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BRIEF COMMUNICATIONS

K_{ATP} Channel Activity Is Required for Hatching in *Xenopus* Embryos

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ABSTRACT **A growing body of work suggests that the activity of ion channels and pumps is an important regulatory factor in embryonic development. We are beginning to identify functional roles for proteins suggested by a survey of expression of ion channel and pump genes in *Xenopus* and chick embryos (Rutenberg et al. [2002] Dev Dyn 225, this issue). Here, we report that the ATP-sensitive K^+ channel protein is present in the hatching gland of *Xenopus* embryos; moreover, we show that its activity is necessary for hatching in *Xenopus*. Pharmacologic inhibition of K_{ATP} channels not only specifically prevents the hatching process but also greatly reduces the endogenous expression of *Connexin-30* in the hatching gland. Based on recent work which showed that gap-junctional communication mediated by Cx30 in the hatching gland was required for secretion of the hatching enzyme, we propose that K_{ATP} channel activity is upstream of *Cx30* expression and represents a necessary endogenous step in the hatching of the *Xenopus* embryo.**
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Key words: K_{ATP} channels; K^+ ion; *Xenopus*; embryogenesis; hatching

INTRODUCTION

Electrical activity due to ion channel and pump function has been suggested to have a causal role in embryonic development (Lund, 1947; Nuccitelli, 1988, 1992). Recently, we conducted a survey to determine what electrogenic genes are expressed in early *Xenopus* and chick embryos with an emphasis on possible non-neuronal roles (Rutenberg et al., 2002). We are now beginning to carry out functional analyses of several such genes and here report the involvement of the K_{ATP} channel in the hatching process in *Xenopus*.

The K_{ATP} ion channel protein is an octamer consisting of four subunits of the K^+ rectifier (KIR6.1 or KIR6.2) surrounded by four regulatory subunits. K_{ATP} channels are found in the pancreatic β cells, cardiac myocytes, pituitary, skeletal and smooth muscles, brain, and kidney (Ashcroft, 1988). K_{ATP} channels couple cell metabolism with membrane electrical excitability and are a key step in the control of glucose-induced insulin release (Koster et al., 2000). K_{ATP} channels also control membrane voltage; this is an important aspect of neoplasm because of its ability to regulate proliferation, cell cycle control, and cell migration (Arcangeli et al., 1996; Knutson et al., 1997; Kamleiter et al., 1998; Wang et al., 1998; MacFarlane and Sontheimer, 2000; Wohlrab et al., 2000). K_{ATP} channel subunits are often misexpressed in tumor tissue (Zhu et al., 1998), and drug-induced inhibition of tumor cell proliferation is, in some contexts, due to the inhibition of K_{ATP} channel activity (Woodfork et al., 1995; Wang et al., 1998; Wondergem et al., 1998). K_{ATP} channels are also believed to be a key aspect of ischemic cardioprotection (Cohen et al., 2000; Gomma et al., 2001). Because of their direct involvement in mechanisms of diabetes, neoplasm, and cardioprotection, K_{ATP} channels are an important biomedical target in adult tissue (Inagaki and Seino, 1998; Day et al., 1999; Lawson, 2000). Interestingly, we detected expression of K_{ATP} channels in early embryonic cells not related to heart or pancreas (Rutenberg et al., 2002), and investigated the novel embryonic role for this ion channel family.

RESULTS AND DISCUSSION

Immunohistochemistry with an antibody against Kir6.1, the main subunit of the K_{ATP} channel, reveals the characteristic Y-shape staining on the embryo's face, indicative of the hatching gland (Fig. 1A,B). No signal for Kir6.2 was detected (data not shown). We then asked which of the two possible accessory sub-

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units (SUR1 and SUR2) might be expressed together with Kir6.1. Immunohistochemistry with antibodies against either subunit revealed that SUR1 was widely expressed in the embryonic head, but specifically absent from the hatching gland (Fig. 1C). In contrast, SUR2 was present in the hatching gland only (Fig. 1D). These expression patterns suggest that the K_{ATP} channel present in the hatching gland is composed of Kir6.1+SUR2, a combination that is believed to constitute cardiac and vascular smooth muscle-type K_{ATP} channels (Dorschner et al., 1999). Moreover, the complementary expression of the regulatory subunits SUR1 and SUR2 with respect to the hatching gland is consistent with tight embryonic control over the precise functional properties of K_{ATP} channels located in the head (Babenko et al., 1998; Inagaki and Seino, 1998).

