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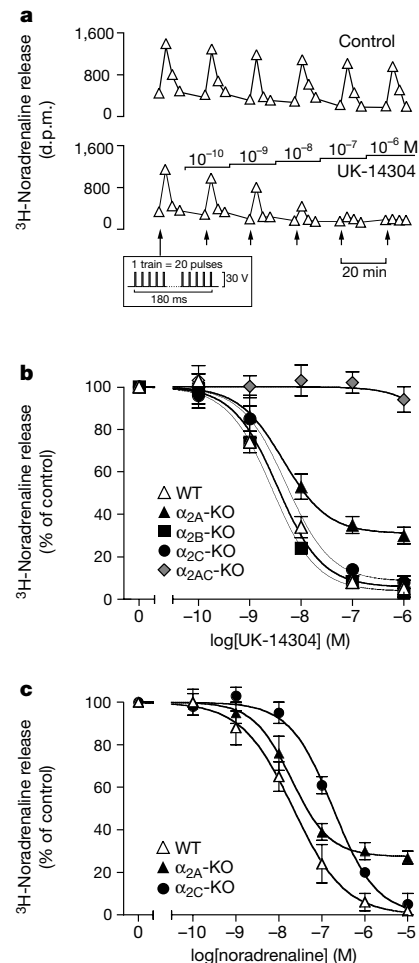
## Two functionally distinct $\alpha_2$ -adrenergic receptors regulate sympathetic neurotransmission

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The sympathetic nervous system regulates cardiovascular function by activating adrenergic receptors in the heart, blood vessels and kidney<sup>1</sup>.  $\alpha_2$ -Adrenergic receptors are known to have a critical role in regulating neurotransmitter release from sympathetic nerves and from adrenergic neurons in the central nervous system<sup>2–5</sup>; however, the individual roles of the three highly homologous  $\alpha_2$ -adrenergic-receptor subtypes ( $\alpha_{2A}$ ,  $\alpha_{2B}$ ,  $\alpha_{2C}$ ) in this process are not known. We have now studied neurotransmitter release in mice in which the genes encoding the three  $\alpha_2$ -adrenergic-receptor subtypes were disrupted. Here we show that both the  $\alpha_{2A}$ - and  $\alpha_{2C}$ -subtypes are required for normal pre-synaptic control of transmitter release from sympathetic nerves in the heart and from central noradrenergic neurons.  $\alpha_{2A}$ -Adrenergic receptors inhibit transmitter release at high stimulation frequencies, whereas the  $\alpha_{2C}$ -subtype modulates neurotransmission at lower levels of nerve activity. Both low- and high-frequency regulation seem to be physiologically important, as mice lacking both  $\alpha_{2A}$ - and  $\alpha_{2C}$ -receptor subtypes have elevated plasma noradrenaline



**Figure 1** Inhibition of noradrenaline release in atria by  $\alpha_2$ -receptor stimulation. **a**, Atria from wild-type mice (WT) were electrically stimulated (1 train of 20 pulses; 50 Hz) at 20-min intervals. In control conditions, transmitter release was constant over six stimulation periods (upper trace). Increasing concentrations of the  $\alpha_2$ -agonist UK-14304 inhibited the electrically evoked release in WT atria (lower trace). **b**, Concentration–response curves for inhibition by the  $\alpha_2$ -agonist UK-14304 of electrically evoked <sup>3</sup>H-noradrenaline release from isolated atria. **c**, Inhibition of <sup>3</sup>H-noradrenaline release from atria by noradrenaline. Data shown are means  $\pm$  s.e.m. from 6–8 atria.

