

TASK (TWIK-Related Acid-Sensitive K⁺ Channel) Is Expressed in Glomerulosa Cells of Rat Adrenal Cortex and Inhibited by Angiotensin II

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The present study was conducted to explore the possible contribution of a recently described leak K⁺ channel, TASK (TWIK-related acid-sensitive K⁺ channel), to the high resting K⁺ conductance of adrenal glomerulosa cells. Northern blot analysis showed the strongest TASK message in adrenal glomerulosa (capsular) tissue among the examined tissues including heart and brain. Single-cell PCR demonstrated TASK expression in glomerulosa cells. In patch-clamp experiments performed on isolated glomerulosa cells the inward current at -100 mV in 30 mM [K⁺] (reflecting mainly potassium conductance) was pH sensitive ($17 \pm 2\%$ reduction when the pH changed from 7.4 to 6.7).

In *Xenopus* oocytes injected with mRNA prepared from adrenal glomerulosa tissue the expressed K⁺ current at -100 mV was virtually insensitive to tetraethylammonium (3 mM) and 4-aminopyridine (3 mM). Ba²⁺ (300 μ M) and Cs⁺ (3 mM) induced voltage-dependent block. Lidocaine (1 mM) and extracellular acidification from pH 7.5 to 6.7 inhibited the current (by 28% and 16%, respectively). This inhibitory profile is similar (although it is not identical) to that of TASK expressed by injecting its cRNA. In oocytes injected with adrenal glomerulosa mRNA, TASK antisense oligonucleotide reduced significantly the expression of K⁺ current at -100 mV, while the sense oligonucleotide failed to have inhibitory effect. Application of angiotensin II (10 nM) both in isolated glomerulosa cells and in oocytes injected with adrenal glomerulosa mRNA inhibited the K⁺ current at -100 mV. Similarly, in oocytes coexpressing TASK and AT1a angiotensin II receptor, angiotensin II inhibited the

TASK current. These data together indicate that TASK contributes to the generation of high resting potassium permeability of glomerulosa cells, and this background K⁺ channel may be a target of hormonal regulation. (*Molecular Endocrinology* 14: 863–874, 2000)

INTRODUCTION

The most important physiological activators of adrenal glomerulosa cells are angiotensin II (ang II), elevated extracellular (EC) [K⁺], and ACTH. While ACTH acts primarily via cAMP, the other two stimuli elevate cytoplasmic [Ca²⁺]. Binding of ang II to AT1 receptors rapidly activates phospholipase C, which is followed by inositol 1,4,5 trisphosphate (InsP₃) generation and release of Ca²⁺ from intracellular stores. This early phase of the signal is followed by a sustained stimulation that is dependent on Ca²⁺ influx. While observations by different groups indicate the role of capacitative Ca²⁺ entry in the sustained phase (1, 2), in addition to this store-operated mechanism, a further dihydropyridine-sensitive influx component has also been well documented (cf Ref. 3). In addition, InsP₃ generation ang II depolarizes glomerulosa cells (4, 5), which explains the activation of voltage-dependent calcium channels and partial dihydropyridine sensitivity of the calcium and aldosterone response (3, 6, 7).

Whereas after ang II stimulation the source of calcium is both the intracellular store and the EC space, the calcium signal evoked by K⁺ depends exclusively on the influx of Ca²⁺ (8, 9). Voltage-dependent T- and L-type channels have been detected electrophysiologically (6–8, 10, 11) and also by molecular biological methods (12). Their contribution to the calcium signal and activation of steroid synthesis is widely

accepted both during ang II and K^+ stimulation. Considering the significance of voltage-dependent mechanisms, the membrane potential and its alteration during stimulation are of particular interest.

The negative resting membrane potential of glomerulosa cells derives mainly from the high K^+ permeability (13). There were several attempts to characterize the K^+ channel that contributes to the high resting permeability. Inward rectifiers (14) and more recently a weakly voltage-dependent current (15) were suggested as possible candidates. Inhibition of these potassium currents by ang II was demonstrated (15, 16), but the channels responsible for the currents were not identified or further characterized beyond electrophysiological properties.

Recently, a new class of K^+ channels with two pore domains has been described (17–21). Since the permeability of these channels is independent or only slightly dependent on the membrane potential, these background (leak) channels are also good candidates for being involved in the determination of resting membrane potential.

In the present study we demonstrate that TASK, a member of the two-pore domain potassium channel family, is expressed abundantly in glomerulosa cells. Patch clamp results on isolated glomerulosa cells indicated partial pH sensitivity of the potassium conductance. Analysis of the potassium current developing after injection of *Xenopus* oocytes with rat glomerulosa mRNA suggests that this background channel contributes to the potassium conductance of glomerulosa cells. We show that ang II, via AT1 receptors, inhibits TASK substantially. This

mechanism may have a role in the depolarizing effect of ang II in glomerulosa cells.

RESULTS

pH and Angiotensin II Sensitivity of the Potassium Conductance of Glomerulosa Cells

The potassium conductance of adrenal glomerulosa cells was studied by the patch clamp technique. The membrane potential of cells showing 10 G Ω (gigaohm) or higher seal resistance was -82 ± 1 mV ($n = 10$). To study only those channels that operate around the resting membrane potential, the cells were clamped at -100 mV. In normal EC medium (3.6 mM $[K^+]$), the inward current was 56 ± 20 pA ($n = 10$). When the EC $[K^+]$ was elevated to 30 mM, the inward current increased to 307 ± 80 pA ($n = 10$). Acidification of the EC medium from pH 7.4 to 6.7 reduced this current by $17 \pm 2\%$ ($n = 10$). However, changing the EC pH to 6.7 failed to evolve any further inhibition in cells being challenged with ang II (10 nM), which caused significant ($61 \pm 7\%$) inward current inhibition at -100 mV (in 30 mM EC $[K^+]$, pH 7.4, $n = 6$). A biramp voltage protocol (from -100 to $+40$ mV) was applied in each bath solution. The current-voltage curves obtained both at lowered pH (pH 6.7, $n = 10$) and in the presence of ang II (10 nM, $n = 6$) crossed over at $-38 \pm 2\%$ mV with the control curve (in 30 mM EC K^+ , Fig. 1), which corresponds to the calculated K^+ equilibrium potential (-39 mV).

