Biochemical and Biophysical Research Communications **272,** 316–319 (2000)
doi:10.1006/bbrc.2000.2780, available online at http://www.idealibrary.com on IDEAL®

Direct Photoaffinity Labeling of Kir6.2 by [γ-^{32}P]ATP-[γ]4-Azidoanilide

Kouichi Tanabe,* Stephen J. Tucker,* Frances M. Ashcroft,† Peter Proks,† Noriyuki Kioka,* Teruo Amachi,* and Kazumitsu Ueda*,[1]

**Laboratory of Biochemistry, Division of Applied Life Sciences, Kyoto University Graduate School of Agriculture, Kyoto 606-8502, Japan; and †University Laboratory of Physiology, Oxford OX1 3PT, United Kingdom*

Received April 28, 2000

ATP-sensitive potassium (K_{ATP}) channels are under complex regulation by intracellular ATP and ADP. The potentiatory effect of MgADP is conferred by the sulfonylurea receptor subunit of the channel, SUR, whereas the inhibitory effect of ATP appears to be mediated via the pore-forming subunit, Kir6.2. We have previously reported that Kir6.2 can be directly labeled by 8-azido-[γ-^{32}P]ATP. However, the binding affinity of 8-azido-ATP to Kir6.2 was low probably due to modification at 8′ position of adenine. Here we demonstrate that Kir6.2 can be directly photoaffinity labeled with higher affinity by [γ-^{32}P]ATP-[γ]4-azidoanilide ([γ-^{32}P]ATP-AA), containing an unmodified adenine ring. Photoaffinity labeling of Kir6.2 by [γ-^{32}P]ATP-AA is not affected by the presence of Mg^{2+}, consistent with Mg^{2+}-independent ATP inhibition of K_{ATP} channels. Interestingly, SUR1, which can be strongly and specifically photoaffinity labeled by 8-azido-ATP, was not photoaffinity labeled by ATP-AA. These results identify key differences in the structure of the nucleotide binding sites on SUR1 and Kir6.2.

Key Words: **ATP-sensitive potassium channel; Kir6.2; photoaffinity labeling.**

ATP-sensitive potassium (K_{ATP}) channels play important roles in many tissues, by linking the metabolic status of the cell to its membrane potential (1, 2). In pancreatic β-cells, K_{ATP} channels are critical for the regulation of glucose-induced insulin secretion (3) and have recently been shown to be an octameric complex of two subunits, which coassemble with a 4:4 stoichiometry (4–8). The pore-forming subunit, Kir6.2, is a member of the inwardly rectifying K^+ channel family (9, 10), while the other subunit, the sulfonylurea receptor (SUR1), is a member of the ATP-binding cassette (ABC) transporter superfamily (11, 12). Although wild-type K_{ATP} channels require both types of subunit (Kir6.2 and SUR) for functional activity, a mutant form of Kir6.2 with a C-terminal truncation of 26 or 36 amino acids (Kir6.2ΔC) is capable of independent expression (13).

We have demonstrated that Kir6.2 can be directly photoaffinity labeled by 8-azido-[γ-^{32}P]ATP, and that mutations in the NH_2-terminus (R50G) and the COOH-terminus (K185Q), which significantly reduce ATP inhibition of Kir6.2ΔC currents (14), reduce photoaffinity labeling of Kir6.2 (15). However, because no saturation of photoaffinity labeling was observed even at the highest concentration tested (200 μM) (15), we were not able to analyze 8-azido-ATP binding to Kir6.2 in detail, and it remains unclear whether the effect of these mutations reflects a direct interaction of these residues with ATP, or whether their effects on ATP binding are mediated allosterically.

The inhibitory nucleotide binding site on Kir6.2 is highly selective for the purine moiety of ATP (14). It therefore seems likely that modification of the adenine ring in 8-azido ATP may be responsible for its reduced affinity. In this study, we show that Kir6.2 can be directly photoaffinity labeled by [γ-^{32}P]ATP-[γ]4-azidoanilide ([γ-^{32}P]ATP-AA), which is modified at the γ-phosphate and contains an unmodified adenine ring, with higher affinity than 8-azido-[γ-^{32}P]ATP. Interestingly, SUR1, which can be strongly and specifically photoaffinity labeled by 8-azido-ATP (17–19), was not photoaffinity labeled by [γ-^{32}P]ATP-AA.