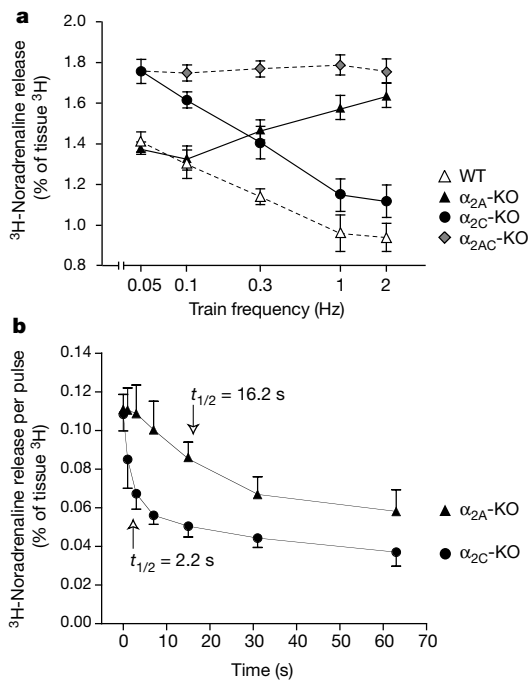
### concentrations and develop cardiac hypertrophy with decreased left ventricular contractility by four months of age.

The release of neurotransmitters is modulated by presynaptic autoreceptors that are activated by the released transmitter. Presynaptic inhibitory autoreceptors were first described for noradrenergic neurons and subsequently for other neurotransmitters, including dopamine, acetylcholine,  $\gamma$ -aminobutyric acid, histamine and serotonin<sup>2–5</sup>. Within the sympathetic nervous system, nine adrenergic receptor subtypes<sup>6</sup> (three  $\alpha_1$ ,  $\alpha_2$  and  $\beta$ -receptors) can be activated by adrenaline and noradrenaline, but only  $\alpha_2$ -adrenergic receptors have been implicated as inhibitory presynaptic autoreceptors. The lack of drugs that are sufficiently selective for the three subtypes has meant that their physiological roles and therapeutic potential have not been fully elucidated. An alternative approach to pharmacological characterization has been the deletion of  $\alpha_{2B}$ - or  $\alpha_{2C}$ -receptor genes, or *in vivo* site-directed mutagenesis of the  $\alpha_{2A}$ -receptor gene by gene targeting in mice<sup>7–11</sup>. We recently disrupted the gene for the  $\alpha_{2A}$ -adrenergic-receptor subtype in mice<sup>12</sup>.  $\alpha_{2A}$ -receptor-deficient mice ( $\alpha_{2A}$ -KO) are produced at the expected mendelian ratios from heterozygote intercrosses. They develop normally and show no gross pathological abnormalities<sup>12</sup>.

To investigate the role of the three  $\alpha_2$ -receptor subtypes in presynaptic autoinhibition, we measured <sup>3</sup>H-noradrenaline release

in tissues containing sympathetic (heart atria) or central (brain cortex) noradrenergic axons from normal (WT) and  $\alpha_2$ -receptor-deficient animals. Electrical stimulation with short pulse trains (20 pulses, 50 Hz) elicited the release of  $^3\text{H}$ -noradrenaline in the atria of WT and  $\alpha_{2A}$ -,  $\alpha_{2B}$ - and  $\alpha_{2C}$ -KO mice, with no difference in the magnitude of evoked release (Fig. 1). In these conditions, autoinhibition does not affect release because the single pulse trains are too short for the released noradrenaline to activate presynaptic  $\alpha_2$ -receptors<sup>13,14</sup>. In WT atria, the  $\alpha_2$ -adrenoceptor agonist UK-14304 caused concentration-dependent inhibition of  $^3\text{H}$ -noradrenaline release up to  $95 \pm 3\%$  (Fig. 1a, b). Similar results were obtained in mice lacking the  $\alpha_{2B}$ - or  $\alpha_{2C}$ -receptor subtype (Fig. 1b). In mice lacking the  $\alpha_{2A}$ -receptor, however, the maximal inhibitory effect of UK-14304 was reduced to  $70 \pm 4\%$  (Fig. 1b). We obtained similar results in brain cortex slices, although the effect of the  $\alpha_2$ -agonist in brain cortex from mice lacking the  $\alpha_{2A}$ -receptor was smaller than in atria (data not shown). These results indicate that, as has been suggested from pharmacological experiments<sup>15–18</sup>, the  $\alpha_{2A}$ -adrenergic receptor is the principal autoreceptor in the presynaptic feedback loop regulating noradrenaline release. However, the residual effect of UK-14304 in  $\alpha_{2A}$ -deficient mice indicates that a second  $\alpha_2$ -autoreceptor operates in both sympathetic neurons and central adrenergic neurons, although the role of this second subtype is much greater in sympathetic neurons.

