Localization of ATP-sensitive K⁺ channel subunits in rat pituitary gland

Ming Zhou¹, Ryoji Suzuki¹, Akimitsu Ishizawa¹, Osamu Tanaka², Yukiko Yasuoka³,⁴, Yoshinori Kanatsu¹, Katsumasa Kawahara³,⁴, Hiroshi Abe¹

¹Department of Anatomy, Akita University Graduate School of Medicine, 1-1-1 Hondo, Akita, 010-8543, Japan, ²Department of Anatomy, Tokai University School of Medicine, 143 Shimokasuya, Isehara, 259-1193, Japan, ³Department of Physiology, Kitasato University Graduate School of Medical Sciences, 1-15-1 Kitasato, Minami-ku, Sagamihara 252-0374, Japan, ⁴Department of Cellular & Molecular Physiology, Kitasato University Graduate School of Medical Sciences, 1-15-1 Kitasato, Minami-ku, Sagamihara 252-0374, Japan.

Summary. ATP-sensitive K⁺ (KATP) channel subunits Kir6.1, Kir6.2, SUR1, SUR2A, and SUR2B in the rat pituitary gland were first investigated by RT-PCR assay and immunohistochemical staining. The results of RT-PCR analysis showed that the rat pituitary gland expressed the five KATP channel subunits mentioned above. Immunohistochemical staining showed that these KATP channel subunits were widely localized in the anterior lobe, intermediate lobe, and posterior lobe at different intensities. Immunofluorescence double and triple staining showed that these KATP channel subunits colocalized with cells containing adrenocorticotropic hormone (ACTH) in the anterior lobe of the pituitary gland. Interestingly, neither Kir6.1 nor Kir6.2 colocalized with cells containing prolactin (PRL), follicular stimulating hormone (FSH), and growth hormone (GH). These results suggest that ACTH cells contain four types of KATP channels: Kir6.1/SUR2A, Kir6.2/SUR2A, Kir6.1/SUR2B, and Kir6.2/SUR2B. KATP channels may play some important roles in ACTH cells in the pituitary gland. The different compositions of KATP channel subunits in corticotrophs but not in the PRL, FSH, and GH cells might be due to the different metabolic situations of these cells.

Key words: ATP-sensitive K⁺ channel; Kir6.x; SUR2; immunohistochemistry; pituitary gland; rat

Introduction

ATP-sensitive K⁺ (KATP) channels, discovered originally in cardiac muscle (Noma 1983), have been found ubiquitously distributed in various cells and tissues (Inagaki et al., 1995b), such as pancreatic β-cells (Cook et al., 1988; Ashcroft and Kakei, 1989), pituitary GH3 cells (Wu et al., 2000), skeletal muscle (Allard and Lazdunski, 1993), neurons and glial cells of brain (Zhou et al., 2002; Thomzig et al., 2005), kidney (Hurst et al., 1993; Zhou et al., 2007b; Zhou et al., 2008) and testis (Acevedo et al., 2006; Zhou et al., 2011). KATP channels close at high intracellular ATP concentrations and open at lower concentrations during ischemia (Yokoshiki et al., 1998; Yuan et al., 2004). They
are hetero-octameric in composition, consisting of four pore-forming subunit molecules, Kir6.x (Kir6.1 or Kir6.2), and four regulatory subunit molecules, sulfonylurea receptors (SURs: SUR1, SUR2A, or SUR2B), to form functional K<sub>ATP</sub> channels (Inagaki <i>et al.</i>, 1996; Clement <i>et al.</i>, 1997).

In pancreatic β-cells, K<sub>ATP</sub> channels are considered to be responsible for insulin secretion. When extracellular glucose concentrations are high, the ratio of intracellular ATP/ADP increases, which inhibits the K<sub>ATP</sub> channel activity, triggering Ca<sup>2+</sup> to enter the β-cells, resulting in insulin release (Philipson and Steiner, 1995).