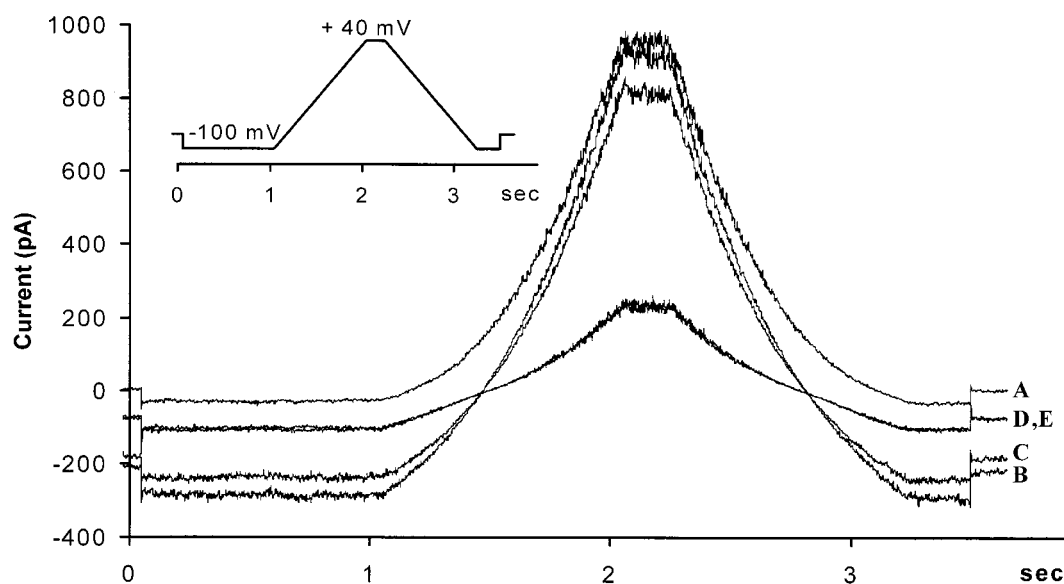


Fig. 1. Inhibition of the currents of a rat adrenal glomerulosa cell by acidification and/or angiotensin II

A, 3.6 mM EC $[K^+]$, pH 7.4; B, 30 mM EC $[K^+]$, pH 7.4; C, 30 mM EC $[K^+]$, pH 6.7; D, 30 mM EC $[K^+]$, pH 7.4, 10 nM angiotensin II; E, 30 mM EC $[K^+]$, pH 6.7, 10 nM angiotensin II. (Biramp depolarizations from -100 mV to $+40$ mV (see inset), the holding potential was -80 mV).

Expression of High Resting K^+ Permeability in *Xenopus* Oocytes

The resting membrane potential (E_m) of oocytes injected with mRNA of rat glomerulosa tissue was more negative than that of water-injected or noninjected oocytes (-80.9 ± 1.6 mV and -44.3 ± 1.7 mV for the 10 most polarized oocytes of the respective group). To characterize the expressed ion conductances responsible for driving E_m close to the K^+ equilibrium potential (E_K), current-voltage (I - V) relationship was measured in solutions containing 2 and 80 mM $[K^+]$. Changing of EC $[K^+]$ from 2 to 80 mM induced the appearance of a noninactivating current in oocytes injected with mRNA (Fig. 2). In oocytes having very negative E_m (-87 mV, or below) in 2 mM $[K^+]$, the shift of reversal potential was 90 ± 1.8 mV ($n = 4$) when EC $[K^+]$ was increased from 2 mM to 80 mM. This value is close to the 93 mV shift predicted for K^+ -selective channels, indicating that potassium channels dominate the membrane conductance. (In oocytes having less negative membrane potential, probably as a consequence of relatively higher leak current or lower level of expression, the contribution of other ions to the

membrane conductance was more notable.) To correct for leak and currents carried by ions other than K^+ , the difference of inward currents in 80 and 2 mM EC $[K^+]$ at -100 mV (I_{D80-2}) was calculated. Control I_{D80-2} (what can be considered as the current of the endogenous K^+ channels of the oocyte) was 111 ± 8 nA in the 10 water-injected or noninjected oocytes having the largest current differences. I_{D80-2} was 1882 ± 120 nA in the 10 mRNA injected oocytes of highest expression.

Comparison of Pharmacology of I_{mRNA} and I_{TASK}

The effect of inhibitors on the inward K^+ current in oocytes injected with glomerulosa mRNA or TASK cRNA was measured in 80 mM $[K^+]$ at -100 mV. Inhibition was expressed in per cent of the control current (80 mM $[K^+]$ at -100 mV (Table 1), and currents in the presence and absence of the inhibitor were corrected for the nonspecific current measured in 2 mM $[K^+]$ (as described above). Neither I_{mRNA} (I_{D80-2} in mRNA injected oocytes) nor I_{TASK} (I_{D80-2} in TASK cRNA injected oocytes) was inhibited significantly by the classical K^+ -channel blockers tetraethylammonium (3 mM) and 4-aminopyridine (3 mM). Slight inhibition was observed with Ba^{2+} (100 μ M) at -100 mV, which was absent at more positive potentials. To study further the characteristics of the voltage-dependent inhibition, the effect of higher concentration of Ba^{2+} (300 μ M) was also tested. Since the Ba^{2+} block developed slowly, long (2 sec) voltage steps were applied to attain steady state at each membrane potential. Ba^{2+} inhibited I_{mRNA} and I_{TASK} with similar kinetics (Fig. 3), and the voltage dependence of steady state inhibition was also similar (Fig. 4, A and B). Applying the model of open pore channel blockers (22) for I_{TASK} results in a dissociation constant (K_D) of 16.8 ± 2.2 mM and $z\delta$ of 0.98 ± 0.03 (the product of the charge of Ba^{2+} and the fraction of the electrical field it has to traverse to reach its binding site), which suggests that the binding site is about halfway in the electrical field. Cs^+ (3 mM) also exerted a voltage-dependent block of I_{mRNA} and I_{TASK} (Fig. 4, C and D). Steady state inhibition by Cs^+ was almost instantaneous. In 3 mM $[Cs^+]$, the inhibition reached its maximum below -110 mV but the inhibition was not complete. I_{mRNA} was reduced by lidocaine (1 mM) or by changing EC pH from 7.5 to 6.7; however, the degree of inhibition was weaker than that of I_{TASK} (Table 1).

Inhibition of I_{mRNA} Expression by TASK Antisense Oligonucleotide

The contribution of TASK to the potassium conductance induced by injection of glomerulosa mRNA into *Xenopus* oocytes was addressed also by an antisense method. The antisense (TASK5'a, 22-mer) oligonucleotide was designed to anneal with the start ATG codon of the TASK mRNA extending 4 bases upstream and 18 bases downstream. The 25-nucleotide long sense

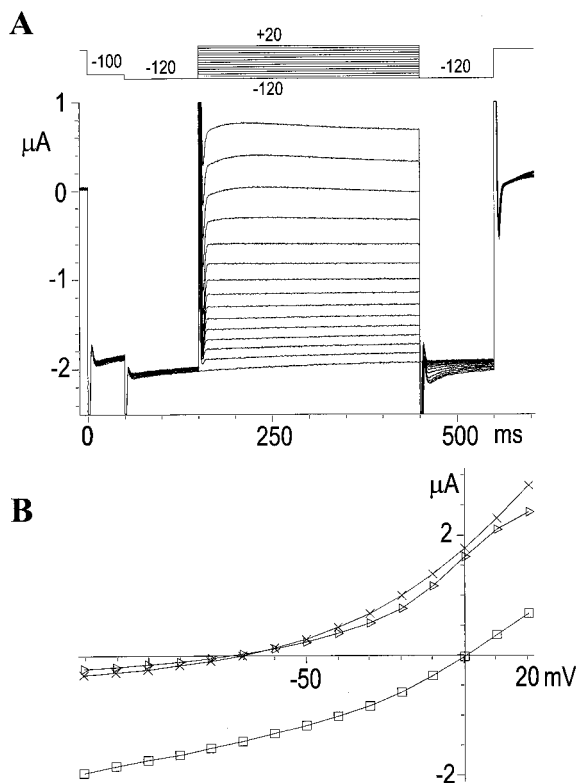


Fig. 2. Currents of *Xenopus* Oocytes Injected with mRNA Purified from Adrenal Capsular Tissue

A, Currents elicited by 300-msec depolarizing voltage steps from -120 to $+20$ mV in 10-mV increments in 80 mM EC $[K^+]$. B, Steady-state current voltage relationship of I_{mRNA} (measured at the end of the 300-msec voltage steps) in 2 mM (\square), 80 mM (\square) EC $[K^+]$ and again in 2 mM EC $[K^+]$ (X) after washout of the high potassium solution.