MATERIALS AND METHODS

Transfection and preparation of membranes. COS-7 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum in a humidified atmosphere of 95% air, 5% CO_2 at 37°C. COS-7 cells were transfected with expression vectors encoding full-length wild-type Kir6.2, tagged with the Flag epitope at the NH_2-terminus and with a hexahistidine tag at the COOH-terminus (Flag-Kir6.2) using LipofectAMINEplus (Life Technologies) according to

[1] To whom correspondence should be addressed. Fax: 81 (JAPAN)-75-753-6104. E-mail: uedak@kais.kyoto-u.ac.jp.

0006-291X/00 $35.00

the manufacturer's directions. Rat Kir4.1 was tagged with the Flag epitope at the COOH-terminus (Kir4.1-Flag). Addition of these epitopes did not significantly affect the functional properties of the channel (data not shown). Rat SUR1 was transfected as described above. Two days after transfection, cells were broken in isotonic buffer by nitrogen cavitation and crude cellular membranes were prepared as described previously (16) without sucrose gradient centrifugation. These membranes probably include all the cellular membranes except nuclear envelope. Immunoblotting was carried out with an anti-Flag M2 monoclonal antibody (Eastman Kodak).

Photoaffinity labeling of Flag-Kir6.2 with [γ][32P]ATP-AA. [γ-^{32}P]-ATP-AA (300–500 GBq/mmol) was purchased from ALT. Membrane proteins (20 μg) were incubated with 50 μM [γ-^{32}P]ATP-AA, 2 mM ouabain, 0.1 mM EGTA, 4 mM $MgSO_4$, and 40 mM Tris-Cl (pH 7.5) in a total volume of 6 μl for 10 min on ice. After UV irradiation (at 254 nm, 4.4–8.2 mW/cm^2) for 15 sec, 500 μl of TE buffer (40 mM Tris-HCl (pH 7.5), 0.1 mM EGTA) was added to the mixture, and free [γ-^{32}P]ATP-AA was removed by centrifugation (15,000*g*, 10 min, 4°C). The pellet was solubilized with 100 μl RIPA buffer (20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 0.15 M NaCl, 10 μg/ml leupeptin, 100 μg/ml pAPMSF). The lysate was kept on ice for 15 min, and centrifuged to remove insoluble material. Flag-Kir6.2 was immunoprecipitated from the supernatant with the anti-Flag M2 antibody. Samples were electrophoresed on a 10% SDS-polyacrylamide gel and autoradiographed. Bound [γ-^{32}P]ATP-AA to Kir6.2 was measured by scanning with a radioimaging analyzer (BAS2000, Fuji Photo Film Co.). Experiments were carried out at least in duplicate.

Photoaffinity labeling of SUR1. Membrane proteins (20 μg) were incubated with 5 μM 8-azido-[γ-^{32}P]ATP, 2 mM ouabain, 0.1 mM EGTA, and 40 mM Tris-Cl (pH 7.5) in a total volume of 6 μl for 10 min at 37°C. The reaction was stopped by the addition of 400 μl of ice-cold Tris-EGTA buffer (40 mM Tris-Cl (pH 7.5), 0.1 mM EGTA), and free ATP was removed after centrifugation (15,000*g*, 10 min, 4°C). Pellets were washed in the same buffer, resuspended in 8 μl of Tris-EGTA buffer, and irradiated for 5 min (at 254 nm, 5.5 mW/cm^2) on ice. Samples were electrophoresed on a 7% SDS–polyacrylamide gel, and autoradiographed.

Electrophysiology. Macroscopic currents were recorded from giant inside-out patches excised from *Xenopus* oocytes expressing Kir6.2ΔC36, as previously described (14). Currents were recorded at a holding potential of 0 mV in response to repetitive voltage ramps from −110 mV to +100 mV, at 20–24°C. The pipette solution contained (mM): 140 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, 10 HEPES (pH 7.4 with KOH) and the internal (bath) solution contained (mM): 110 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 30 KOH, 10 EGTA, 10 HEPES (pH 7.2 with KOH) and ATP-AA as indicated. Dose-response relationships for ATP-AA were measured by alternating the control solution with a test ATP-AA solution and the extent of inhibition was expressed as a fraction of the mean of the value obtained in the control solution before and after ATP-AA application. Dose-response curves were fit to the Hill equation $G/Gc = 1/(1 + ([ATP\text{-}AA]/IC_{50})h)$, where [ATP-AA] is the ATP-AA concentration, IC_{50} is the ATP-AA concentration at which inhibition is half maximal and *h* is the slope factor (Hill coefficient).