The pituitary gland secretes various hormones with important functions and has close connections with adrenal glands, thyroid gland, and reproductive systems. There is an important axis called the hypothalamic-pituitary-adrenal (HPA) axis among the hypothalamus, the pituitary gland, and the adrenal gland. This axis is important for an organism's survival in changing environmental conditions (Xiong and Zhang, 2013) and controls reactions to stress and regulates many body processes, including digestion, the immune system, mood and emotions, sexuality, and energy storage and expenditure (Xiong and Zhang, 2013). There have been some reports that found that SUR gene expression was recognized in the pituitary gland in adult mice and several stages of mouse embryo (Hernandez-Sanchez <i>et al.</i>, 1997). There have been no reports about localization of K<sub>ATP</sub> channel subunits in the pituitary gland, not to mention colocalization with corticotrophs (ACTH cells), lactotrophs (PRL cells), somatotrophs (GH cells), and follicle stimulating hormone (FSH) cells. Here, we report for the first time localization of K<sub>ATP</sub> channel subunits in the pituitary gland and also reveal their colocalization with ACTH cells but not with the other cells examined, such as PRL, FSH, and GH cells. These new data will help us understand the relationship of K<sub>ATP</sub> channels with hormone secretion in the pituitary gland other than pancreatic β-cells.

**Materials and Methods**

**Animals and tissue preparation**

Male Sprague-Dawley (SD) rats (4–8 weeks) were supplied by CLEA Japan Inc. (Tokyo, Japan). This study was conducted according to the protocols for the Animal Research Committee of Akita University. All animals were handled according to the Guidelines for Animal Experimentation of Akita University. Rats used in this study were kept under constant environmental conditions in group cages (2 to 3 rats per cage) with 12h light/dark cycles and given food and water <i>ad libitum</i>.

Samples for RT-PCR were taken quickly from rats under anesthetized conditions and freshly frozen with dry ice and stored at -80°C until use. Samples for immunohistochemistry were taken from those fixed by transcardial perfusion with cold Zamboni fixatives (2g paraformaldehyde dissolved in 15 ml of saturated picric acid with addition of Karasson Schwlt's phosphate buffer solution to make 100 ml) and immersed in the same fixative for 6 hr at 4°C, and then changed into 30% sucrose in PBS. Cryosections (4–6 μm thick) were cut with a Leica CM1950 cryostat, thaw-mounted on MAS-coated glass slides (Matsunami Industries, Kishiwada, Japan), and stored at 4°C for use.

**Reverse transcriptase-polymerase chain reaction (RT-PCR)**

Pituitary gland RNA was extracted using an RNaseasy mini kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription reactions were carried out as described previously (Zhou <i>et al.</i>, 2008).

Primers for rat Kir6.1 (GenBank ID: D42145) were designed to reside 212 bp from 1323 to 1534; primers for rat Kir6.2 (GenBank ID: D86039) were designed to reside 297 bp from 942 to 1238; primers for SUR1 (GenBank ID: L40624) were designed to reside 329 bp from 2047 to 2375; primers for SUR2A (GenBank ID: D83598) were designed to reside 155 bp from 4646 to 4800; primers for SUR2B (GenBank ID: AF019628) were designed to reside 152 bp from 4646 to 4797. PCR reaction was performed with a Taq kit (Takara, Biotech. Inc., Tokyo) under the following conditions:

Specimens were heated to 94°C for 1 min, and then subjected to 30 cycles of denaturation (94°C, 30 s), annealing (30 s; 55°C for Kir6.1 and SUR2A; 58°C for Kir6.2, SUR1, and SUR2B), and extension (72°C, 30 s). A final extension phase (72°C, 3–5 min) was included for all samples. Finally, 1.5% agarose gels were used to separate the PCR amplification, and the bands of PCR product were...
observed by ethidium bromide staining under ultraviolet light.

**Immunohistochemistry**

After air-drying for 30 min, cryosections of pituitary gland were treated with 0.3% Tween-20 for 45 min. In order to reduce endogenous peroxidase reaction, the sections were treated with 0.3% H$_2$O$_2$/methanol for 10 min. Sections were pre-incubated with 5% normal goat serum or 5% normal rabbit serum for 30 min, and then incubated with goat anti-human Kir6.1 (sc11224, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), goat anti-human Kir6.2 (sc11228, Santa Cruz Biotechnology, Inc.), and rabbit anti-rat SUR2A and rabbit anti-rat SUR2B (Zhou et al., 2007a), each in 1:500 dilution. After washing in PBS three times, 5 min each, the sections were incubated with biotinylated rabbit anti-goat IgG (BA5000; Vector Laboratories, Inc., Burlingame, CA), or biotinylated goat anti-rabbit IgG (BA1000; Vector Laboratories, Inc.), 1:200 each for 30 min, and then with ABC complex (Vectastain ABC Kit; Vector Laboratories, Inc.) following the description in the manufacturer’s instructions. Immunoreaction was developed with 3, 3-diaminobenzidine (DAB) in the presence of 0.003% H$_2$O$_2$, followed by counterstaining with methyl green and coverslipping. As a negative control, an absorption test was performed, being pre-incubated with peptide antigens with which the antibodies were generated.