Table 1. Pharmacology of I_{mRNA} and I_{TASK}

Treatment	Inhibition of I_{mRNA} (%)	n	Inhibition of I_{TASK} (%)	n
TEA (3 mM)	8 ± 3	5	6 ± 2	5
4-AP (3 mM)	2 ± 1	5	6 ± 5	6
Ba ²⁺ (100 μM)	23 ± 2	5	N.D.	0
Ba ²⁺ (300 μM)	41 ± 3	6	39 ± 2	6
Cs ⁺ (3 mM)	71 ± 3	5	82 ± 4	6
Lidocaine (1 mM)	28 ± 2	10	38 ± 6	5
pH 6.7 from 7.5	16 ± 2	14	60 ± 3	3

TEA, Tetraethylammonium; 4-AP, 4-aminopyridine.

oligonucleotide (TASK5's) was used as the negative control being complementary with the antisense nucleotide in a 18-bp segment.

To avoid possible nonspecific effects of the oligonucleotides, they were administered in a second 50-nl injection 2–3 h after the injection of glomerulosa mRNA. The antisense oligonucleotide reduced the expression of I_{mRNA} almost to the small current of the control oocytes injected with water only. The sense oligonucleotide did not have any effect on its own; furthermore, when the same amount of sense and antisense oligos were mixed and injected, the inhibitory effect was partially reverted, confirming the specificity of the antisense approach (Fig. 5).

Ang II inhibits I_{mRNA} and I_{TASK}

Oocytes injected with glomerulosa mRNA or ang II receptor cRNA responded to ang II (10 nM) challenge with a calcium signal that was detected by following the activation of the endogenous Ca²⁺-activated Cl⁻ channel of the oocyte. In standard EC solution ([K⁺] = 2 mM) at negative membrane potentials (–80 or –100 mV), the activation of the channel appears only as a small inward current, while it is represented as a much larger increase of the outward current at +20 mV (Fig. 6A). In oocytes perfused with high potassium solution ([K⁺] = 80 mM) alteration of the K⁺ permeability is expected to influence the current minimally at +20 mV (as this potential is close to E_K). Hence, current changes at +20 mV were dominated by the Ca²⁺-dependent Cl⁻ current.

On the other hand, in oocytes having robust I_{mRNA} or I_{TASK} expression, the major component of the inward current at –100 mV in high [K⁺] solution was the potassium influx, since the Ca²⁺-activated Cl⁻ current was relatively small at –100 mV (even during ang II stimulation; see Fig. 6A). Thus, during stimulation the Ca²⁺ signal and the changes of the K⁺ current could be detected simultaneously and fairly separately by measuring the current alternately at –100 and +20 mV in high [K⁺] solution. Using this experimental protocol I_{mRNA} (measured at –100 mV in 80 mM [K⁺]) was slightly reduced by ang II mainly in those oocytes that showed the largest inward current before stimulation, indicating high level expression (Fig. 6B), while in many cells the inhibition was barely detectable.

To address the question whether the inhibitory effect of ang II can be related to the modulation of TASK activity, angiotensin (AT1a) receptor and TASK cRNAs were coinjected into oocytes. Ang II (10 nM) was applied in the superfusion medium, and its effect on the currents at –100 and +20 mV was followed for 3–5 min. Ang II reduced I_{TASK} by 77 ± 2.7% (n = 5, Fig. 7). Inhibition of TASK was maintained and only slowly diminished in time after ang II had been withdrawn while activation of the Ca²⁺-activated Cl⁻ current had a transient component and after reaching a peak value it was quickly reduced to a sustained level.

Molecular Biological Verification of TASK Expression in Glomerulosa Cells

The PCR product generated by TASK1s and TASK1a (two degenerate primers for both TASK and TWIK, flanking a nonconserved region of the two mRNAs) was cloned, sequenced, and identified as the appropriate fragment of rat TASK. In addition to this fragment the total coding region of TASK in two separate overlapping (5' and 3') parts was amplified (TASK5's-TASK1a, 1–608 n and TASK1s-TASK3'a 249–1233 n) and sequenced. The sequence of these products corresponded to that of rat TASK. TASK-specific PCR product was amplified (in two independent experiments) from single glomerulosa and fasciculata-reticularis cells, which derived from capsular and decapsular cell cultures, respectively. Under identical conditions in nested PCR reaction, the ratio of TASK-positive cells was higher in glomerulosa (28 positive of 29 cells) than in fasciculata-reticularis cells (21 positive of 29 cells, Fig. 8), which difference was statistically significant ($P < 0.05$).

Northern blots were performed for comparing the TASK mRNA content of rat glomerulosa tissue to that of other rat tissues using the labeled TASK1s-TASK1a fragment (249–608 nucleotides) as a probe. Since the heart was found to be the most abundant source of rat TASK (23), and TASK in the mouse was shown to be expressed in the atria rather than in the ventricles of the heart (18), we prepared RNA from rat atria. TASK expression in rat adrenal capsule was the highest among the examined tissues (Fig. 9). In one experiment another TASK-specific probe (*Pst*I-SacI fragment of human TASK), homologous to 1016–1228 nu-