RESULTS

Photoaffinity labeling of Kir6.2 by ATP-AA. We investigated the direct interaction of Kir6.2 with ATP, using the photoaffinity ATP analog, [γ-^{32}P]ATP-AA. Flag-Kir6.2 and Kir4.1-Flag were transiently expressed in COS-7 cells and expression levels were monitored by immunoblot analysis of membrane fraction preparations (Fig. 1A). Membranes were incubated with 10 μM and 50 μM of [γ-^{32}P]ATP-AA on ice and irradiated with UV light to analyze ATP binding. Flag-Kir6.2 and Kir4.1-Flag were immunoprecipitated with an anti-Flag M2 antibody and subjected to electrophoresis. The autoradiogram in Fig. 1B shows an approximately 43-kDa photoaffinity-labeled protein to be immunoprecipitated from Flag-Kir6.2 transfected cells (lanes 3 and 6). The molecular weight of this photoaffinity-labeled membrane protein is identical to that of Flag-Kir6.2, identified by Western blotting (Fig. 1A). Only faintly labeled bands were observed by labelling with membrane proteins from Kir4.1-Flag transfected cells (Fig. 1B, lane 5).

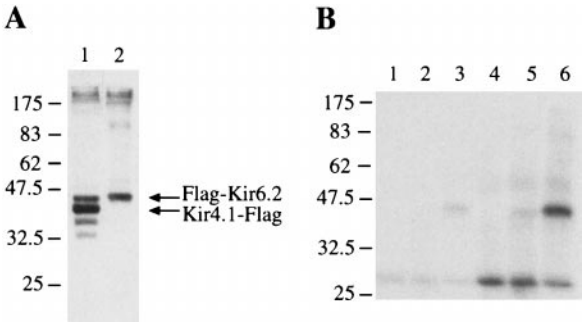

FIG. 1. Photoaffinity labeling of Kir6.2 with [γ-^{32}P]ATP-AA. (A) Immunoblot analysis. Membrane proteins (5 μg) from COS-7 cells expressing Kir4.1-Flag (lane 1) and Flag-Kir6.2 (lane 2) were separated on a 10% SDS–polyacrylamide gel and detected by immunoblotting with anti-Flag M2 monoclonal antibody. (B) Photoaffinity labeling. Membrane proteins (20 μg) from untransfected Cos-7 cells (lanes 1, 4) or Cos-7 cells expressing Kir4.1-Flag (lanes 2, 5) and Flag-Kir6.2 (lanes 3, 6) were photoaffinity-labeled with 10 μM (lanes 1–3) or 50 μM (lanes 4–6) [γ-^{32}P]ATP-AA. Kir4.1-Flag and Flag-Kir6.2 were immunoprecipitated with antibody M2 after solubilization, and analyzed as described under Materials and Methods.

Inhibition of Kir6.2ΔC36 currents by ATP-AA. Figure 2 shows that ATP-AA blocks Kir6.2ΔC36 currents with about the same potency as ATP. Half-maximal inhibition was found with 127 ± 7 μM ATP-AA (n = 4) compared with ~100 μM for ATP (13, 14). The Hill coefficient was unchanged at 0.97 ± 0.05.

Concentration dependence. Membranes from Flag-Kir6.2 transfected cells were incubated with different concentrations (10 to 100 μM) of [γ-^{32}P]ATP-AA in the absence or presence of Mg^{2+} and photoaffinity-labeled (Fig. 3). Photoaffinity-labeling increased with increasing concentrations of [γ-^{32}P]ATP-AA and saturated at 50 μM either in the absence or presence of Mg^{2+}. Mg^{2+} did not affect photoaffinity labeling of Flag-Kir6.2 by [γ-^{32}P]ATP-AA.