To test which developmental events may depend on the function of K_{ATP} channels, we used the pharmacologic reagent Nicorandil (Kukovetz et al., 1992; Sato et al., 2000), a powerful and specific drug that causes the opening of K_{ATP} channels and hyperpolarizes cells that express significant levels of this protein. Embryos cultured in the presence of Nicorandil from stage 11 onward developed normally but were unable to hatch from the vitelline membrane. Compared with control embryos at stage (st.) 39 (Fig. 1E, which were >95% hatched by approximately st. 28, n = 52), Nicorandil-exposed embryos were still in their vitelline membranes (Fig. 1F, n = 48). When compared with a control embryo (Fig. 1G), Nicorandil-exposed embryos that were manually released from the vitelline membrane show fairly complete development of the head, eyes, and somites (Fig. 1H). Embryos freed at this stage were alive and mobile, but the outer epidermis showed signs of disintegration, possibly due to the increased pressure against the vitelline membrane resulting from confinement within the membrane at these late stages. To confirm specificity of the Nicorandil exposure, we performed several control experiments. Agonists of other pathways (nitric oxide donors such as sodium nitroprusside, serotonergics such as RS67333, V-ATPase blockers such as concanamycin, and V-ATPase activators such as fusicoccin) as well as vehicle (dimethyl