**Immunofluorescence double and triple staining**

Cryosections of pituitary gland were washed in PBS and then fixed in cold acetone for 10 to 15 min. To determine whether the Kir6.1 or Kir6.2 is colocalized with SUR2A or SUR2B in the pituitary cells, sections were incubated with a mixture of goat anti-human Kir6.1 and rabbit anti-rat SUR2A, or a mixture of goat anti-human Kir6.2 with rabbit anti-rat SUR2A antibody, or a mixture of goat anti-human Kir6.1 with rabbit anti-rat SUR2B, or a mixture of goat anti-human Kir6.2 and rabbit anti-rat SUR2B antibody, diluted to 1:400 each, respectively, for 12 hr at room temperature. After thoroughly washing with PBS, the sections were incubated with Alexa555-conjugated donkey anti-rabbit IgG (Molecular Probes, Inc., Eugene, OR) and Alexa488-conjugated donkey anti-rabbit IgG (Molecular Probes, Inc.), diluted to 1:200 each.

To determine whether the K$_{\text{ATP}}$ channel subunit molecules colocalize with ACTH cells, PRL cells, FSH cells, and GH cells, sections were incubated with a mixture of each K$_{\text{ATP}}$ channel subunit’s antibodies with mouse anti-human ACTH (Bio-Rad AbD Serotec. Ltd., Tokyo, Japan), 1:400, or mouse anti-human prolactin (Santa Cruz Biotechnology, Inc.), 1:200, or mouse anti-human FSH (Abova, Taipei), 1:300, and/or mouse anti-rat GH (R&D Systems, Inc., Minneapolis, USA), 1:400, respectively. After thoroughly washing with PBS, these sections were incubated with a suitable mixture of secondary antibodies that could distinguish the K$_{\text{ATP}}$ channel subunits (donkey anti-rabbit alexa488 for detecting SUR2A and SUR2B, or donkey anti-goat alexa488 for detecting Kir6.1 and Kir6.2) and related hormone-containing cells (chicken anti-mouse alexa594 for detecting ACTH, FSH, GH, and prolactin) in the anterior lobe of the pituitary gland. Finally, these sections were coverslipped with Fluoromount/Plus aqueous mounting medium (Diagnostic BioSystems, CA).

In order to determine whether the pore-forming subunits and regulator subunits colocalize with corticotrophs, we tried immunofluorescence triple staining, i.e., after incubation with normal donkey serum, the sections were incubated with a mixture of goat anti-Kir6.1 or goat anti-Kir6.2, rabbit anti-SUR2A or anti-SUR2B, and mouse anti-ACTH antibodies, 1:400 each, respectively. After washing with PBS three times, the sections were further incubated with Alexa555 donkey anti-goat IgG for 1 h, washed again with PBS, and then incubated with a mixture of Alexa550-conjugated goat anti-rabbit IgG (Molecular Probes, Inc.) and Alexa488-conjugated goat anti-mouse IgG (Molecular Probes, Inc.), diluted to 1:200 each, for 1 h. Finally, the sections were coverslipped with PermaFluor aqueous mounting medium (Thermo; Pittsburgh, PA).

Fluorescence image signals were acquired with an Olympus DP72 (Tokyo, Japan) and a laser scanning confocal microscope (LSM 510 or LSM 780, Carl Zeiss, Oberkochen, Germany).
Results

Detection of K<sub>ATP</sub> channel subunit mRNAs in rat pituitary gland

The presence of mRNAs for Kir6.1, Kir6.2, SUR1, SUR2A, and SUR2B in adult rat pituitary gland was detected by RT-PCR analysis. The expected amplification of cDNA fragments specific for Kir6.1 is of a 212-bp product (from 1323 to 1534), for Kir6.2 is of a 297-bp product (from 3019 to 1238), for SUR1 is of a 329-bp product (from 2047 to 2375), for SUR2A is of a 155-bp product (from 4646 to 4800), and for SUR2B is of a 152-bp product (from 4646 to 4797) (Fig. 1). These results revealed that all these K<sub>ATP</sub> channel subunit genes were expressed in rat pituitary gland.

