Modulation of the Inward Rectifier Potassium Channel IRK1 by the Ras Signaling Pathway*

Received for publication, October 31, 2001, and in revised form, January 22, 2002
Published, JBC Papers in Press, January 22, 2002, DOI 10.1074/jbc.M110466200

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In this study, we investigated the role of Ras and the mitogen-activated protein kinase (MAPK) pathway in the modulation of the inward rectifier potassium channel IRK1. We show that although expression of IRK1 in HEK 293 cells leads to the appearance of a potassium current with strong inward rectifying properties, coexpression of the constitutively active form of Ras (Ras-L61) results in a significant reduction of the mean current density without altering the biophysical properties of the channel. The inhibitory effect of Ras-L61 is not due to a decreased expression of IRK1 since Northern analysis indicates that IRK1 mRNA level is not affected by Ras-L61 co-expression. Moreover, the inhibition can be relieved by treatment with the mitogen-activated protein kinase/ERK kinase (MEK) inhibitor PD98059. Confocal microscopy analysis of cells transfected with the fusion construct green fluorescent protein-IRK1 shows that the channel is mainly localized at the plasma membrane. Coexpression of Ras-L61 delocalizes fluorescence to the cytoplasm, whereas treatment with PD98059 partially restores the membrane localization. In conclusion, our data indicate that the Ras-MAPK pathway modulates IRK1 current by affecting the subcellular localization of the channel. This suggests a role for Ras signaling in regulating the intracellular trafficking of this channel.

Inwardly rectifying potassium channels play a key role in stabilizing resting membrane potential in both excitable and non-excitable cells. IRK1/Kir 2.1 is a member of this family, showing strong inward rectification properties. It is expressed in a wide variety of tissues and cell types including neurons of the central and peripheral nervous system, glia, muscle, and immune system cells. Phosphorylation of IRK1 protein at both serine/threonine and tyrosine sites modulates its activity. The inhibitory effect of Ras-L61 is not due to a decreased expression of IRK1 since Northern analysis indicates that IRK1 mRNA level is not affected by Ras-L61 co-expression. Moreover, the inhibition can be relieved by treatment with the mitogen-activated protein kinase/ERK kinase (MEK) inhibitor PD98059. Confocal microscopy analysis of cells transfected with the fusion construct green fluorescent protein-IRK1 shows that the channel is mainly localized at the plasma membrane. Coexpression of Ras-L61 delocalizes fluorescence to the cytoplasm, whereas treatment with PD98059 partially restores the membrane localization. In conclusion, our data indicate that the Ras-MAPK pathway modulates IRK1 current by affecting the subcellular localization of the channel. This suggests a role for Ras signaling in regulating the intracellular trafficking of this channel.

* This work was supported by Ministero dell’Università e della Ricerca Scientifica e Tecnologica, Cofinanziamento programmi di ricerca di rilevante interesse nazionale (to A. P. and to E. S.), by Consiglio Nazionale delle Ricerche Target Project of Biotechnology (to E. S.), and Contract BIO4-CT98-0297 from the European Community (to E. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/ERK kinase; Ras-L61, constitutively active form of Ras; HEK, human embryonic kidney; GFP, green fluorescent protein; EGFP, enhanced GFP; EGF, epidermal growth factor; EGFR, EGF receptor; NHS, N-hydroxysuccinimide; h, human; m, mouse.

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Vol. 277, No. 14, Issue of April 5, pp. 12158–12163, 2002
Printed in U.S.A.
sated pipette capacity, cell capacity, and series resistance before each voltage clamp protocol. Currents were not leakage-subtracted before acquisition. Experimental protocols, data acquisition, and analysis were done using pCLAMP 7 (Axon Instruments) and Origin (Microcal, Northampton, MA) software. All experiments were performed at room temperature, and current traces were filtered at 5 kHz. Currents were measured with pipettes having 3–4 meq/m for resistance. All transfections were performed overnight, and fixed. Hybridization was performed at 68°C.