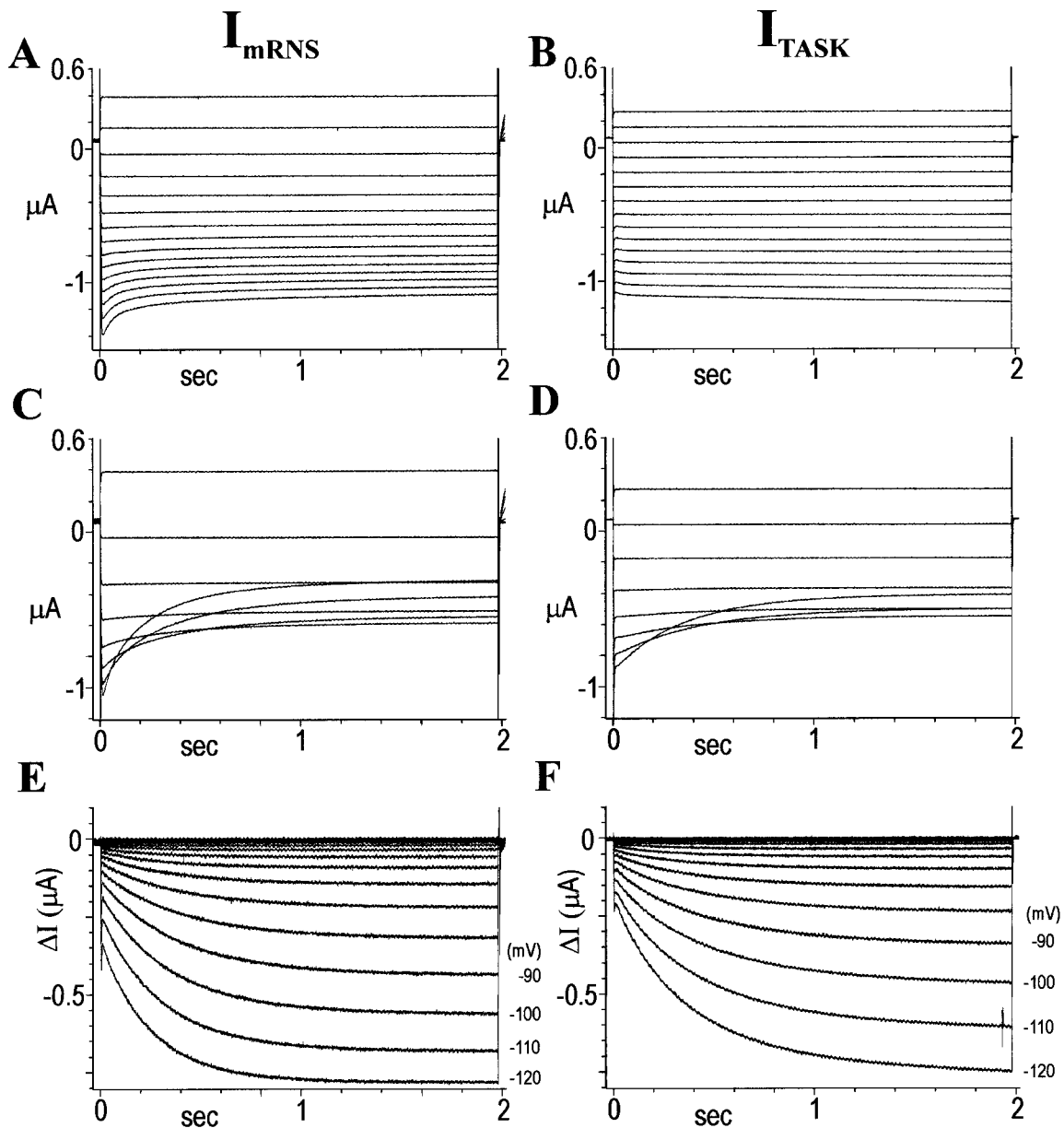


Fig. 3. Time Dependence of the Ba^{2+} Block

I_{mRNS} (A and C) and I_{TASK} (B and D) in high (80 mM) $[\text{K}^+]$ solution in the absence (A and B) and in the presence (C and D) of $300 \mu\text{M}$ Ba^{2+} . Oocytes were held at 0 mV and currents were recorded during 2 s voltage steps from -120 to $+20$ mV in 10-mV increments. (In C and D only every second trace is shown.) The inhibition is calculated by subtracting the currents in the presence of Ba^{2+} (C and D) from the currents in the absence of Ba^{2+} (A and B), respectively, and the difference is plotted [I_{mRNS} (panel E) and I_{TASK} (panel F)].

cleotides of rat TASK coding region was used and gave similar results (data not shown).

DISCUSSION

Sensitivity to physiological changes of EC $[\text{K}^+]$ (24, 25), maintenance of highly negative membrane potential (13, 15, 26), and the importance of voltage-sensitive mechanisms in the action of physiological stimuli on glomerulosa cells (27) urged several researchers to

study the potassium channels of this tissue. The first detailed analysis of electrical properties of rat adrenal glomerulosa cells was performed by Quinn and co-workers (13) using whole-cell patch-clamp methods (13). While around and below the resting membrane potential the voltage-current relationship followed the constant field equation, it showed an outward rectification under depolarized conditions. The increased conductance in the depolarized state was assigned to opening of voltage- and/or Ca^{2+} -activated K^+ channels. In addition to the noninactivating delayed rectifier

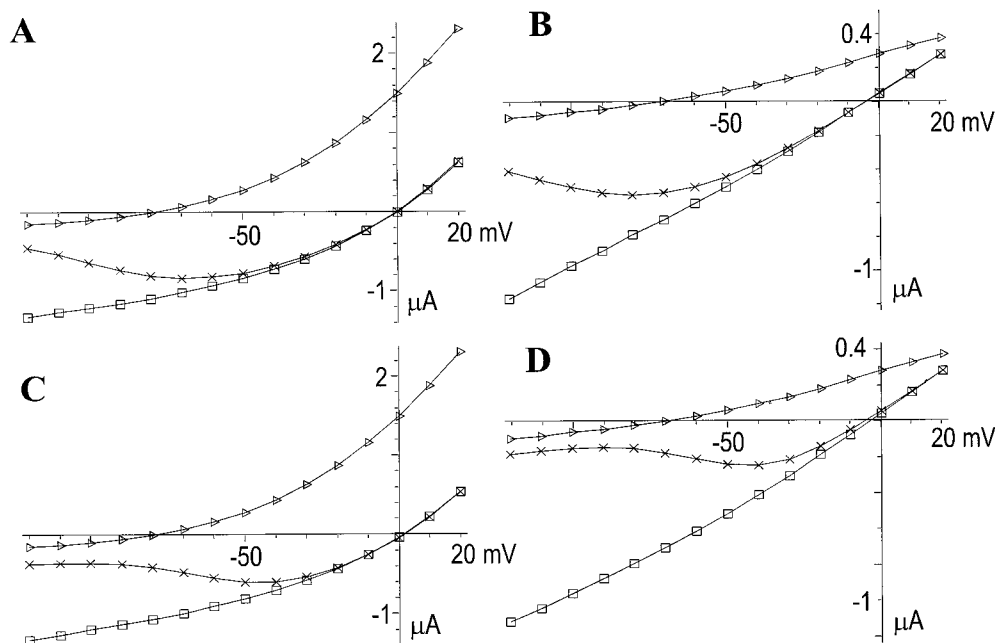


Fig. 4. Voltage Dependence of Ba^{2+} and Cs^+ Block

Steady state current-voltage relationship was measured at the end of 2 s voltage steps (see in the legend to Fig. 3) in low (2 mM, \triangleleft) and high (80 mM) $[\text{K}^+]$ solutions in the presence (X) and absence (\square) of the blocking ion. (I_{mRNA} , 300 μM Ba^{2+} (A); I_{TASK} , 300 μM Ba^{2+} (B); I_{mRNA} , 3 mM Cs^+ (C); I_{TASK} , 3 mM Cs^+ (D).

K^+ currents (which were present in all species) a transient (A type) current was also detected in human (16, 28) and bovine (16) glomerulosa cells. Studies at the single-channel level revealed further members of the channel repertoire. Inward rectifier K^+ channels were observed frequently in membrane patches of rat (and bovine) glomerulosa cells (29). Delayed rectifiers (29) and Ca^{2+} -activated maxi- K^+ channels (15) were also well distinguished.

While these channels may have a role in restoring the resting condition from a stimulated state, they presumably are not involved in generation of the negative E_{m} , since they require depolarization and/or high intracellular $[\text{Ca}^{2+}]$ for opening. Only the inwardly rectifying K^+ channel would be appropriate for maintaining the very negative E_{m} ; however, it could not be detected at the macroscopic current level (15, 29). A challenging question is what is behind the discrepancy between single-channel and macroscopic current results. Lotshaw (15) described a weakly voltage-dependent (background) potassium channel in nystatin-perforated cells and suggested that it has a major role in the control of the resting membrane potential. Rapid run down may have hampered the detection of this current in single-channel measurements; nevertheless, the characteristics of a single-channel conductance described and interpreted as delayed rectifier (29) are consistent with this background channel. Our results also provide evidence that during hyperpolarization (at -100 mV) potassium channels, which do not show inward rectification, are mainly responsible for the membrane conductance. This conductance is

pH sensitive, in accordance with the previously observed stimulation of aldosterone production by acidic pH (30, 31). In an attempt to gather more information about the channels responsible for this conductance, more detailed characterization was performed in a heterologous expression system.

