonist (*Right*) dominates the kinetics of inhibition (τ_3 = 0.7 s) and proceeds significantly faster than inhibition induced by 100 μM forskolin (τ_4 = 2.08 s). Scale bar represents 3 nA for (3) and 0.4 nA for (4). (*B*) Bar histogram shows time constants of the onset of inhibition for receptor agonist (5-HT, 8-OH DPAT), forskolin, 8-bromo-cAMP, and 5-HT plus 8-bromo-cAMP applied together.

Whereas voltage-step protocols documented the inhibition of wild-type IRK1 channels in the presence of catalytic PKA subunits (Fig. 5*A*), no sign of current inhibition through mutant $IRK1_{S425N}$ channels was observed that was significantly different from cells monitored in the absence of PKA subunits (n = 12; Fig. 5*B*). Thus, we tentatively conclude that IRK1 channels are subject to potential inhibition by direct PKA-mediated phosphorylation.

When COS cells expressing mutant $IRK1_{S425N}$ channels were subjected to internal perfusion with GTP[γ-S] (100–400 μM) I_{Kir} currents decreased in amplitude by only 15 ± 6% (n = 6) during the first 10 min of whole-cell recording. This value was comparable with control recordings in the presence of 200 μM GTP (6 ± 5%; n = 8), but was significantly smaller than the GTP[γ-S]-induced I_{Kir} reduction in cells transfected with native IRK1 channels. This observation may lead to the conclusion that under the experimental conditions used in our experiments, constitutively activated G proteins affect IRK channels primarily through PKA-mediated protein phosphorylation. Recent reports also suggest PKC to be involved in the inhibition of K^+ inward rectifiers (18, 23). In preliminary experiments using both the potent PKC-inhibitors staurosporine (n = 8) and bisindolylmaleimide (n = 9) as well as the PKC-activator SC-10 (n = 9), no effect was found on I_{IRK1}.

DISCUSSION

Slow inhibition by 5-HT receptor agonists, GTP[γ-S], forskolin, 8-bromo-cAMP, and catalytic subunits of PKA suggests that recombinant IRK1 (Kir2.1) channels expressed in COS-7 cells are negatively controlled by a cAMP-dependent phosphorylation process. The predicted amino acid sequence of IRK1 harbors four potential PKC phosphorylation sites, two tyrosine kinase phosphorylation sites, and a single putative cAMP/cGMP phosphorylation site at the third last serine residue (S425). When S425 was replaced by an asparagine residue in a mutant channel, the above substances no longer exerted their specific effects; thus, to our knowledge, we have demonstrated for the first time that Kir channels may function as a substrate for direct PKA-mediated protein phosphorylation. Because the apparent phosphorylation site is located at the very end of the C-terminal cytoplasmic tail of the protein ≈250 amino acids distant from the second putative transmembrane segment (M2), a direct conformational transition within the channel pore region induced by S425 phosphorylation seems unlikely. In analogy to the "ball and chain" inactivation model developed for transient current flow through A-type K^+ channels (37, 38), we suggest that a C-terminal portion of IRK1 might itself serve as the closing gate for the channel pore.

Recent studies on cholinergic nucleus basalis neurons in the basal forebrain (19) and dopaminergic neurons from the substantia nigra (20) report on the peptidergic inhibition of native Kir channels of unknown molecular identity mediated by PKC. Furthermore, the activity of recombinant IRK1 (Kir 2.1; ref. 23) and HRK1 (Kir 2.3; ref. 39) channels expressed in *Xenopus* oocytes were found to be decreased by PKC, although evidence for a direct PKC/channel interaction process has not been provided yet. It is conceivable that the N-terminal channel portion also plays a role in channel gating if the aforementioned serine and threonine residues in IRK1 (at positions 3 and 6, respectively) represent functional phosphorylation sites for PKC. However, our preliminary data using PKC-inhibitors and -activators are not yet conclusive as to whether in addition to a PKA-mediated signaling pathway IRK1 channels may be modulated by a phosphorylation/dephosphorylation process through PKC.