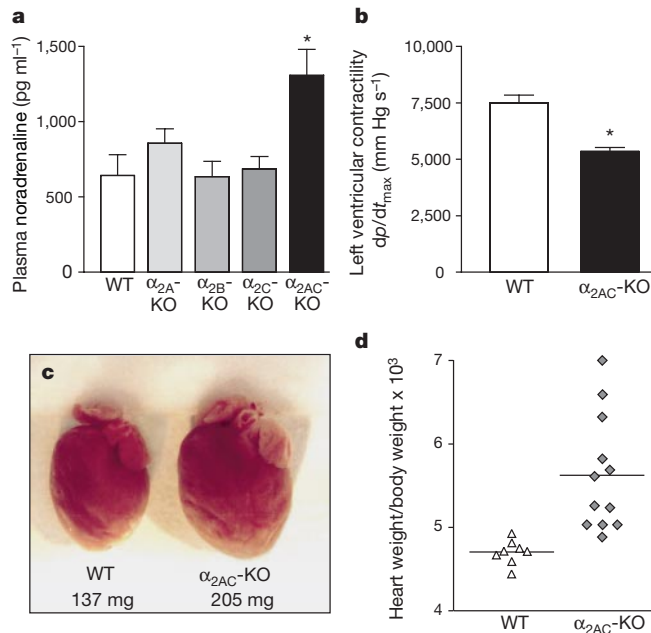
To determine the identity of the second subtype, we crossed mouse strains lacking  $\alpha_{2A}$ -,  $\alpha_{2B}$ - or  $\alpha_{2C}$ -subtypes to generate mice



**Figure 2** Autoinhibition of noradrenergic neurotransmission in isolated atria is mediated by  $\alpha_{2A}$ - and  $\alpha_{2C}$ -receptors. **a**, Frequency dependence of noradrenaline release. Isolated atria were stimulated with 16 pulse trains (10 pulses per train at 50 Hz) and the frequency of stimulation was varied between 0.05 and 2 Hz. In wild-type mice, transmitter release decreased with higher stimulation frequencies, whereas release increased in  $\alpha_{2A}$ -KO atria. In atria lacking  $\alpha_{2A}$ - and  $\alpha_{2C}$ -receptors, noradrenaline release was independent of the stimulation frequency. In atria from  $\alpha_{2C}$ -deficient mice, the frequency release curve was shifted to the right compared with results for WT mice, indicating that  $\alpha_{2C}$ -adrenergic receptors operate to regulate noradrenaline release at low stimulation frequencies. **b**, Time course of inhibition of noradrenaline release from mouse atria by  $\alpha_{2A}$ - and  $\alpha_{2C}$ -receptors. Isolated atria were stimulated with 1, 2, 3, 4, 8, 16, 32 or 64 pulse trains (10 pulses per train at 50 Hz; train frequency, 1 Hz) and the amount of transmitter released during the first and the following pulse trains was plotted against time. Inhibition mediated by the  $\alpha_{2A}$ -subtype was much faster ( $\alpha_{2C}$ -KO;  $t_{1/2} = 2.2$  s) than inhibition by the  $\alpha_{2C}$ -receptor ( $\alpha_{2A}$ -KO;  $t_{1/2} = 16.2$  s). Data shown are means  $\pm$  s.e.m. from 8–10 atria.

lacking two receptor subtypes. The only viable animals lacking two  $\alpha_2$ -receptor subtypes that could be produced from these crosses were mice lacking both  $\alpha_{2A}$ - and  $\alpha_{2C}$ -receptors ( $\alpha_{2AC}$ -KO). In these mice, the inhibitory effect of UK-14304 was completely abolished (Fig. 1b), indicating that the  $\alpha_{2C}$ -subtype is also involved in regulating noradrenaline release.