Competition of photoaffinity labeling by ATP. In order to determine whether photoaffinity-labeling of Flag-Kir6.2 was specific, competition by ATP was examined (Fig. 4). Membranes were pre-incubated with 0, 2, 5, and 7.5 mM ATP on ice for 10 min, and then 50 μM [γ-^{32}P]ATP-AA was then added. Photoaffinity-labeling of Flag-Kir6.2 was reduced as the concentra-

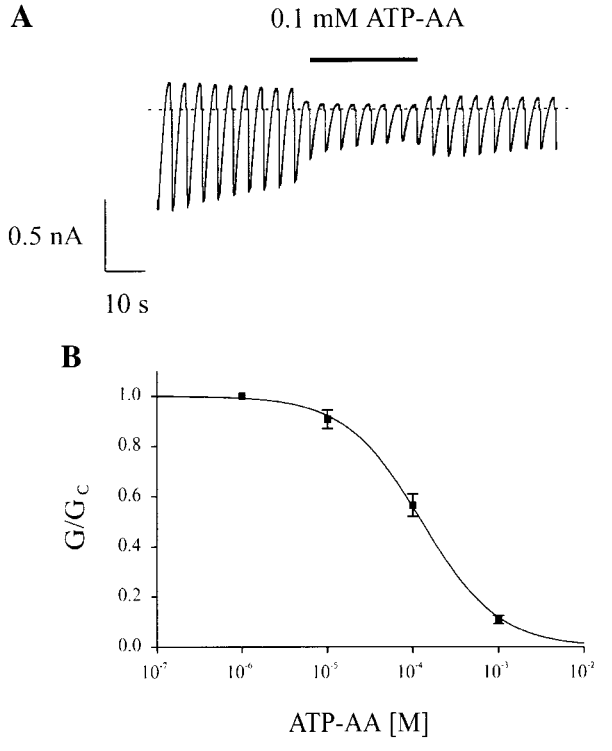

FIG. 2. Inhibition of Kir6.2ΔC36 currents by ATP-AA. (A) Macroscopic currents recorded from a giant inside-out patch on an oocyte injected with mRNA encoding Kir6.2ΔC36. Currents were elicited in response to a series of voltage ramps from −110 to +100 mV. ATP-AA (100 μM) was added to the internal solution as indicated by the bar. (B) Mean ATP-AA dose-response relationship for Kir6.2ΔC36 currents (n = 4). Test solutions were alternated with control solutions and the slope conductance (G) is expressed as a fraction of the mean (Gc) of that obtained in control solution before and after exposure to ATP. The solid lines is the best fit of the data to the Hill equation (Eq. 1) using the mean values for IC_{50} and h given in the text.

tion of ATP was increased. Quantitation by radioimaging analysis revealed that photo-labeling of Flag-Kir6.2 was reduced by 50% with approximately 1.7 mM ATP.

Photoaffinity labeling of SUR1 by 8-azido-ATP and ATP-AA. We have demonstrated that SUR1 can be specifically photoaffinity-labeled with high affinity by 8-azido-[^{32}P]ATP (17–19) in crude membrane preparations. Because ATP-AA can be used to photoaffinity label Kir6.2, we examined if [γ-^{32}P]ATP-AA binds to SUR1. Membranes prepared from cells expressing Flag-Kir6.2 and SUR1 were incubated with 8-azido-[γ-^{32}P]ATP or [γ-^{32}P]ATP-AA at 37°C for 5 min, because high affinity 8-azido ATP binding of SUR1 is temperature-dependent, and is more efficient at 37°C than at 0°C (17). Proteins were UV-irradiated after free nucleotides were removed by centrifugation (Fig. 5). SUR1 was strongly and specifically photoaffinity-labeled with 5 μM 8-azido-[γ-^{32}P]ATP (lane 1). By contrast, SUR1 was not labeled with 10 μM [γ-^{32}P]ATP-AA (lane 2).

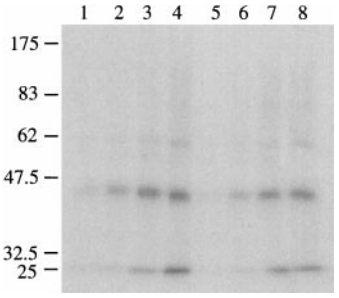

FIG. 3. Concentration and Mg^{2+} dependence of photoaffinity labeling of Kir6.2 with [γ-^{32}P]ATP-AA. Membrane proteins (20 μg) from COS-7 cells expressing Flag-Kir6.2 were photoaffinity-labeled with 10 μM (lanes 1, 5), 20 μM (lanes 2, 6), 50 μM (lanes 3, 7), or 100 μM (lanes 4, 8) [γ-^{32}P]ATP-AA in the absence (lanes 1–4) or presence (lanes 5–8) of Mg^{2+}.

Even with 50 μM [γ-^{32}P]ATP-AA, no specific labeling of SUR1 was observed (lane 3).