A wide range of K^+ channels, including several inwardly rectifying ones, were successfully expressed and studied in oocytes. Although the rate and the time dependence of expression of distinct channels may be different, this system provides significant advantages and may be optimal for characterizing the dominant K^+ conductance of a cell type by injecting its mRNA into oocytes. It should be recalled, however, that endogenous *Xenopus* channels may influence the membrane conductance. Oocytes have usually only small endogenous currents at membrane potentials below -40 mV; however, Cl^- channels, activating slowly in response to hyperpolarization and sometimes conducting substantial currents, were described to be present occasionally in particular oocyte batches (32). In our experiments, some oocyte batches also showed this type of current; however, it could be corrected for by calculating the difference of the inward currents in 80 and 2 mM EC $[\text{K}^+]$ ($I_{\text{D80-2}}$). The effect of inhibitors was measured in oocytes, where the hyperpolarization-activated current was negligible.

The K^+ current measured at -100 mV in oocytes injected with mRNA prepared from adrenal glomerulosa or with TASK cRNA (I_{mRNA} and I_{TASK} , respectively) was not affected by the conventional K^+ channel blockers. It was minimally inhibited by 100 μM Ba^{2+} ,

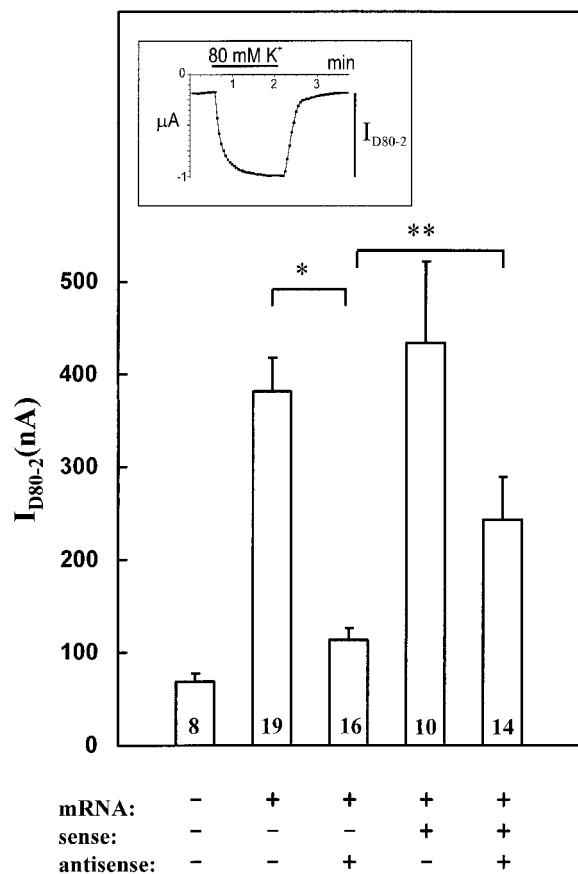


Fig. 5. Effects of the Sense and Antisense Oligonucleotides on the Expression of I_{mRNA}

Currents were recorded at the end of 300-msec voltage pulses to -100 mV from a holding potential of -30 mV in every 3 sec. I_{D80-2} was estimated as the difference of the maximum of current in 80 mM $[K^+]$ and the average of the currents in 2 mM $[K^+]$ before and after the high K^+ solution (see inset). Numbers in the bars represent number of oocytes deriving from two frogs. (*, $P < 10^{-5}$; **, $P < 0.0015$).

which argues against the significant contribution of most of the inwardly rectifying K^+ channels to the current. Voltage dependence of inhibition by higher concentration of Ba^{2+} ($300 \mu M$) and Cs^+ (3 mM) was similar in the case of I_{mRNA} and I_{TASK} . A further similarity between I_{TASK} and I_{mRNA} is their slowly developing inhibition by $300 \mu M$ Ba^{2+} , the time dependence of which is not general even among the members of the tandem pore domain K^+ channel family (33). While the parameters of the Ba^{2+} binding site can be calculated according to the original open-channel block model (22), the steep voltage dependence of the Cs^+ inhibition, which cannot be explained by this model, indicates a more compound mechanism of blockage (34). Accordingly, TASK probably has a multion conducting pore (35) similar to many other K^+ channels (34). Local anesthetics and decreased EC pH inhibit human and rat TASK (18, 23). Calculation with the inhibition of I_{mRNA} and I_{TASK} by lidocaine (1 mM) or by EC acidification indicates that I_{TASK} is responsible for at least

25% of I_{mRNA} . If we consider this minimum contribution of TASK to I_{mRNA} suggested by the EC acidification, then the additional component of I_{mRNA} shows significant pharmacological similarities to the two-pore domain background potassium channels.

The TASK antisense oligonucleotide prevented the expression of I_{TASK} (data not shown) and also reduced the expression of I_{mRNA} by 85%. Control experiments confirmed the specificity of the antisense effect. The sense oligo failed to inhibit the expression of I_{mRNA} ; moreover, when coinjected with the antisense one, it reduced the inhibitory effect of the latter. Considering that the sense and the antisense oligonucleotides were complementary, this means that only the single-stranded form of the antisense was effective, which indicates its specificity. Partial reversal of the inhibition is probably due to incomplete formation of oligonucleotide dimers (although theoretically a limited nonspecific effect cannot be ruled out). Almost complete inhibition of I_{mRNA} by TASK antisense oligonucleotide raises the possibility that TASK expressed by injection of glomerulosa mRNA might have partially different pharmacological properties from pure I_{TASK} . It would be conceivable if another pore-forming or auxiliary subunit cooperating with TASK was presumed.

Depolarization of glomerulosa cells by ang II has been demonstrated both by fluorimetric (5, 36) and electrophysiological methods (4, 37). The depolarization may be attributed principally to sustained inhibition of K^+ permeability (38, 39), while inhibition of the Na^+, K^+ -ATPase (40), as well as opening of a nonspecific cation channel (41), may contribute to the effect. As to K^+ permeability, patch-clamp studies revealed ang II-induced inhibition of inward rectifier (14), delayed rectifier K^+ (14, 15), and weakly voltage-dependent currents (15). While inhibition of delayed rectifying K^+ channels may prolong but may not initiate depolarization, the significance of inward rectifiers is questionable (see above). Therefore, inhibition of the weakly voltage-dependent K^+ current, which was confirmed in the present experiments, may account predominantly for the depolarizing action of ang II in rat glomerulosa cells.