Exogenous noradrenaline inhibited transmitter release dose dependently (Fig. 1c). However, the concentration–response curve for noradrenaline was shifted to the right in atria from  $\alpha_{2C}$ -deficient mice compared with WT or  $\alpha_{2A}$ -deficient mice (Fig. 1c;  $\text{EC}_{50}$  (50% excitatory concentration) values: WT, 16 nM;  $\alpha_{2A}$ -KO, 20 nM;  $\alpha_{2C}$ -KO, 156 nM). This potency difference for noradrenaline may reflect the difference in affinity for noradrenaline between the  $\alpha_2$ -receptor subtypes: noradrenaline has a higher affinity for the  $\alpha_{2C}$ -subtype than for the  $\alpha_{2A}$ -receptor<sup>19</sup> ( $K_i$  inhibitory constant values:  $\alpha_{2C}$ , 650 nM;  $\alpha_{2A}$ , 5,800 nM). The results of the noradrenaline inhibition studies (Fig. 1c) demonstrate that the  $\alpha_{2C}$ -subtype mediates autoinhibition by low concentrations of noradrenaline in wild-type mice. Sensitivity to low catecholamine concentrations may be important for regulating catecholamine release from sympathetic nerves stimulated at low frequencies. To investigate this hypothesis, we stimulated isolated atria at different frequencies to mimic the physiological range of sympathetic nerve activity<sup>20</sup>. For this experiment, the atria were electrically stimulated with 16 pulse trains and the intervals between the individual trains were varied between 0.5 s (frequency of 2 Hz) and 20 s (frequency of 0.05 Hz). Without presynaptic autoinhibition, one would expect that the amount of transmitter released would be independent of the stimulation frequency. We observed this in  $\alpha_{2AC}$ -KO atria (Fig. 2a). However, if presynaptic receptors are inhibiting further transmitter release, the amount of transmitter overflow should decrease at



**Figure 3** Elevated plasma noradrenaline levels and cardiac phenotype of mice lacking  $\alpha_2$ -adrenergic-receptor subtypes. **a**, Circulating noradrenaline levels were determined in plasma samples obtained from wild-type and  $\alpha_2$ -receptor-deficient mice under anaesthesia. The plasma noradrenaline concentration in  $\alpha_{2AC}$ -KO mice was twice as high as in wild-type animals or mice lacking single  $\alpha_2$ -receptor subtypes ( $P = 0.0021$ , one-way analysis of variance ANOVA followed by Dunnett's tests,  $*P < 0.01$ ). **b**, *In vivo* determination of left ventricular contractility in wild-type and  $\alpha_{2AC}$ -KO mice. Left ventricular maximal contractility ( $\text{dp}/\text{dt}_{\text{max}}$ ) in anaesthetized mice was less in  $\alpha_{2AC}$ -KO mice than in wild-type mice. Data are from six animals per group. **c**, Heart of a non-transgenic mouse (WT; left) and an  $\alpha_{2AC}$ -KO mouse (right) at 14 weeks of age. Compared with the control heart, the  $\alpha_{2AC}$ -KO heart shows significant hypertrophy. **d**, The heart-weight/body-weight ratio was increased in  $\alpha_{2AC}$ -KO mice compared with WT mice.

higher synaptic transmitter concentrations, that is, higher stimulation frequencies. Indeed, noradrenaline release from WT atria was significantly less at high stimulation frequencies than at low frequencies (Fig. 2a). In these conditions, this effect can be attributed to presynaptic inhibition of transmitter release by  $\alpha_{2A}$ - and  $\alpha_{2C}$ -receptors, as noradrenaline overflow from  $\alpha_{2AC}$ -KO atria did not change within the frequency range applied. In  $\alpha_{2A}$ -deficient atria, noradrenaline release was inhibited at low frequencies, but increased at higher stimulation frequencies; this indicates that the  $\alpha_{2C}$ -receptor operates to inhibit noradrenaline release primarily at low frequencies (low noradrenaline concentrations). In contrast, atria from  $\alpha_{2C}$ -deficient mice showed no autoinhibition at low stimulation frequencies, confirming that the  $\alpha_{2C}$ -receptor inhibits transmitter release at low levels of sympathetic nerve activity and that  $\alpha_{2A}$  is required to regulate release at higher frequencies. These results demonstrate that both  $\alpha_{2A}$ - and  $\alpha_{2C}$ -receptors are required for physiological presynaptic autoinhibition.