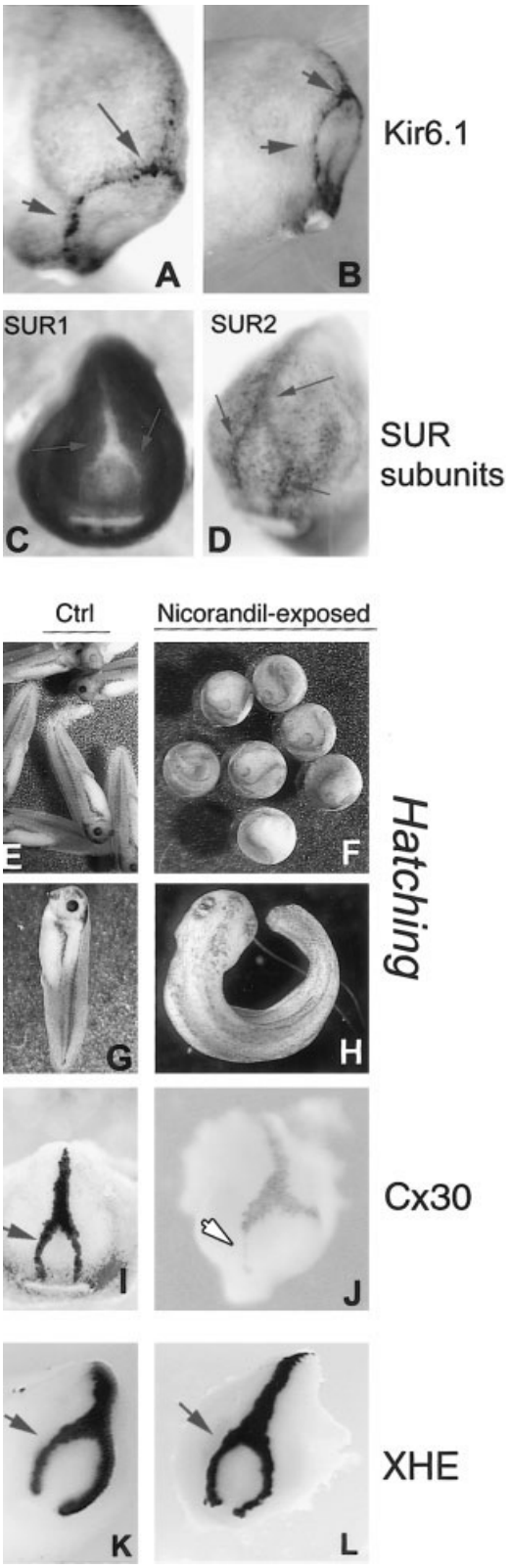

Fig. 1. K_{ATP} channels are involved in hatching gland function. **A,B:** Immunohistochemistry using an antibody to Kir6.1 reveals the presence of K_{ATP} channels in the Y-shaped tissue of the hatching gland. **C:** SUR1 protein is strongly expressed in the head but is strikingly absent from the hatching gland cells, the cement gland, and the tissue between the two frontal arms of the hatching gland Y shape. **D:** SUR2 is present in the hatching gland. **E:** Control embryos hatch by stage 29. **F:** In contrast, embryos exposed to Nicorandil, an opener of K_{ATP} channels, remain in the vitelline membrane and are unable to hatch. **G:** Individual embryo showing morphology at stage 38. **H:** Individual embryo manually freed from the membrane shows normal development to this late stage, despite confinement to the vitelline membrane. **I:** *Connexin30* is normally strongly expressed in the hatching gland. **J:** Embryos exposed to Nicorandil show a much reduced expression of *Cx30* in the same tissue (white arrow). Compared with control embryos (**K**), the expression of the hatching enzyme is equally strong in Nicorandil-exposed embryos (**L**), showing that the tissue is alive and that the cells maintain hatching gland identity. Red arrows indicate expression. Green arrows indicate lack of expression.

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sulfoxide [DMSO]) were without effect on hatching. Moreover, blockers of other types of K^+ transporters (Na^+/K^+-ATPase: ouabain, H^+/K^+-ATPase: lansoprazole, $Na^+/Cl^-/K^+$ cotransporter: bumetanide) had no effect on hatching (not shown). We conclude that the activity of K_{ATP} channels composed of Kir6.1 is likely to play a role in the hatching process.

Release from the vitelline membrane occurs due to the secretion of hatching enzyme XHE (Katagiri et al., 1997) from the hatching gland on the face of the embryo. To gain mechanistic insight into how the hatching process was dependent on the activity of K^+ channels, we examined the expression of mRNA markers expressed in the hatching gland. *Connexin30*, a member of the connexin family of genes that form gap junctions, is expressed in the cells of the hatching gland in *Xenopus* and is likely to be involved in the regulation of hatching enzyme release from gland tissue (Levin and Mercola, 2000). Control embryos showed strong expression of *Cx30* in a Y-shape on the face of the embryo (Fig. 1I). In contrast, embryos exposed to Nicorandil, showed a much-reduced expression of *Cx30* (Fig. 1J, n = 20). To ensure that this effect was not due to cell death in the hatching gland or a respecification of identity in the gland tissue, we examined the expression of the hatching enzyme gene itself (Katagiri et al., 1997). Control embryos showed strong expression in the same characteristic Y shape (Fig. 1K). Similarly, embryos exposed to Nicorandil also showed strong expression of *XHE* (Fig. 1L) identical to the controls, ruling out loss of hatching gland cells and suggesting that the mechanism of Nicorandil action is downstream of hatching enzyme transcription.

In previous work, we presented data showing that pharmacologic and genetic loss of gap-junctional communication in the hatching gland inhibits hatching (Levin and Mercola, 2000). Thus, a down-regulation of endogenous *Cx30* expression in hatching gland cells is a likely mechanism by which opening of K_{ATP} channels prevents hatching. Gating of mature gap-junctional complexes by membrane voltage is a well-known phenomenon (Revilla et al., 2000). Our data are consistent with another level of control of gap-junctional communication by the activity of ion channels: transcription of connexin genes. Gap junctions play a recognized role in regulation of enzymatic secretion in several contexts (Meda, 1996a, b). Thus, we propose a model (Fig. 2) in which (1) K_{ATP} channels consisting of Kir6.1 and SUR2 determine membrane voltage in hatching gland cells, which (2) permits the expression of *Cx30* in the gland tissue, which (3) enables XHE secretion by virtue of gap-junctional synchronization of secretion signals. Our data also suggest the investigation of gap junctions as an intermediate step in the regulation of hormone secretion by K^+ flux and K_{ATP} channels in other contexts (Abraham et al., 1999; Seino et al., 2000). The mechanisms by which membrane voltage is coupled to *Cx30* transcription is a key area for future investigation, because understanding how voltage can be transduced to gene expression is likely to shed crucial light on the mechanisms by which endogenous ion fluxes and voltage gradients control morphogenetic events.