Data from native renal Kir channels (20), as well as heterologously expressed Kir 1.0 (22) and Kir 2.1 channels (23), supported the intriguing hypothesis of channel modulation by both PKA and PKC in an opposite manner. In contrast to our observations on IRK1 channels in COS cells, these studies suggested that channel activity could rather be enhanced by PKA. While the reasons for this discrepancy are not yet understood, most studies both in native cells and in recombinant systems report that I_{Kir} declines over time during intracellular perfusion with the nondiscriminating G protein "activator" GTP[γ-S], i.e., the Kir channel response, even if inversely modulated, is usually governed by the inhibitory process, which our results demonstrate is through PKA. With the putative S425 PKA phosphorylation site destroyed in our experiments, macroscopic currents through $IRK1_{S425N}$ channels are not potentiated, but rather slightly inhibited (≈15%) by GTP[γ-S] perfusion. This indicates that in COS-7 cells a signaling pathway other than cAMP-dependent phosphorylation is unlikely to contribute significantly to the functional state of IRK1 channels.

This study raises several still unsolved questions on the signaling cascade that may be addressed in the specific environment of other host cells. Serotonin effects in IRK1-expressing COS cells were observed only in ≈50% of the cells, and in preliminary experiments coexpressed G_s protein-coupled β2 adrenergic receptors significantly reduced I_{IRK1} only in a minority of the tested cells (data not shown). This could have been due to either a failure/lower rate of receptor expression or an otherwise disturbed coupling process. However, it is less clear why ≈66% of all cotransfected COS cells, as well as IRK1 channels in native rat basophilic leukemia cells (data not shown) failed to strongly respond to the application

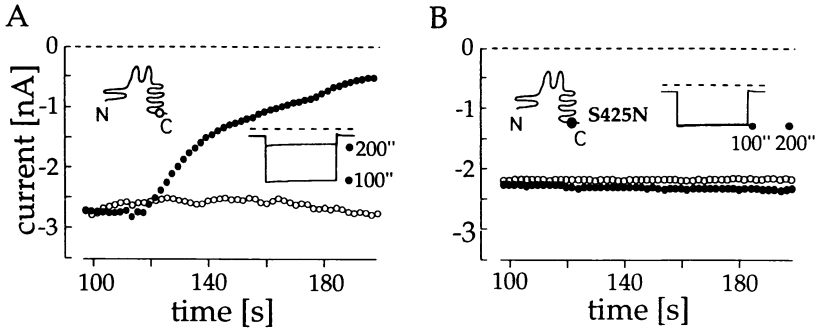

FIG. 5. PKA-mediated current inhibition is abolished in mutant $IRK1_{S425N}$ channels. (*A*) Time course of macroscopic I_{Kir} amplitudes in IRK1/5-HT_{1A}R-cotransfected COS-7 cells during whole-cell recording in the absence (open circles) or presence (solid circles) of 20 units per ml PKA catalytic subunits in the internal solution. Symbols depict steady state current amplitudes determined from voltage jumps to −120 mV (see *Inset*) delivered at intervals of 2 s. Abscissa shows the time after whole-cell break-in, ordinate values depict whole-cell currents (starting current levels set to the same values for illustrative purpose). (*B*) Same experiment performed for a COS cell transfected with a mutant channel $IRK1_{S425N}$ in which a putative PKA serine phosphorylation site at position 425 had been replaced by an Asp residue. No inhibition was found during the equivalent 3-min interval both in the absence and in the presence of internal PKA catalytic subunits.

of 8-bromo-cAMP. Relatedly, a logical consequence of our results in COS cells would be elevation of intracellular cAMP levels through 5-HT_{1A}Rs, which has been reported before in native cells (e.g., ref. 40), but is in contrast to the general idea of their coupling to the inhibition of adenylate cyclase through G_i proteins (41) and not to stimulating G_s proteins (42). A possible source of this discrepancy could be the recent observation that $G\beta\gamma$ subunits liberated by the stimulation of 5-HT_{1A}Rs may increase the activity of adenylate cyclase II most likely in synergy with $G\alpha_s$ subunits (43–45). It should also be noted that in certain batches of COS cells, transfected only with IRK1, application of 5-HT lead to an infrequent (<5%) and small inhibition of I_{IRK1}, which is indicative of endogenous 5-HT receptors. However, even if these receptors should belong to a G_s-coupled subtype, their effects were negligible compared with the pronounced 5-HT/8-OH-DPAT-induced I_{Kir} inhibition mediated by coexpressed 5-HT_{1A}Rs.