Immunolocalization of K<sub>ATP</sub> channel subunit proteins in rat pituitary gland

In pituitary gland, immunoreactivity for Kir6.1 antibodies was expressed weakly to moderately in the anterior lobe, weakly to moderately in the intermediate lobe, and faintly to weakly in the posterior lobe (Fig. 2A). In the anterior lobe, weak immunoreactivity with Kir6.1 was detected in some round or oval cells, and moderate immunoreactivity was shown in some small irregular cells and some large polygonal cells, which were scattered in clumps and around the sinusoids of the blood vascular system (Fig. 2B).

As for Kir6.2, immunoreactivity with anti-Kir6.2 was expressed moderately to intensely in the anterior lobe and intermediate lobe, but faintly in the posterior lobe (Fig. 2C). Those Kir6.2-positive cells in the anterior lobe were small to medium in size with irregular shapes, with some large and polygonal shaped cells, which were distributed in irregular cords or clusters (Fig. 2D).

As for SUR2A, immunoreactivity with anti-SUR2A antibodies was expressed weakly in many cells in the anterior lobe, faintly in the posterior lobe, and almost not at all in the intermediate lobe, but some flat oval cells at the edge of the intermediate lobe were observed showing immunoreactivity to SUR2A (Fig. 3A). Those cells with immunoreactivity to SUR2A were widely distributed in the cellular cord as clumped around sinusoids (Fig. 3B).

As for SUR2B, immunoreactivity with anti-SUR2B
antibodies was expressed moderately to intensely in the anterior lobe, weakly to moderately in the intermediate lobe, and faintly in the posterior lobe (Fig. 3C). At the anterior lobe, some large polygonal cells showed moderate to intense immunoreactivity to SUR2B, some small elongate, irregular-shaped cells showed moderate immunoreactivity, and some round or oval cells were shown faintly to weakly (Fig. 3D). The negative control sections incubated with an antibody that had previously been absorbed with polypeptide antigen with which the antibody was produced showed no reaction or very poor staining under the same condition (Fig. 4).

**Colocalization of Kir6.x with SURs in rat pituitary gland**

To determine whether Kir6.1 or Kir6.2 is colocalized with SUR2A or SUR2B in pituitary gland cells, immunofluorescence double staining against the Kir6.x and SURs was performed. Immunoreactivity with Kir6.1 (Kir6.1⁺) or Kir6.2 (Kir6.2⁺) was detected as red fluorescence (Fig. 5A, D, G, and J), and immunoreactivity of SUR2A (SUR2A⁺) or SUR2B (SUR2B⁺) was detected as green fluorescence (Fig. 5B, E, H, and K). The merged yellow images showed that Kir6.1⁺ or Kir6.2⁺ colocalized with SUR2A and/or SUR2B (Fig. 5C, F, I, and L). The results showed that the Kir6.1⁺ cells were partly colocalized with SUR2A in the anterior lobe of the pituitary gland (Fig. 5C). Some Kir6.2⁺ cells colocalized with SUR2A were also observed (Fig. 5F). As for Kir6.1 and/or Kir6.2 with SUR2B, the Kir6.1⁺ cells were nearly all overlapped by SUR2B (Fig. 5I), while Kir6.2⁺ cells were only partly overlapped by SUR2B (Fig. 5L).
Colocalization of K\textit{ATP} channel subunits with hormone secreting cells

Immunofluorescence double staining for K\textit{ATP} channel subunits with ACTH cells showed that the immunoreactivity of ACTH was detected as red fluorescence (Fig. 6A, D, G, and J), and the immunoreactivity of Kir6.1, Kir6.2, SUR2A, and/or SUR2B was detected as green fluorescence (Fig. 6B, E, H, and K). The merged yellow images showed that each K\textit{ATP} channel subunit colocalized with ACTH (Fig. 6C, F, I, and L). In the anterior lobe of the pituitary gland, the majority of Kir6.1\textsuperscript{+} cells colocalized with ACTH (Fig. 6C), and Kir6.2\textsuperscript{+} cells also colocalized with ACTH (Fig. 6F). As for SUR2A and ACTH, we could only find that SUR2A\textsuperscript{+} cells only partly colocalized with ACTH (Fig. 6I). And SUR2B\textsuperscript{+} cells were almost all colocalized with ACTH (Fig. 6L).