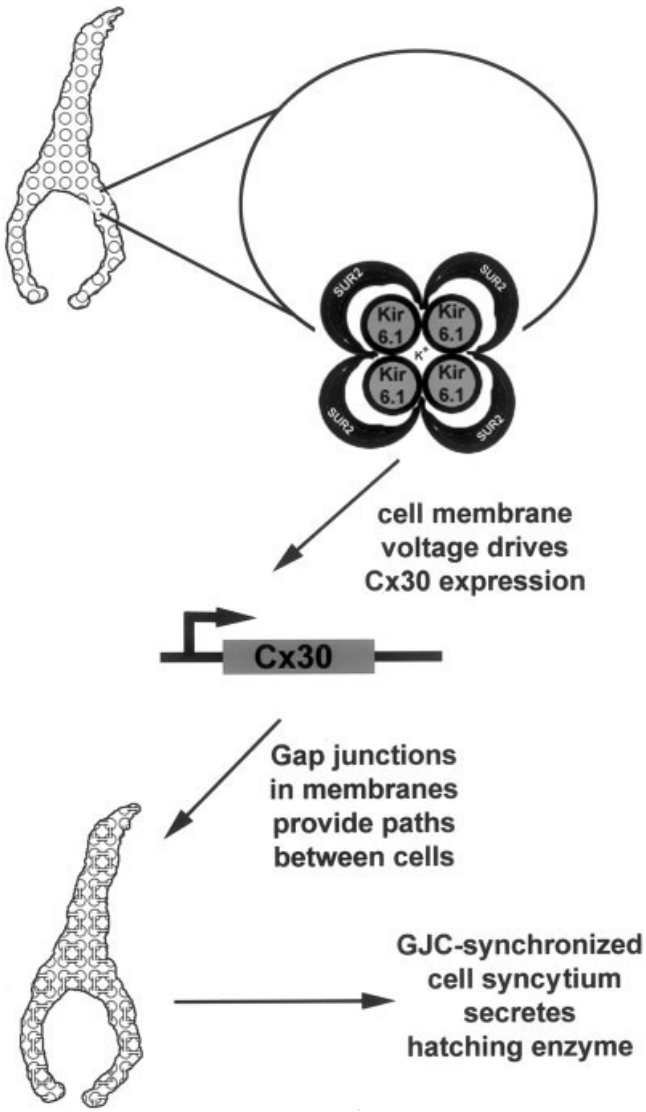

Fig. 2. A model of K_{ATP} channels' involvement in hatching gland function. K_{ATP} channels consisting of Kir6.1 and SUR2 determine membrane voltage in hatching gland cells; this potential is transduced to the transcription machinery (by unknown mechanisms) and permits the expression of *Cx30* in gland tissue. Gap junctions are formed from connexin proteins and result in a syncytium, which underlies the synchronization of secretion signals. This process enables release of XHE by hatching gland cells, leading to breakdown of the vitelline membrane.

EXPERIMENTAL PROCEDURES

In Situ Hybridization

Before in situ hybridization, *Xenopus* embryos were collected and fixed in MEMFA (Harland, 1991). All embryos were washed in phosphate buffered saline (PBS) + 0.1% Tween-20 and then transferred to methanol through a 25%/50%/75% series. In situ hybridization was performed according to a standard protocol (Harland, 1991) by using chromogenic detection with

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alkaline-phosphatase. Probes for in situ hybridization were generated in vitro from linearized templates using digoxigenin-labeling mix from Roche. The *Cx30* probe is described in (Levin and Mercola, 2000). The *XHE* probe is described in (Katagiri et al., 1997).

Whole-Mount Immunohistochemistry

Embryos were fixed overnight in MEMFA and stored at 4°C in PBTr (1× PBS + 0.1% Triton-100). They were then washed 3× in PBTr, blocked with 10% goat serum, and incubated with primary antibody at 1:500 in PBTr overnight, washed 6× with PBTr, and incubated with an alkaline-phosphatase secondary antibody overnight. After six washes in PBTr, detection was carried out by using nitro blue tetrazolium and 5-bromo-4-chloro-3-indoxyl phosphate (X-Phos). Antibodies against Kir6.1, Kir6.2, SUR1, and SUR2 were a generous gift of Dr. Blanche Swappach.

Nicorandil Exposure

Control embryos were exposed to the same level of vehicle (DMSO) as exposed embryos. Nicorandil stocks were made as 100 mg of Nicorandil in 11 ml of water + 1 ml of DMSO and used at 4.7 μM. Nicorandil was a kind gift of Merck KgaA, Darmstadt, Germany. All other drugs were obtained from Sigma and used at standard concentrations.

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