It is generally recognized that the phosphorylation state (and hence the functional state of a target protein) is controlled by the balance of activities of specific protein kinases and protein phosphatases. Consequently, the success of all manipulations performed in this report likely depended on the dephosphorylation of IRK1 before and during the experiment controlled by a still unknown type of protein phosphatase (e.g., ref. 17). As a means to avoid large variabilities in cell responses, future experiments should therefore be directed to more precisely define the level of phosphorylation of the target IRK1 channel.

Because both 5-HT_{1A}Rs (46) and IRK1 inwardly rectifying K^+ channels (47) are abundantly expressed in the mammalian central nervous system, colocalization of both proteins and possibly functional coupling in the same neurons is not unlikely. 5-HT_{1A}R-mediated inhibition of IRK channels would thus render serotonergic synaptic input to result in a depolarization and excitation of the target cells.

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1. Doupnik, C. A., Davidson, N. & Lester, H. A. (1995) *Curr. Opin. Neurobiol.* **5,** 268–277.
2. Clapham, D. E. (1994) *Annu. Rev. Neurosci.* **17,** 441–464.
3. Reuveny, E., Slesinger, P. A., Inglese, J., Morales, J. M., Iñiguez-Lluhi, J. A., Lefkowitz, R. J., Bourne, H. R., Jan, Y. N. & Jan, L. Y. (1994) *Nature (London)* **370,** 143–146.
4. Wickman, K. D., Iñiguez-Lluhi, J. A., Davenport, P. A., Taussig, R., Krapivinsky, G. B., Linder, M. E., Gilman, A. G. & Clapham, D. E. (1994) *Nature (London)* **368,** 255–257.
5. Takao, K., Yoshii, M., Kanda, A., Kokubun, S. & Nukada, T. (1994) *Neuron* **13,** 747–755.
6. Inanobe, A., Morishige, K.-I., Takahashi, N., Ito, K., Yamada, M., Takumi, T., Nishina, H., Takahashi, K., Kanaho, Y., Katada, T. & Kurachi, Y. (1995) *Biochem. Biophys. Res. Commun.* **212,** 1022–1028.
7. Kunkel, M. T. & Peralta, E. G. (1995) *Cell* **83,** 443–449.
8. Pessia, M., Bond, C. T., Kavanaugh, M. P. & Adelman, J. P. (1995) *Neuron* **14,** 1039–1045.
9. North, A. (1989) *Br. J. Pharmacol.* **98,** 13–28.
10. Hille, B. (1992) *Neuron* **9,** 187–195.
11. Lesage, F., Duprat, F., Fink, M., Guillemare, E., Coppola, T., Lazdunski, M. & Hugnot, J. P. (1994) *FEBS Lett.* **353,** 37–42.
12. Duprat, F., Lesage, F., Guillemare, E., Fink, M., Hugnot, J. P., Bigay, J., Lazdunski, M., Romey, G. & Barhanin, J. (1995) *Biochem. Biophys. Res. Commun.* **212,** 657–663.
13. Kofuji, P., Davidson, N. & Lester, H. A. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 6542–6546.
14. Krapivinsky, G., Gordon, E. A., Wickman, K., Velimirovic, B., Krapivinsky, L. & Clapham, D. E. (1995) *Nature (London)* **374,** 135–141.
15. Spauschus, A., Lentes, K.-L., Wischmeyer, E., Dissmann, E., Karschin, C. & Karschin, A. (1996) *J. Neurosci.* **16,** 930–937.
16. Karschin, A., Ho, B. Y., Lebarca, C., Elroy-Stein, O., Moss, B., Davidson, N. & Lester, H. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 5694–5698.
17. Inoue, M. & Imanaga, I. (1995) *J. Gen. Physiol.* **105,** 249–266.