As for FSH, PRL, and GH cells, immunofluorescence double staining showed that both Kir6.1 and Kir6.2 did not colocalize with FSH cells (Fig. 7C and F), not with PRL cells (Fig. 7I and L), and also not with GH cells (Fig. 8C and F).

To determine whether or not the ACTH cells really contain K\textit{ATP} channels formed by Kir6.1/SUR2A, Kir6.2/SUR2A, Kir6.1/SUR2B, and/or Kir6.2/SUR2B, immunofluorescence triple staining was also performed. Immunoreactivity with SUR2A and/or SUR2B was detected as blue (Fig. 9A, E, I, and M), immunoreactivity with Kir6.1 and/or Kir6.2 was detected as red (Fig. C, G, K, and O), and immunoreactivity with ACTH was detected as green (Fig. 9B, F, J, and N). The results showed that four pairs of K\textit{ATP} channel subunits, i.e., Kir6.1/SUR2A (Fig. 9D), Kir6.2/SUR2A (Fig. 9H), Kir6.1/SUR2B (Fig. 9L), and Kir6.2/SUR2B (Fig. 9P), were colocalized with ACTH cells in the
anterior lobe of the pituitary gland, respectively.

**Discussion**

The present study revealed that five kinds of K<sub>ATP</sub> channel subunits were expressed in the pituitary gland as demonstrated by RT-PCR, and immunostaining showed that they were widely distributed in the adenohypophysis. Four types of subunits, Kir6.1, Kir6.2, SUR2A, and SUR2B, were further revealed to be colocalized with ACTH cells. Since we have no suitable anti-SUR1 antibody for immunostaining at hand, there have been at least four types of K<sub>ATP</sub> channels, Kir6.1/SUR2A, Kir6.1/SUR2B, Kir6.2/SUR2A, and Kir6.2/SUR2B, localized in the ACTH cells.

Pituitary adrenocorticotropin hormone (ACTH) is an important component of the hypothalamus-pituitary-adrenal (HPA) axis, which is essential for body homeostasis and survival during stress (Stevens and White, 2010; Fuchs et al., 2013; Uchoa et al., 2014). It is influenced by various stimuli such as cortical release hormone (CRH), and to a lesser extent, by arginine vasopressin (AVP). It receives a negative feedback suppression of cortisol from the adrenal glands (Fuchs et al., 2013). A patch-clamp experiment for membrane potential in single rat corticotrophs showed that CRH triggered a sustained [Ca<sup>2+</sup>]i elevation and membrane depolarization for corticotrophs (Lee and Tse, 1997), and ACTH secretion was related to an increase in the L-type Ca<sup>2+</sup> current (LeBeau et al., 1997). Such an ACTH-releasing process is somehow like that of pancreatic β-cells, which release insulin through the L-type Ca<sup>2+</sup> channel triggered by the K<sub>ATP</sub> channel (Aguilar-Bryan et al., 1995; Philipson and
Fig. 5. Immunofluorescence double staining shows the expression of Kir6.1 and Kir6.2 as red (Alexa 555, A, D, G, J) and SUR2A and SUR2B as green (Alexa 488, B, E, H, K) in the anterior lobe of the pituitary gland. The merged images (C, F, I, L) indicate that SUR2A colocalizes with Kir6.1 (C) and/or Kir6.2 (F); SUR2B colocalizes with Kir6.1 (I) and/or Kir6.2 (L). Scale bar: 50 μm.
Fig. 6. Immunofluorescence double staining shows the expression of ACTH as red (Alexa 594, A, D, G, J) and Kir6.1, Kir6.2, SUR2A, and SUR2B as green (Alexa 488, B, E, H, K) in the anterior lobe of the pituitary gland. The merged images (right column, C, F, I, L) indicate that Kir6.1 (C), Kir6.2 (F), SUR2A (I), and/or SUR2B (L) colocalize with ACTH. Scale bar: 50 μm.
Fig. 7. Immunofluorescence double staining shows the expression of FSH (A, D) and PRL (G, J) as red and Kir6.1 and Kir6.2 as green (B, E, H, K). There are no colocalization images that could be seen in the FSH with Kir6.1 (C) and Kir6.2 (F). Also, there are no colocalization images that could be seen in the prolactin (PRL) with Kir6.1 (I) and Kir6.2 (L). Scale bar: 40 μm.
K<sub>ATP</sub> channel subunits in rat pituitary gland

Fig. 8. Immunofluorescence double staining shows the expression of GH as red (A, D) and Kir6.1 and Kir6.2 as green (B, E). It could be seen that both Kir6.1 and Kir6.2 do not colocalize with GH (C, F). Scale bar: 40 μm.