In oocytes injected with mRNA prepared from glomerulosa tissue, ang II also reduced the inward current at -100 mV in 80 mM $[K^+]$, which indicates the inhibition of the expressed I_{mRNA} . The possible reason why the degree of the detected inhibition was smaller than that observed in glomerulosa cells could be the concomitant activation of the calcium-activated Cl^- and/or K^+ channels (the latter possibly introduced by adrenal glomerulosa mRNA). This may have partially masked the inhibitory effect mainly in oocytes where the expression of I_{mRNA} was moderate. Toxicity of glomerulosa mRNA limited the achievable I_{mRNA} expression, and coinjection of ang II receptor cRNA with mRNA failed to increase the apparent inhibition at -100 mV by ang II (result not shown). We examined whether such regulation could be exerted via inhibition of TASK. When TASK and AT1a angiotensin II receptor

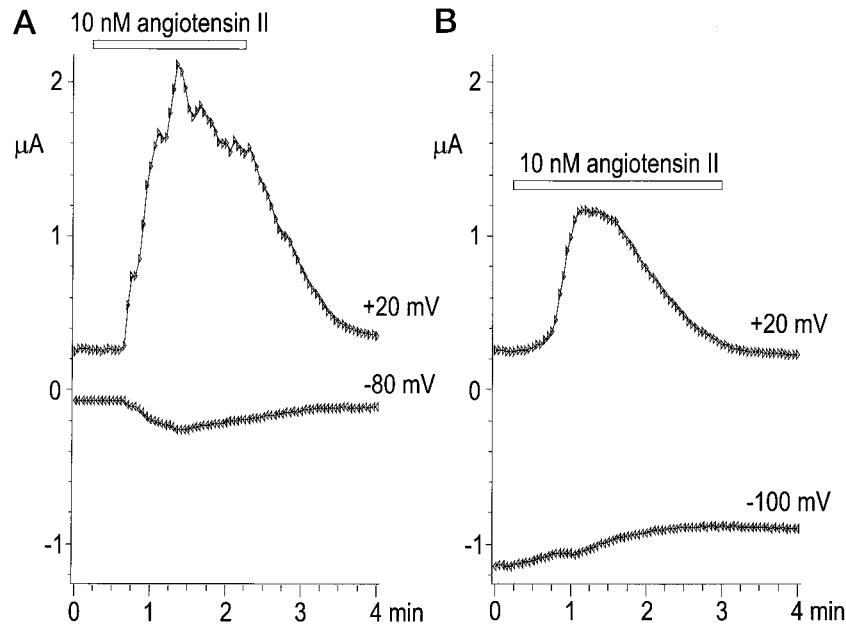


Fig. 6. Effect of Angiotensin II (10 nM) on the Current Measured at -100 (or -80) and $+20$ mV in 2 and 80 mM EC $[K^+]$ in Oocytes Injected with Adrenal Capsular mRNA

A, EC $[K^+]$ was 2 mM. The holding potential was -80 mV, and 300-msec depolarizing voltage steps (to $+20$ mV) were applied every 3 sec. Currents at -80 mV measured before the step and currents measured at the end of the step were plotted. B, EC $[K^+]$ was 80 mM. Every 3 sec the voltage protocol (-100 mV for 300 msec, 0 mV for 250 msec, $+20$ mV for 300 msec, depicted in Fig. 7) was applied from a holding potential of 0 mV. Currents at the end of the steps to -100 mV and $+20$ mV were plotted.

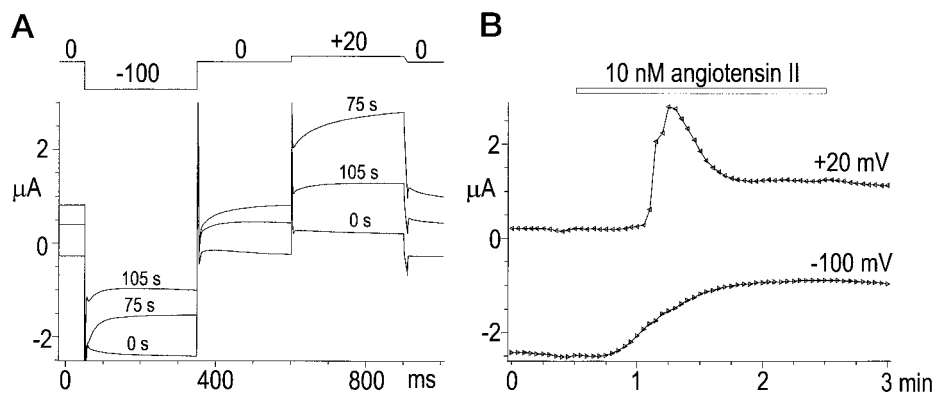


Fig. 7. Inhibition of TASK by Angiotensin II (10 nM) in an Oocyte Coinjected with TASK and Angiotensin II Receptor cRNAs

A, Every 3 sec the voltage protocol (see *inset*) was applied from a holding potential of 0 mV. Currents before ang II stimulus (0 sec), at the peak of the Ca^{2+} -activated current (75 sec) and after the developing of the maximum inhibition of I_{TASK} (105 sec) are shown. B, Currents at the end of the step to -100 mV and at the end of the step to $+20$ mV were plotted as the function of time.

were coexpressed, a dramatic inhibition of I_{TASK} was observed as a result of ang II stimulation.

In conclusion, we demonstrated that TASK, a background potassium channel, is abundantly expressed in adrenal glomerulosa cells. TASK is a significant component of the potassium conductance expressed in oocytes after injection of glomerulosa mRNA; thus, it may contribute to the maintenance of the highly negative membrane potential in adrenal glomerulosa cells. Activation of angioten-

sin II (AT1a) receptor inhibits TASK; therefore, this channel is a target for the depolarizing action of ang II and it may be a component of the complex signal transduction routes used by the peptide *in vivo*. To our knowledge this is the first demonstration that a K^+ channel of the tandem pore domain family is inhibited by a Ca^{2+} -mobilizing hormone. The signaling pathway of this inhibition and its contribution to the physiological function of intact glomerulosa cells remain to be established.