18. Takano, K., Stanfield, P. R., Nakajima, S. & Nakajima, Y. (1995) *Neuron* **14,** 999-1008.
19. Wu, T. & Wang, H.-L. (1995) *Neurosci. Lett.* **184,** 121–124.
20. Wang, W. & Giebisch, G. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 9722–9725.
21. Tabcharani, J. A., Boucher, A., Eng, J. W. L. & Hanrahan, J. W. (1994) *J. Membrane Biol.* **142,** 255–266.
22. McNicholas, C. M., Wang, W., Ho, K., Hebert, S. & Giebisch, G. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 8077–8081.
23. Fakler, B., Brändle, U., Glowatzki, E., Zenner, H. P. & Ruppersberg, J. P. (1994) *Neuron* **13,** 1413–1420.
24. Numann, R., Catterall, W. A. & Scheuer, T. (1991) *Science* **254,** 115–118.
25. Sculptoreanu, A., Scheuer, T. & Catterall, W. A. (1993) *Nature (London)* **364,** 240–243.
26. White, R. E., Lee, A. B., Shcherbatko, A. D., Lincoln, T. M., Schonbrunn, A. & Armstrong, D. L. (1993) *Nature (London)* **361,** 263–266.
27. Moss, S. J., Smart, T. G., Blackstone, C. D. & Huganir, R. L. (1992) *Science* **257,** 661–665.
28. Wang, L.-Y., Taverna, F. A., Huang, X.-P., MacDonald, J. F. & Hampson, D. R. (1993) *Science* **259,** 1173–1175.
29. Raymond, L. A., Blackstone, C. D. & Huganir, R. L. (1993) *Nature (London)* **361,** 637–641.
30. Huganir, R. L., Delcour, A. H., Greengard, P. & Hess, G. P. (1986) *Nature (London)* **321,** 774–776.
31. Wischmeyer, E., Lentes, K.-U. & Karschin, A. (1995) *Pflügers Arch.* **429,** 809–819.
32. Kubo, Y., Baldwin, T. J., Jan, Y. N. & Jan, L. Y. (1993) *Nature (London)* **362,** 127–133.
33. Kobilka, B. K., Frielle, T., Collins, S., Yang-Feng, T., Kobilka, T. S., Francke, U., Lefkowitz, R. J. & Caron, M. G. (1987) *Nature (London)* **329,** 75–79.
34. Hamill, O. P., Marty. A., Neher. E., Sakmann. B. & Sigworth, F. J. (1981) *Pflügers Arch.* **391,** 85–100.
35. McCloskey, M. A. & Cahalan, M. D. (1990) *J. Gen. Physiol.* **95,** 205–227.
36. Hoshi, T., Garber, S. S. & Aldrich, R. W. (1988) *Science* **240,** 1652–1655.
37. Armstrong, C. M. & Bezanilla, F. (1977) *J. Gen. Physiol.* **70,** 567.
38. Hoshi, T., Zagotta, W. & Aldrich, R. W. (1990) *Science* **250,** 533–538.
39. Henry, P., Pearson, W., Melton, R., Makhina, E. N. & Nichols, C. G. (1995) *Biophys. J.* 68, A47 (abstr.).
40. Lucas, J. J., Mellström, B., Colado, M. I. & Naranjo, J. R. (1993) *Neuron* **10,** 599–611.
41. Albert, P. R., Zhou, Q. Y., van Tol, H. H. M., Bunzow, J. R. & Civelli, O. (1990) *J. Biol. Chem.* **265,** 5825–5832.
42. Raymond, J. R., Olsen, C. L. & Gettys, T. W. (1993) *Biochemistry* **32,** 11064–11073.
43. Federman, A. D., Conklin, B. R., Schrader, K. A., Reed, R. R. & Bourne, H. E. (1992) *Nature (London)* **356,** 159–161.
44. Andrade, A. (1993) *Neuron* **10,** 83–88.
45. Uezono, Y., Bradley, J., Min, C., McCarty, N. A., Quick, M., Riordan, J. R., Chavkin, C., Zinn, K., Lester, H. A. & Davidson, N. (1993) *Receptors Channels* **1,** 233–241.
46. Pompeiano, M., Palacios, J. M. & Mengod, G. (1992) *J. Neurosci.* **12,** 440–453.
47. Morishige, K. I., Takahashi, N., Findlay, I., Koyama, H., Zanelli, J. S., Peterson, C., Jenkins, N. A., Copeland, N. G., Mori, N. & Kurachi, Y. (1993) *FEBS Lett.* **336,** 375–380.