DISCUSSION

Electrophysiological studies with Kir6.2ΔC, which can produce functional K_{ATP} channel in the absence of SUR, have suggested that the site at which ATP mediates channel inhibition resides on Kir6.2 (13) and that both the β-phosphate and moieties within the adenine ring are critical for K_{ATP} channel inhibition by ATP (14). We examined photoaffinity analogs [γ-^{32}P]ATP-AA and [γ-^{32}P]ATP-[γ]benzophenone (data not shown), which have an intact adenine ring, for their ability to label Kir6.2, and demonstrated here that Kir6.2 can be specifically labeled by [γ-^{32}P]ATP-

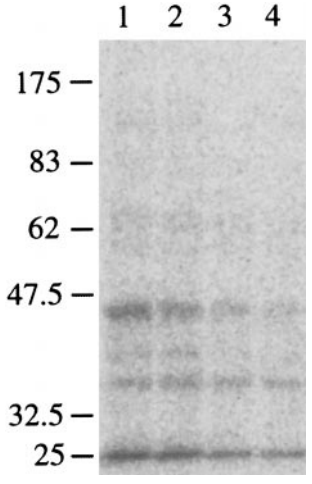

FIG. 4. Inhibition of [γ-^{32}P]ATP-AA photoaffinity labeling of Kir6.2 by cold ATP. Membrane proteins (20 μg) from COS-7 cells expressing Flag-Kir6.2 were photoaffinity-labeled with 50 μM [γ-^{32}P]ATP-AA in the presence of 0 (lane 1), 2 (lane 2), 5 (lane 3), or 7.5 mM (lane 4) ATP. Flag-Kir6.2 was immunoprecipitated with antibody M2 after solubilization, and analyzed as described under Materials and Methods.

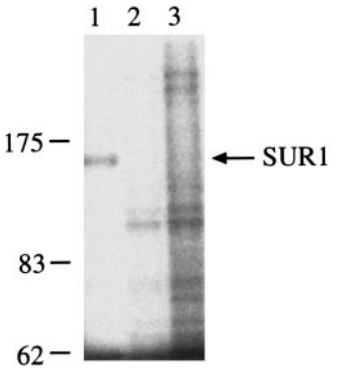

FIG. 5. Photoaffinity labeling of SUR1 with 8-azido-[γ-^{32}P]ATP and [γ-^{32}P]ATP-AA. Membrane proteins (20 μg) from COS-7 cells expressing SUR1 and Kir6.2 were incubated with 5 μM 8-azido-[γ-^{32}P]ATP (lane 1), 10 μM (lane 2) or 50 μM (lane 3) [γ-^{32}P]ATP-AA. Proteins were UV irradiated after free ATP was removed, and directly analyzed by electrophoresis as described under Materials and Methods.

AA and with a little less efficiency by [γ-^{32}P]ATP-[γ]benzophenone (data not shown). The efficiency of labeling affinity of Kir6.2 by ATP-AA is, as expected, higher than that of 8-azido-ATP, because photoaffinity labeling of Kir6.2 [γ-^{32}P]ATP-AA is saturated at 50 μM, while that by 8-azido [γ-^{32}P]ATP is not saturated even at 200 μM (15).

Photoaffinity-labeling of Kir6.2 by [γ-^{32}P]ATP-AA is not affected by the presence of Mg^{2+}. This is consistent with electrophysiological studies showing that both Kir6.2 (13) and native β-cell K_{ATP} channel (20) activity is inhibited by ATP in a Mg^{2+}-independent manner. The ability of ATP-AA to bind to, and block, Kir6.2 is in agreement with studies showing that Ap4A (14) inhibits K_{ATP} channels. It appears that the γ phosphate is not essential phosphate for nucleotide interaction with Kir6.2, a view that is confirmed by the fact that ADP blocks Kir6.2ΔC almost as effectively as ATP (14).

It is noteworthy that the concentration of ATP-AA required to produce half-maximal inhibition of K_{ATP} channel activity (130 μM) was similar to that found for ATP (100 μM), whereas the ATP concentration required to produce half-maximal displacement of [γ-^{32}P]ATP-AA binding to Kir6.2 was significantly higher (K_i = 1.7 mM). There are a number of possible reasons for this difference, but it seems most likely to relate to the fact that photolabeling with [γ-^{32}P]ATP-AA is irreversible and in order to visualize labeled proteins significant proportion have to be covalently crosslinked. Thus cold ATP does not act as a true competitive ligand.