3T3 cells plated in 100-mm dishes were transfected with 6 μg of plasmid DNA per plate. Western blots using antibodies against the Myc epitope-tagged protein were performed to check for expression of the transfectants as specified in the figure legends. Western blots using antibodies against the Myc epitope-tagged protein were performed to check for expression of the transfectants as specified in the figure legends. Western Blot Analysis—For Northern analysis, HEK 293 and NIH 3T3 cells plated in 100-mm dishes were transfected with 6 μg of plasmid DNA per plate. Western blots using antibodies against the Myc epitope-tagged protein were performed to check for expression of the transfectants as specified in the figure legends. Western Blot Analysis—For Northern analysis, HEK 293 and NIH 3T3 cells plated in 100-mm dishes were transfected with 6 μg of plasmid DNA per plate. Western blots using antibodies against the Myc epitope-tagged protein were performed to check for expression of the transfectants as specified in the figure legends. Western Blot Analysis—For Northern analysis, HEK 293 and NIH 3T3 cells plated in 100-mm dishes were transfected with 6 μg of plasmid DNA per plate. Western blots using antibodies against the Myc epitope-tagged protein were performed to check for expression of the transfectants as specified in the figure legends. Western Blot Analysis—For Northern analysis, HEK 293 and NIH 3T3 cells plated in 100-mm dishes were transfected with 6 μg of plasmid DNA per plate. Western blots using antibodies against the Myc epitope-tagged protein were performed to check for expression of the transfectants as specified in the figure legends. Western Blot Analysis—For Northern analysis, HEK 293 and NIH 3T3 cells plated in 100-mm dishes were transfected with 6 μg of plasmid DNA per plate. Western blots using antibodies against the Myc epitope-tagged protein were performed to check for expression of the transfectants as specified in the figure legends. Western Blot Analysis—For Northern analysis, HEK 293 and NIH 3T3 cells plated in 100-mm dishes were transfected with 6 μg of plasmid DNA per plate. Western blots using antibodies against the Myc epitope-tagged protein were performed to check for expression of the transfectants as specified in the figure legends. Western Blot Analysis—For Northern analysis, HEK 293 and NIH 3T3 cells plated in 100-mm dishes were transfected with 6 μg of plasmid DNA per plate. Western blots using antibodies against the Myc epitope-tagged protein were performed to check for expression of the transfectants as specified in the figure legends. Western Blot Analysis—For Northern analysis, HEK 293 and NIH 3T3 cells plated in 100-mm dishes were transfected with 6 μg of plasmid DNA per plate. Western blots using antibodies against the Myc epitope-tagged protein were performed to check for expression of the transfectants as specified in the figure legends. Western Blot Analysis—For Northern analysis, HEK 293 and NIH 3T3 cells plated in 100-mm dishes were transfected with 6 μg of plasmid DNA per plate. Western blots using antibodies against the Myc epitope-tagged protein were performed to check for expression of the transfectants as specified in the figure legends. Western Blot Analysis—For Northern analysis, HEK 293 and NIH 3T3 cells plated in 100-mm dishes were transfected with 6 μg of plasmid DNA per plate. Western blots using antibodies against the Myc epitope-tagged protein were performed to check for expression of the transfectants as specified in the figure legends. Western Blot Analysis—For Northern analysis, HEK 293 and NIH 3T3 cells plated in 100-mm dishes were transfected with 6 μg of plasmid DNA per plate. Western blots using antibodies against the Myc epitope-tagged protein were performed to check for expression of the transfectants as specified in the figure legends. Western Blot Analysis—For Northern analysis, HEK 293 and NIH 3T3 cells plated in 100-mm dishes were transfected with 6 μg of plasmid DNA per plate. Western blots using antibodies against the Myc epitope-tagged protein were performed to check for expression of the transfectants as specified in the figure legends. Western Blot Analysis—For Northern analysis, HEK 293 and NIH 3T3 cells plated in 100-mm dishes were transfected with 6 μg of plasmid DNA per plate. Western blots using antibodies against the Myc epitope-tagged protein were performed to check for expression of the transfectants as specified in the figure legends. Western Blot Analysis—For Northern analysis, HEK 293 and NIH 3T3 cells plated in 100-mm dishes were transfected with 6 μg of plasmid DNA per plate. Western blots using antibodies against the Myc epitope-tagged protein were performed to check for expression of the transfectants as specified in the figure legends.
untranslated region of about 3.7 kb. A band with the expected 5.4-kb length was evident although faint in transfected HEK 293 cells, whereas a more intense band of about 1.7 kb was evident in the same lanes. No bands were detected by the mIRK1 probe in HEK 293 cells transfected with the control plasmid. The intensity of the two bands was not modified either by cotransfection with Ras-L61 or by a 3-h pretreatment with PD98059, indicating that neither Ras-L61 nor PD98059 modify the expression of mIRK1.