The role of the  $\alpha_{2A}$ -receptor in mediating autoinhibition at high frequencies (high noradrenaline concentrations) can be attributed to the lower affinity of this subtype for noradrenaline and perhaps a resistance to desensitization. The higher affinity of the  $\alpha_{2C}$ -subtype for noradrenaline makes it better suited to respond to the lower concentrations of catecholamines at sympathetic nerve terminals stimulated at low frequencies. To test whether the failure of the  $\alpha_{2C}$ -subtype to mediate autoinhibition at high frequencies in this experiment was due to desensitization of this subtype or to slower onset of inhibition, we determined the time course of presynaptic inhibition (Fig. 2b). Transmitter release was elicited with 1 to 64 pulse trains at 1-s intervals and the amount of transmitter released during each pulse was determined. There was no difference in the amount of transmitter released with a single pulse train in atria from  $\alpha_{2A}$ -KO or  $\alpha_{2C}$ -KO mice. However, the amount of transmitter released during the subsequent pulses declined much faster in atria from  $\alpha_{2C}$ -KO mice than in those from  $\alpha_{2A}$ -KO mice (Fig. 2b;  $t_{1/2}$  (time to half-maximal inhibition) = 2.2 s and 16.2 s, respectively). Thus, coupling of the  $\alpha_2$ -receptor to inhibition of catecholamine release seems to be much faster for the  $\alpha_{2A}$ -subtype than for the  $\alpha_{2C}$ -subtype. There is no apparent desensitization of either subtype over this time interval.

Several lines of evidence suggest that  $\alpha_{2A}$ - and  $\alpha_{2C}$ -subtypes are also required for autoinhibition of noradrenaline release *in vivo*. Deletion of the  $\alpha_{2A}$ -receptor gene causes tachycardia<sup>12</sup>, and increased noradrenaline turnover was observed in mice expressing a mutant  $\alpha_{2A}$ -receptor ( $\alpha_{2A}$ -D79N)<sup>21</sup>. Thus, we measured plasma noradrenaline levels in mice deficient in  $\alpha_2$ -receptors, and found that, in anaesthetized animals, these levels were dramatically increased in  $\alpha_{2AC}$ -KO mice compared with wild-type mice or mice lacking single  $\alpha_2$ -receptor subtypes (Fig. 3a).

To determine the long-term physiological consequences of the altered sympathetic transmitter release in  $\alpha_2$ -receptor-deficient mice, we examined cardiac haemodynamics and the ratios of heart weight to body weight in WT and  $\alpha_{2AC}$ -receptor knockout mice. The heart is very sensitive to chronic elevations of catecholamines, and abnormal activity of the sympathetic nervous system has been implicated in the pathogenesis of heart failure<sup>22–24</sup>. Moreover, cardiac hypertrophy can be induced by chronic infusion of catecholamines in experimental animals. In  $\alpha_{2AC}$ -KO mice, left ventricular maximal contractility ( $dp/dt_{max}$ ; the maximal change in pressure per unit time) decreased to 70% of the wild-type value at four months of age (Fig. 3b). Mice lacking single  $\alpha_2$ -receptor subtypes showed no impairment of cardiac function at this age (data not shown). In addition, cardiac hypertrophy developed in  $\alpha_{2AC}$ -KO mice (Fig. 3c, d). Heart-weight/body-weight ratios were increased in these mice (Fig. 3d), but not in mice lacking only one  $\alpha_2$ -receptor subtype (data not shown). These findings suggest that the heart is exposed to significantly higher levels of catecholamines in  $\alpha_{2AC}$ -KO mice than in either  $\alpha_{2A}$ -KO or  $\alpha_{2C}$ -KO mice. Thus,

inhibition of catecholamine release mediated by both subtypes is physiologically important. □

## Methods

### Deletion of the $\alpha_{2A}$ -adrenergic-receptor gene

The genomic DNA for the mouse  $\alpha_{2A}$ -receptor (*Adra2a*) was cloned from a 129/Sv mouse genomic library (Stratagene). For the gene-targeting vector, a 2.7-kilobase (kb) fragment and an 8-kb fragment were placed around a neomycin-resistance gene, and a herpes simplex virus thymidine kinase gene was added to the 5' end of the plasmid. R1 embryonic stem cells were electroporated and screened for homologous targeting events as described<sup>12,25</sup>. Chimaeric mice were produced by aggregation of morulae with embryonic stem cells and two chimaeric male mice transmitted the  $\alpha_{2A}$ -mutation through the germ line. Homologous recombination at the *Adra2a* locus was identified by Southern blotting using an external genomic probe.