SUR1 can be strongly and specifically photoaffinity-labeled by 8-azido-[α or γ-^{32}P]ATP but not by [γ-^{32}P]-ATP-AA. It is possible that the azidoanilide moiety at the γ-phosphate may prevent ATP-AA from accessing or stably binding to NBF1 of SUR1. The ability of ATP-AA to label Kir6.2 but not with SUR1 provides further evidence that the structure of the inhibitory binding site on Kir6.2 is very different from that of the stimulatory site located on the NBDs of SUR1. Importantly, the findings we present in this study also suggest that ATP-AA may be used to analyze ATP binding to Kir6.2 and to discriminate the effect of ATP binding to Kir6.2 from that to SUR1.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas "ABC proteins" (No. 10217205) from the Ministry of Education, Science, Sports, and Culture of Japan, and in the UK by the Wellcome Trust and the Royal Society.

REFERENCES

1. Ashcroft, S. J., and Ashcroft, F. M. (1990) *Cell Signal* **2**(3), 197–214.
2. Nichols, C. G., and Lederer, W. J. (1991) *Am. J. Physiol.* **261**(6 Pt 2), H1675–H1686.
3. Ashcroft, F. M., and Gribble, F. M. (1998) *Trends Neurosci.* **21,** 288–294.
4. Inagaki, N., Gonoi, T., and Seino, S. (1997) *FEBS Lett.* **409**(2), 232–236.
5. Clement, J. P., IV, Kunjilwar, K., Gonzalez, G., Schwanstecher, M., Panten, U., Aguliar-Bryan, L., and Bryan, J. (1997) *Neuron* **18,** 827–838.
6. Shyng, S.-L., and Nichols, C. G. (1997) *J. Gen. Physiol.* **110,** 655–664.
7. Aguilar-Bryan, L., Clement, J. P., IV, Gonzalez, G., Kunjilwar, K., Babenko, A., and Bryan, J. (1998) *Physiol. Rev.* **78,** 227–245.
8. Babenko, A. P., Aguilar-Bryan, L., and Bryan, J. (1998) *Annu. Rev. Physiol.* **60,** 667–687.
9. Sakura, H., Ämmäla, C., Smith, P. A., Gribble, F. M., and Ashcroft, F. M. (1995) *FEBS Lett.* **377,** 338–344.
10. Inagaki, N., Gonoi, T., Clement, J. P., IV, Namba, N., Inazawa, J., Gonzalez, G., Aguilar-Bryan, L., Seino, S., and Bryan, J. (1995) *Science* **270,** 1166–1169.
11. Inagaki, N., Gonoi, T., Clement, J. P., IV, Wang, C.-Z., Aguilar-Bryan, L., Bryan, J., and Seino, S. (1996) *Neuron* **16,** 1011–1017.
12. Aguilar-Bryan, L., Nichols, C. G., Wechsler, S. W., Clement, J. P. T., Boyd, A. E. R., Gonzalez, G., Herrera-Sosa, H., Nguy, K., Bryan, J., and Nelson, D. A. (1995) *Science* **268**(5209), 423–426.
13. Tucker, S. J., Gribble, F. M., Zhao, C., Trapp, S., and Ashcroft, F. M. (1997) *Nature* **387,** 179–183.
14. Tucker, S. J., Gribble, F. M., Proks, P., Trapp, S., Ryder, T. J., Haug, T., Reimann, F., and Ashcroft, F. M. (1998) *EMBO J.* **17,** 3290–3296.
15. Tanabe, K., Tucker, S. J., Matsuo, M., Proks, P., Ashcroft, F. M., Seino, S., Amachi, T., and Ueda, K. (1999) *J. Biol. Chem.* **274,** 3931–3933.
16. Ueda, K., Okamura, N., Hirai, M., Tanigawara, Y., Saeki, T., Kioka, N., Komano, T., and Hori, R. (1992) *J. Biol. Chem.* **267,** 24248–24252.
17. Ueda, K., Inagaki, N., and Seino, S. (1997) *J. Biol. Chem.* **272,** 22983–22986.
18. Matsuo, M., Kioka, N., Amachi, T., and Ueda, K. (1999) *J. Biol. Chem.* **274,** 37479–37482.
19. Matsuo, M., Tucker, S. J., Ashcroft, F. M., Amachi, T., and Ueda, K. (1999) *FEBS Lett.* **458,** 292–294.
20. Kakei, M., Kelly, R. P., Ashcroft, S. J., and Ashcroft, F. M. (1986) *FEBS Lett.* **208**(1), 63–66.