Interestingly, when the same construct was transfected in NIH 3T3 fibroblasts (of mouse origin), three different transcripts were detected, and the 5.4-kb band was also highly represented. The finding obtained in NIH 3T3 fibroblasts confirms that, as observed in HEK 293 cells, transfected mIRK1 cDNA gives rise, in addition to the 5.4-kb band, also to shorter RNA products possibly due to alternative polyadenylation and/or early termination of the transcription.

Effects of Ras-L61 on the Human IRK1 Channel—To further analyze the modulatory effect of the Ras/MAPK pathway on IRK1 and to study the cellular distribution of the channel, we utilized an EGFP-hIRK1 construct (7) coding for the green fluorescent protein fused in its C-terminal to the human Kir2.1 channel. Electrophysiological measurements (Fig. 4A) allowed the detection of a Ba$^{2+}$-sensitive current, which showed the typical inward rectification properties as already described (13). The kinetics of the whole cell currents exhibited by the human isoform fused to GFP were very similar to those produced by the mouse isoform (compare Figs. 2A and 4A). The differences in shape of the I/V curves are probably due to intrinsic properties of the two channel isoforms; the human channel has a characteristic outward component (13) not present in mIRK1. The shift of the curves toward more positive potentials (compare Figs. 2B and 4B) is caused instead by the reduction of [K$^+$] in the extracellular solution (see “Experimental Procedures”).
TABLE I

| Transfection/treatment | Mean $I_{\text{mem}}$ (pA/pF) | S.E. | n  |
|------------------------|-----------------------------|------|----|
| EGFP-hIRK1             | $-107$                      | $\pm 11.736$ |    |
| EGFP-hIRK1/PD98059     | $-115$                      | $\pm 11.535$ |    |
| EGFP-hIRK1 + Ras-L61   | $-53.2^a$                   | $\pm 8.139$  |    |
| EGFP-hIRK1 + Ras-L61/PD98059 | $-87$                  | $\pm 13.235$ |    |

$^a$ the mean that is statistically significant from the others ($p < 0.01$).

As already reported for mIRK1, also for human IRK1 construct, cotransfection with Ras-L61 largely reduced, by 50%, the mean current density (see Table I). A 2-h pretreatment with PD98059 reverted the current density to values close to that observed in cells transfected with EGFP-hIRK1 alone. On the other hand, PD98059 had no significant effect on EGFP-hIRK1-transfected cells. To investigate whether Ras modifies the level of IRK1 protein, equal amounts of protein from cells transfected with EGFP-hIRK1, with or without Ras-L61 and treated or not with PD98059, were analyzed by Western blotting with anti-hIRK1 antibodies.

Fig. 5 shows that similar levels of EGFP-hIRK1 protein were present in all conditions. As an internal standard, the expression of the endogenous protein ERK2 was analyzed, confirming comparable loading in all lanes. Immunoblot analysis using anti-Myc antibodies detected a protein with the expected 21 kDa molecular size only in cells transfected with the Ras-L61 construct. The activation state of ERKs was also investigated: antibodies against the phosphorylated form of ERK1 and ERK2 showed that Ras-L61 causes a strong activation of MAPKs and that treatment with the MEK inhibitor completely blocks this activation.

These data clearly indicate that RasL61, which activates ERKs, does not alter the expression of the EGFP-hIRK1 protein. Moreover, a 3-h pretreatment with PD98059 has no effect on the level of EGFP-hIRK1.

Effects of Ras-L61 on the Cellular Localization of EGFP-hIRK1—We then analyzed the subcellular localization of the GFP construct by confocal microscopy. In cells transfected with EGFP-hIRK1 alone, fluorescence was mainly localized at the plasma membrane with rare cytoplasmic spots probably related to a Golgi complex localization (Fig. 6A).