### <sup>3</sup>H-Noradrenaline release from mouse heart atria and brain cortex

This was determined as described<sup>14,26</sup>, with minor modifications. Briefly, halves of left and right atria or round brain cortex slices (2 mm diameter) were incubated with <sup>3</sup>H-noradrenaline (0.1  $\mu$ M) in physiological buffer<sup>26</sup> or noradrenaline-free medium containing 1  $\mu$ M desipramine at 1.8 ml min<sup>-1</sup>. Transmitter release was elicited by rectangular electrical pulses of 2 ms width and 15 V cm<sup>-1</sup> voltage. There were six periods of stimulation in each experiment, applied at 20-min intervals. Drugs were added 15 min before stimulation periods. At the end of the experiments, tissues were solubilized and tritium was determined in superfusate samples and tissues. Electrically evoked overflow of total tritiated compounds was calculated as the difference between total tritium outflow and estimated basal outflow, and was expressed as a percentage of tissue tritium at the time of stimulation. The electrically evoked overflow of total tritium reflects exocytotic release of <sup>3</sup>H-noradrenaline<sup>27</sup> and is termed thus in this paper. Results are presented as means  $\pm$  s.e.m.

### Noradrenaline plasma levels

Blood samples were obtained from mice anaesthetized with tribromoethanol (13  $\mu$ l of 2.5% solution per g body weight) and placed on a 37 °C table. After 20-min anaesthesia, 400  $\mu$ l blood was drawn from the left carotid artery and centrifuged. Plasma noradrenaline was quantified by HPLC combined with electrochemical detection<sup>28</sup>. Results are displayed as means  $\pm$  s.e.m. from 8–12 mice.

### Cardiac haemodynamics

Mice were anaesthetized with tribromoethanol and catheterized with a 1.8 French high-fidelity catheter-tip micromanometer (Millar Instruments) as described<sup>29</sup>.

### Cardiac pathology

Hearts from WT and KO animals were fixed in 4% paraformaldehyde in PBS as described<sup>30</sup>. Mid-ventricular cross-sections were used for computerized morphometric analysis of myocyte cross-sectional areas<sup>30</sup>. For determination of heart weights, the ventricles of 14-week-old animals were separated from the atria and surrounding connective tissue, rinsed in physiological buffer and briefly blotted dry. Heart weight was normalized to body weight and tibia length.

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**Rapid gating and anion permeability of an intracellular aquaporin**

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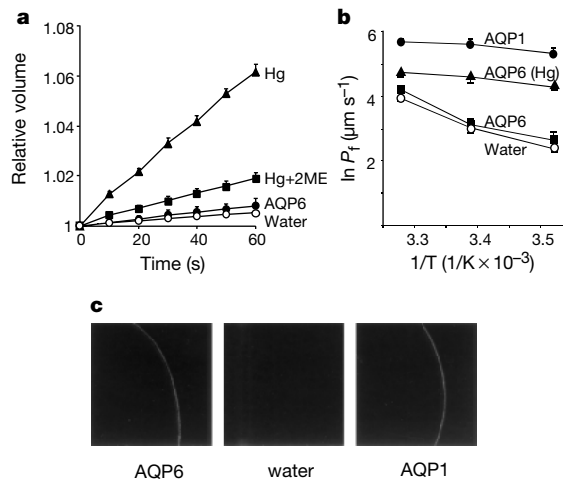
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**Aquaporin (AQP) water-channel proteins are freely permeated by water but not by ions or charged solutes<sup>1</sup>. Although mammalian aquaporins were believed to be located in plasma membranes, rat AQP6 is restricted to intracellular vesicles in renal epithelia<sup>2</sup>. Here we show that AQP6 is functionally distinct from other known**