Thus, K<sub>ATP</sub> channels found in corticotrophs might play an important role in controlling ACTH release. Interestingly, the HPA axis regulation is closely associated with noninsulin-dependent diabetes mellitus (NIDDM), type 2 diabetes (Yi et al., 2010), which in turn has a close relationship with sulfonylureas, the target of the K<sub>ATP</sub> channel for treatment of NIDDM (Ashfield and Ashcroft, 1998). In contrast to such a mechanism in insulin secretion, there was also a report that stated that ACTH release was not associated with the L-type voltage-dependent Ca<sup>2+</sup> channel, stimulated by cortical release hormone (CRH) and/or vasopressin, but rather via ER Ca<sup>2+</sup> store refilling (Yamashita et al., 2009). Thus, further studies are needed to ascertain whether or not K<sub>ATP</sub> channels work in the corticotrophs.

It is well known that K<sub>ATP</sub> channels have different types based on different combinations of Kir6.x and SURs in native tissues and cells (Seino and Miki, 2003). Kir6.1 and SUR2B form a vascular smooth-muscle-type K<sub>ATP</sub> channel (Yamada et al., 1997), Kir6.2 and SUR1 form a pancreatic β-cell-type K<sub>ATP</sub> channel (Sakura et al., 1995; Inagaki et al., 1997), Kir6.2 and SUR2A form a cardiac-type K<sub>ATP</sub> channel (Inagaki et al., 1996), and Kir6.2 and SUR2B form a smooth-muscle-type K<sub>ATP</sub> channel (Isomoto et al., 1996). In addition, different combinations of Kir6.x and SURs will have distinct electrophysiological properties and pharmacological sensitivities (Seino and Miki, 2003), although SUR2A and SUR2B differ only in terms of the last 42 amino acids (Aguilar-Bryan et al., 1998a; Chutkow et al., 1999). For example, the K<sub>ATP</sub> channel formed by Kir6.2 and SUR2A, which is considered to be the cardiomyocyte type, is sensitive to two potassium channel openers (KCOs), pinacidil and cromakalim, but only weakly to diazoxide, while the K<sub>ATP</sub> channel formed by Kir6.2 and SUR2B, which is considered to be the vascular smooth muscle type, is activated by all three KCOs mentioned above (Aguilar-Bryan et al., 1998b). As for the response to potassium channel blockers, glibenclamide blocks the Kir6.2/SUR1 channel, but only slightly inhibits the Kir6.2/SUR2A channel (Inagaki et al., 1995a; Gribble et al., 1998), whereas
tolbutamide highly inhibits the Kir6.2/SUR1 channel current, but it does not inhibit the Kir6.2/SUR2A channel current (Inagaki et al. 1995a; Gribble et al. 1998). These different types of \( \text{K}_\text{ATP} \) channels in corticotrophs observed in the present study might correspond to different metabolic and physiological states. The diversity of the \( \text{K}_\text{ATP} \) channels in corticotrophs might also be due to the different mechanism in utilizing Ca\(^{2+}\) ions by the L-type Ca\(^{2+}\) channel or via ER Ca\(^{2+}\) store refilling.

In pancreatic \( \beta \)-cells, increasing the intracellular ATP closes the \( \text{K}_\text{ATP} \) channels, followed by opening the voltage-dependent calcium channels and the calcium influx (Seino and Miki, 2003). As in the corticotrophs, there is no such explanation of the ACTH delivery mechanism based on biological and physiological data, but the localization of \( \text{K}_\text{ATP} \) channel subunits in those cells suggest that \( \text{K}_\text{ATP} \)
channels have some relationship connected to their hormone secretion. Further studies are needed to shed light on the KATP channel functions correlated with the HPA axis.

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