Conversely, cotransfection with Ras-L61 led to a redistribution of the fluorescence signal to the whole cytoplasm (Fig. 6B). In addition, PD98059 reverted this effect since the fluorescence signal returned mainly at the level of the plasma membrane (Fig. 6D). PD98059 treatment of cells transfected with the channel alone (Fig. 6C) did not lead to any change in the fluorescence distribution pattern.

To further verify the specific effect of the Ras-MAPK pathway on IRK1 trafficking, cells were transfected with a construct coding for the EGFR receptor (EGFR) fused in C-terminal to GFP (8) that is able to undergo ligand-induced endocytosis (14). As shown in Fig. 6, E and F, coexpression of Ras-L61 did not alter the localization of the fluorescence signal exhibited by EGFR-GFP, which remained mainly localized at the plasma membrane level.

To confirm the data reported above, we compared the level of surface-associated IRK1 channels and endogenously expressed EGFR under different conditions. Cells were transfected with hIRK1 with or without Ras-L61, and 48 h later, cell surface proteins were biotinylated with sulfo-NHS-biotin. Biotinylated proteins were affinity-purified with immobilized streptavidin and analyzed. Immunoblotting with anti-hIRK1 antibodies shows that the amount of biotinylated (cell surface-associated) hIRK1 is higher in cells transfected with EGFP-hIRK1 alone than in cells cotransfected with Ras-L61. Conversely, no reduction of cell surface-associated EGFR could be detected in the presence of Ras. An equivalent amount of the two proteins was detected in total cell extracts (Fig. 7). These data further support a selective role for the Ras-MAPK pathway on the IRK1 channel without generally affecting membrane trafficking.
Modulation of IRK1 by the Ras pathway

Interestingly, our results show that activation of MAPK does not affect endocytosis of the EGFR. We have not analyzed whether MAPK activity alters ligand-induced EGF receptor internalization.

**DISCUSSION**

We investigated the role of the Ras-MAPK pathway in regulating IRK1/Kir 2.1 channel coexpressing in HEK 293 cells Kir 2.1 (either the mouse isoform or the human one fused to GFP) and constitutively active Ras. The expression of the active form of Ras (Ras-L61) reduces IRK1 current, identified as a Ba"-sensitive potassium component, without affecting the kinetic properties of the channels. Inhibition of the MAPK cascade by PD98059 restores the level of IRK1 current, suggesting that Ras modulates the current through the downstream pathway.

Our data rule out an inhibitory effect of Ras-L61 on IRK1 at the transcriptional level since mIRK1 mRNA level is not affected by Ras-L61 expression. Even though a 5.4-kb cDNA has been used and endogenous IRK1 has been reported to give rise to a 5.4-kb message (6), we found in HEK 293 cells a predominant 1.7-kb message and a fainter one at 5.4 kb. Moreover, when the same plasmid was transfected in mouse NIH 3T3 fibroblasts, three different transcripts were detected, one of which is 5.4 kb. These results suggest that, depending on the cell type, alternative polyadenylation and/or early termination of the transcription can occur.

To study the effect of Ras-L61 on the level of channel expression and its cellular localization, we used the EGFP-hIRK1 fusion protein. We could demonstrate that neither expression of Ras-L61 nor PD98059 treatment alters the level of the EGFP-hIRK1 protein.

Confocal microscopy shows that although EGFP-hIRK1, when expressed alone, is mainly localized at the cell surface, in the presence of activated Ras, it becomes diffusely distributed in the cytoplasm. This situation can be partially reversed by a 2-h treatment with PD98059. This inhibitor acts mainly on channel redistribution rather than on the de novo synthesis of IRK1 since treatment with cycloheximide together with PD98059 did not significantly alter the fluorescence signal at the plasma membrane (data not shown). These findings, together with the reduced level of biotinylated IRK1 protein found in Ras-L61-transfected cells, indicate that in HEK 293 cells, the Ras-MAPK pathway reduces the channel molecules present on the plasma membrane.