**aquaporins. When expressed in *Xenopus laevis* oocytes, AQP6 exhibits low basal water permeability; however, when treated with the known water channel inhibitor, Hg<sup>2+</sup>, the water permeability of AQP6 oocytes rapidly rises up to tenfold and is accompanied by ion conductance. AQP6 colocalizes with H<sup>+</sup>-ATPase in intracellular vesicles of acid-secreting  $\alpha$ -intercalated cells in renal collecting duct. At pH less than 5.5, anion conductance is rapidly and reversibly activated in AQP6 oocytes. Site-directed mutation of lysine to glutamate at position 72 in the cytoplasmic mouth of the pore changes the cation/anion selectivity, but leaves low pH activation intact. Our results demonstrate unusual biophysical properties of an aquaporin, and indicate that anion-channel function may now be explored in a protein with known structure.**

The function of AQP6 was previously unknown. The amino-acid sequence of AQP6 (ref. 3) is most closely related to those of AQP2 from principal cells of renal collecting duct<sup>4</sup> and AQP0 (major intrinsic protein) from lens<sup>5</sup>. Unlike AQP2 (ref. 6), AQP6 does not travel to the plasma membrane<sup>2</sup>. Like AQP0 (ref. 7), AQP6 has been reported to have low water permeability<sup>8</sup>. When compared with water-injected *X. laevis* oocytes (water permeability ( $P_f$ ) =  $5.3 \pm 0.3 \text{ cm s}^{-1} \times 10^{-4}$ ), osmotic water permeability of oocytes expressing rat AQP6 is only slightly increased ( $P_f = 12.0 \pm 0.9 \text{ cm s}^{-1} \times 10^{-4}$ ) (Fig. 1a).

Mercury ions inhibit the water permeability of most aquaporins<sup>1</sup>; HgCl<sub>2</sub> was also reported to inhibit AQP6 (ref. 8), but, unexpectedly, we found that oocytes expressing rat AQP6 are activated by Hg<sup>2+</sup> (Fig. 1a). Within seconds, exposure to 300  $\mu\text{M}$  HgCl<sub>2</sub> markedly increased the osmotic water permeability of AQP6 oocytes ( $P_f = 93.0 \pm 4.3 \text{ cm s}^{-1} \times 10^{-4}$ ), which was reversed with 5 mM  $\beta$ -mercaptoethanol ( $P_f = 20.0 \pm 3.9 \text{ cm s}^{-1} \times 10^{-4}$ ). Other aquaporins have low activation energies,  $E_A < 4 \text{ kcal mol}^{-1}$ , which rise after treatment with Hg<sup>2+</sup> (ref. 9). For AQP6,  $E_A$  shifted from 12.5 to 3.6 kcal mol<sup>-1</sup> when the oocytes were preincubated with HgCl<sub>2</sub> (Fig. 1b). Hg<sup>2+</sup> could not have activated water permeability by inducing vesicle trafficking to the cell surface, because



**Figure 1** Water permeability of AQP6. **a**, Time-dependent osmotic swelling of water-injected oocytes (open circles), AQP6 oocytes without treatment (filled circles), AQP6 oocytes incubated with 300  $\mu\text{M}$  HgCl<sub>2</sub> (triangles), and oocytes incubated with HgCl<sub>2</sub> followed by 5 mM  $\beta$ -mercaptoethanol (squares). **b**, Arrhenius activation energy ( $E_A$ ) of osmotic water permeabilities of water-injected oocytes (open circles), untreated AQP1 oocytes (filled circles), untreated AQP6 oocytes (squares) and AQP6 oocytes incubated with 300  $\mu\text{M}$  HgCl<sub>2</sub> (triangles). Osmotic-swelling assays were then performed at 11, 22 or 32 °C (means  $\pm$  s.e.,  $n = 5$ ). **c**, Confocal microscopy of oocytes injected with AQP6 cRNA, water or AQP1 cRNA after fixation, and incubation with anti-AQP6 (left and middle panels) or anti-AQP1 (right panel).