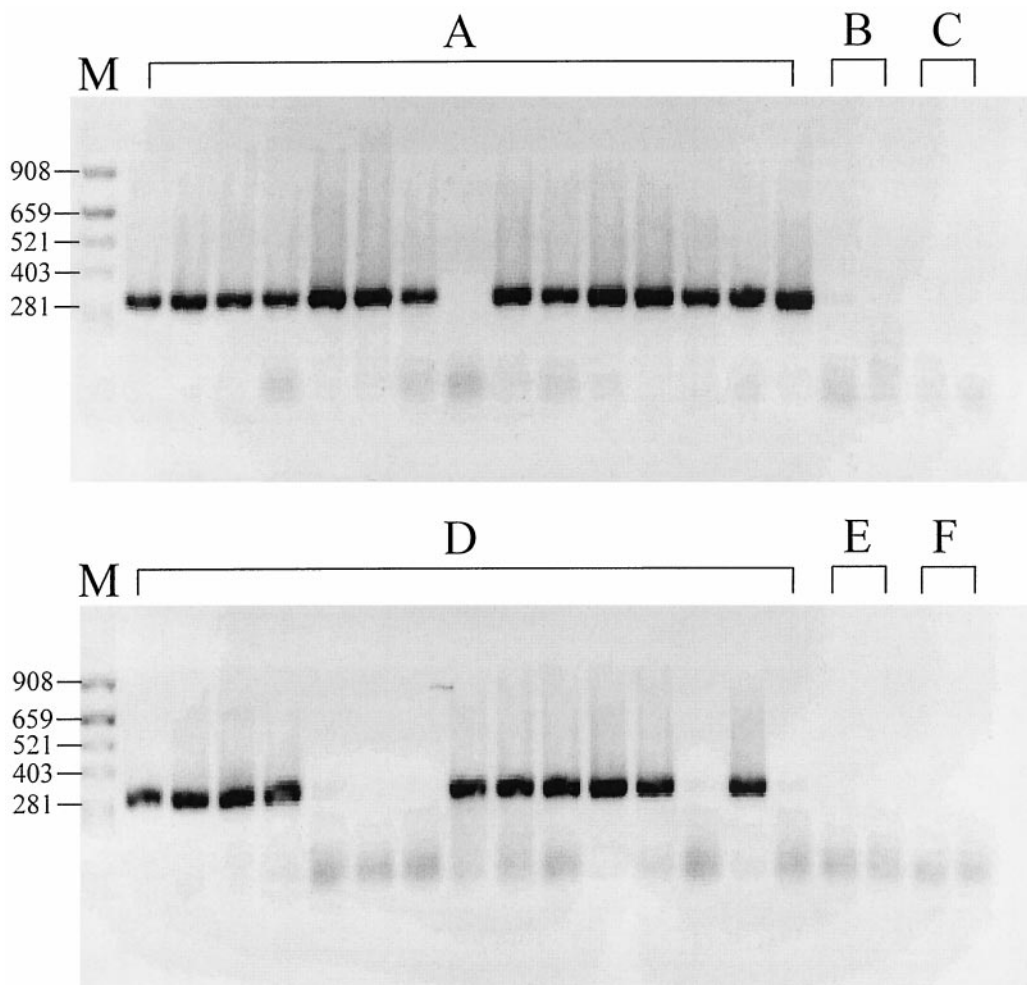


Fig. 8. Single-Cell PCR for Detection of TASK mRNA

Nested RT-PCR was performed from single adrenal glomerulosa cells (A), tissue culture medium of glomerulosa cells (B), single adrenal glomerulosa cells without reverse transcription (C), single adrenal fasciculata/reticularis cells (D), tissue culture medium of fasciculata/reticularis cells (E) and single adrenal fasciculata/reticularis cells without reverse transcription (F). The expected size of the PCR product is 302 bp. (Representative one of two experiments).

MATERIALS AND METHODS

Chemicals

Enzymes and kits for molecular biological studies were purchased from Ambion, Inc. (Austin, TX), Amersham Pharmacia Biotech (Little Chalfont, UK), Fermentas (Vilnius, Lithuania), New England Biolabs, Inc. (Beverly, MA), Pharmacia Biotech (Uppsala, Sweden), and Promega Corp. (Madison, WI). Fine chemicals of analytical grade were obtained from Fluka Chemical Co. (Buchs, Switzerland), Promega Corp., and Sigma (St. Louis, MO). [α - 32 P]dCTP, [α - 35 S]dCTP, and [α - 32 P]dATP were from Izinta (Budapest, Hungary).

Animals and Tissue Preparation

Mature female *Xenopus laevis* frogs were obtained from Amrep Reptielen (Breda, The Netherlands). Frogs were anesthetized by immersing them into benzocaine solution (0.03%). Ovarian lobes were removed, the tissue was dissected and treated with collagenase (1.45 mg/ml, 148 U/mg, type I, Worthington Biochemical Corp. (Freehold, NJ) followed by

continuous mechanical agitation in Ca^{2+} -free OR2 solution containing (in mM): NaCl, 82.5; KCl, 2; MgCl_2 , 1; HEPES, 5; pH 7.5) for 1.5–2 h. Stage V and VI oocytes were defolliculated manually and kept at 19 C in modified Barth's saline containing (in mM): NaCl, 88; KCl, 1; NaHCO_3 , 2.4; MgSO_4 , 0.82; $\text{Ca}(\text{NO}_3)_2$, 0.33; CaCl_2 , 0.41; HEPES, 20, buffered to pH 7.5 with NaOH and supplemented with penicillin (100 U/ml), streptomycin (100 $\mu\text{g}/\text{ml}$), sodium pyruvate (4.5 mM), and theophyllin (0.5 mM).

Wistar rats (250–350 g, Charles River Kft., Budapest, Hungary) were stunned before decapitation, and the adrenal glands were removed. Capsular tissue (containing the zona glomerulosa with the fibrous capsule) and decapsulated tissue (containing the inner cortical zones and the adrenal medulla) were separated macroscopically according to standard methods (42). Glomerulosa and fasciculata-reticularis cells, respectively, were prepared using a collagenase digestion technique as previously described (43). The contamination of this type of glomerulosa cell preparation was tested previously by electron microscopic analysis and was found to be less than 5%. Isolated cells were plated onto poly-L-lysine-coated (1 $\mu\text{g}/\text{cm}^2$) Petri dishes and were kept in a CO_2 (5%) incubator at 37 C in a mixture (38:62, vol/vol) of modified Krebs-

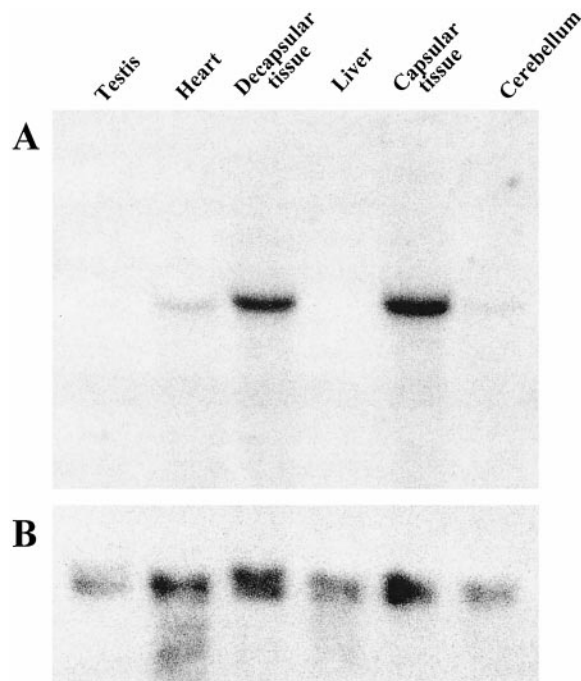


Fig. 9. Expression of TASK mRNA in Different Rat Tissues. A, Representative Northern blot (10 μ g RNA) with the rat TASK1s-TASK1a probe. B, GAPDH was used as the reference signal. After subtracting background counts of the TASK bands were divided by the count of the corresponding GAPDH band (\Rightarrow quotient). Dividing the quotients of different tissues by the quotient of capsular tissue in the same blot resulted in the following values: capsular tissue (1); decapsular tissue (0.52 ± 0.07); cerebellum (0.08 ± 0.03); atria of heart (0.07 ± 0.01); liver and brain below 0.05 (n = 5 Northern blots).

Ringer-bicarbonate-glucose solution and Medium 199 (K^+ , 3.6 mM; Ca^{2+} , 1.2 mM; Mg^{2+} , 0.5 mM). The medium was completed with 100 U/ml penicillin and 100 μ g/ml streptomycin. Glomerulosa and fasciculata-reticularis cells for molecular biological studies were selected according to standard criteria [size and lipid droplets (44)] from the respective capsular and decapsular preparations after one-day culturing.