The redistribution of IRK1 does not seem to be due to a general effect of active Ras on membrane trafficking; in fact, we did not detect any significant reduction of an endogenous surface-associated protein (EGFR). Moreover, cotransfection of Ras with EGFR-EGFP (a chimeric construct in which the GFP moiety does not affect the EGFR functions (10)) did not alter its fluorescence distribution.

Our experimental protocols do not allow a determination of how much of the membrane is internalized since capacitance measurements have been done in a steady state condition. However, the determined mean cell capacitance did not show significant differences in the different conditions (data not shown). Thus, the Ras-MAPK pathway appears to act on the trafficking of IRK1 molecules and does not have a generalized effect on membrane proteins.

One of the mechanisms employed by cells to regulate the activity of ion channels is to modulate their localization (15–17). In fact, cell surface expression is the result of the balance of insertion of de novo synthesized proteins, of internalization through endocytosis, and of recycling (18).

Our results suggest that a MAPK-dependent phosphorylation event rather than protein synthesis is required to reduce the level of cell surface-associated IRK1. We also show that a 2-h treatment with PD 98059 (which completely blocks MAPK activation) is sufficient to inhibit RasL61-mediated effects. This suggests that MAPK activity induces a rapid cycling of the IRK1 channel, although we cannot define whether phosphorylation accelerates internalization or whether it reduces the rate of the surface expression of IRK1 channels. In addition, we do not know whether IRK1 itself is the substrate of this phosphorylation or whether other proteins are involved in this process. However, it is worthwhile to recall that a consensus sequence for MAPKs is present in the C-terminal region of IRK1 that might be important for channel trafficking. Mechanisms for endo- and exocytosis of this channel are yet poorly understood. This point has been studied, for instance, by acute exposure to either extracellular signaling molecules or specific tyrosine phosphatase inhibitors (3). To this regard, Tong et al. (5) have shown that IRK1 endocytosis requires Tyr-242, part of a motif recognized by clathrin adaptor proteins, suggesting that internalization is mediated by clathrin. In addition, recent findings have revealed that forward trafficking (19) of the channel, which is dependent on a C-terminal sequence of IRK1, also contributes to the regulation of the number of surface-associated channel. Further studies are required to fully elucidate the mechanisms of IRK1 channel trafficking.

Modulation of the availability of cell surface ion channels and channel trafficking may be particularly suitable to shape the electrophysiological response (7) and may represent one of the events that control the basal electrical activity of the cell, an issue that seems important for the cell fate, proliferative versus differentiative (20, 21). We have previously shown that expression of CDC25M/Ras-GRF1 in the SK-N-BE neuroblastoma, induced to differentiate with retinoic acid, led to a significant increase in the number of cells showing the IRK1-like current (12). The opposite modulation reported here, exerted by the Ras-MAPK pathway on the IRK1 channel, may depend on the cellular system used. The two model lines may differ in the inventory of expressed protein or in the cross-talk of signaling pathways. The occurrence of opposite effects in different systems under the same stimulus is not a new issue. For example, protein kinase A can exert a positive or negative effect on Kir2.1 if the channel is expressed in Xenopus oocytes or in COS7 cells, respectively (1, 22).

Ras and MAPK pathway play a pivotal role in cell prolifer-
ation, survival, and differentiation, acting on many different target proteins. Modulation of calcium, potassium, and sodium channels by the Ras signaling pathway has already been demonstrated (23–25). Moreover, it has been shown that in oligodendrocytes, inhibition of the inward rectifying potassium current, induced by ceramide, is mediated by a Ras- and Raf-1-dependent pathway (26). With our present data, we suggest that the Ras-MAPK cascade modulates the inward rectifying potassium channel by reducing the cell surface channel availability. This may be relevant for the ion channel function in the context of its contribution to cell growth activity in non-excitatory cell or to the electrical activity in excitable cells.

Acknowledgments—We thank Dr. P. Camelliti for valuable technical help and Dr. L. Y. Jan (Howard Hughes Medical Institute, San Francisco, California), Dr D. C. Johns (Johns Hopkins University, Baltimore, Maryland), and Dr. T. M. Jovin (Max Planck Institute for Biophysical Chemistry, Gottingen, Germany) for the gift of the plasmids.

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