The treatment of animals was conducted in accordance with state laws and institutional regulations. The experiments were approved by the Animal Care and Ethics Committee of the Semmelweis University.

Injection of *Xenopus laevis* Oocytes

Oocytes were injected 1 day after defolliculation. Fifty nanoliters of the appropriate RNA solution were delivered with Nanoliter Injector (World Precision Instruments, Sarasota, FL). In experiments designed to test the effect of sense and antisense TASK oligonucleotides on the expression of glomerulosa tissue mRNA-induced inward current, the oligonucleotides were administered in a second injection (TASK5'a: 5'-CACATTCTGCCGCTTCATCGTC-3' (70 μ M, 50 nl) and TASK5's: 5'-GGCATATGAAGCGGCAGAATGTGCG-3' (90 μ M, 50 nl) 2–3 h after the injection of mRNA. Currents were measured 3 or 4 days after the injection(s).

Electrophysiology

Patch-Clamp Recordings For ion current measurements on adrenal glomerulosa cells, the whole-cell patch-clamp

technique (45) was applied. The standard EC solution had the following composition (mM): NaCl, 137; KCl, 3.6; $MgCl_2$, 0.5; $CaCl_2$, 2; glucose, 11; HEPES, 10; piperazine-*N,N'*-bis[2-ethanesulfonic acid] (PIPES), 3.3 [pH 7.4 or pH 6.7 (NaOH)]. Pipettes were pulled from borosilicate glass Clark GC120TF-10 (Clark Electromedical, Pangburne, Reading, UK) by a P-87 puller (Sutter Instrument Co., Novato, CA) and fire polished. Pipette resistance ranged between 4 and 6 M Ω when filled with the intracellular solution, containing (mM): KCl, 135; $MgCl_2$, 2; $CaCl_2$, 0.05; EGTA, 1; Na-ATP, 2; HEPES, 10; pH 7.3 (KOH). The pipette was connected to the headstage of a patch-clamp amplifier [Axopatch-1D (Axon Instruments, Inc., Foster City, CA) or RK-400 (Biological, Claix, France)] which was mounted on a PCS-750/1000 manipulator (Burleigh Instruments, Inc., Fishers, NY). Seal resistance was about 10 G Ω . The capacitance of the selected glomerulosa cells amounted to 5–10 pF. Series resistance was about 10 M Ω . Data were filtered at 1 kHz (-3 dB; 4-pole, low-pass Bessel filter) and digitally sampled at 4 kHz by a Digidata 1200 interface board (Axon Instruments, Inc.), stored, and later analyzed by PC/AT computer. Experiments, data storage, and analysis were performed with pClamp software, version 6.0 (Axon Instruments, Inc.). Solutions were applied by a gravity-driven perfusion system.

Two-Electrode Voltage Clamp Membrane currents of oocytes were recorded by two-electrode voltage clamp (OC-725-C, Warner Instrument Corp., Hamden, CT) using microelectrodes made of borosilicate glass (Clark Electromedical Instruments, Pangbourne, UK) with resistance of 0.3–3 M Ω when filled with 3 M KCl. Currents were filtered at 1 kHz, digitally sampled at 1–2.5 kHz with a Digidata Interface (Axon Instruments, Inc.), and stored on a PC/AT computer. Recording and data analysis were performed using pCLAMP software 6.0.4 (Axon Instruments, Inc.). Experiments were carried out at room temperature, and solutions were applied by a gravity-driven perfusion system. Low [K^+] solution contained (in mM): NaCl, 95.4; KCl, 2; $CaCl_2$, 1.8; HEPES, 5. High [K^+] solution contained 80 mM K^+ (78 mM Na^+ of the low [K^+] solution was replaced with K^+). Unless otherwise stated, the pH of every solution was adjusted to 7.5 with NaOH.

mRNA Purification and cRNA Synthesis

Total RNA was extracted from different rat tissues as previously described, using the phenol-chloroform-guanidium isothiocyanate method (12). mRNA was purified on oligo dT-cellulose (Pharmacia Biotech or New England Biolabs, Inc.), aliquoted, and stored at -70 C. TASK and ang II receptor cRNA were synthesized *in vitro* according to the manufacturer's instructions (Ambion, Inc. mMESSAGE mMACHINE T7 *In vitro* Transcription Kit) using the *Xho*I-linearized pEXO-TASK construction (18), which contained the total coding region of human TASK and a *Not*I-linearized plasmid construction comprising the coding sequence and 5'-untranslated region of rat AT1a receptor (a gift from Dr. L. Hunyady).

RT-PCR and Single-Cell PCR

TASK cDNA fragments were amplified by *Taq* DNA polymerase using TASK1s (5'-SYTCTWCTTCGCCAKACCG-3') or TASK5's sense and TASK1a (5'-CCSARGCCRATGGTGST-SAG-3') or TASK3'a (CACKGAGCTCCTGCGCTTCATG) antisense oligonucleotides after reverse transcription (MMLV-RT, random hexamers from Promega Corp.) of 1 μ g of total RNA prepared from rat glomerulosa tissue. TASK1s and TASK1a were designed to amplify not only TASK but also the cDNA of TWIK (17). The first denaturing step (94 C, 120 sec) was followed by 35 cycles of denaturation (30 sec at 94 C), annealation (60 sec at 50 C), and extension (90 sec at 72 C). The TASK1s-TASK1a product was cloned into pBluescript KS- (Stratagene, La Jolla, CA) vector with blunt ends and

sequenced according to standard methods (Sequenase II kit, United States Biochemical Corp., Cleveland, OH). 5'- and 3'-parts of the mRNA were amplified by TASK5's-TASK1a and TASK1s-Task3'a primers, respectively. For single-cell PCR, individual glomerulosa or fasciculata-reticularis cells were selected microscopically from the appropriate capsular or decapsular cell preparation, respectively, and were placed into RT reactions after freezing and thawing. Nested PCR was performed with primer pairs TASK1s and 5'-TCCTTCT-GCAGCGCCACGTAG-3' in the first, and 5'-ACGGACGGAG-GCAAGGTGTTTC-3' and TASK1a in the second reaction. Tissue culture medium after RT or cells without RT were used as controls. PCR conditions were the same as above both in the first and the second reaction.

Northern Blot Analysis

Ten micrograms of total RNA from different tissues were loaded and run on 1% agarose formaldehyde gel after denaturation. Electrophoretic separation of the RNA was followed by its transfer to Hybond nylon membrane (Amersham Pharmacia Biotech). TASK probe was generated by random primer labeling the 360-bp TASK1s-TASK1a PCR product or the 212-bp *Pst*I-SacI fragment of the human TASK clone (18) with [³²P]dCTP. Hybridization was carried out at 42 C for 24 h. After hybridization the blot was washed successively in buffers of 1× SSPE + 0.1% SDS twice for 30 min at room temperature and 0.1× SSPE + 0.1% SDS twice for 30 min at 65 C (46). After detection of the radioactivity by Phosphorimager (model GS-525, Bio-Rad Laboratories, Inc. Hercules, CA), the membrane was stripped and reprobed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reference signal as previously described (12).

Statistics

Data are expressed as means ± SEM. Statistical significance was estimated by the nonparametric Mann-Whitney *U* test, or the nonparametric Fisher exact test [STATISTICA program package (StatSoft, Tulsa, OK)].

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