A-Type K⁺ Current in Neurons Cultured From Neonatal Rat Hypothalamus and Brain Stem: Modulation by Angiotensin II

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Wang, Desuo, Colin Sumners, Philip Posner, and Craig H. Gelband. A-type K⁺ current in neurons cultured from neonatal rat hypothalamus and brain stem: modulation by angiotensin II. J. Neurophysiol. 78: 1021–1029, 1997. The regulation of A-type K⁺ current (Iₐ) and the single channel underlying Iₐ in neonatal rat hypothalamus/brain stem cultured neurons were studied with the use of the patch-clamp technique. Iₐ had a threshold of activation between −30 and −25 mV (n = 14). Steady-state inactivation of Iₐ occurred between −80 and −70 mV and had a membrane voltage at which Iₐ was half-maximum at −52.2 mV (n = 14). The mean values for the activation and inactivation (decay) time constants during a voltage step to +20 mV were 2.1 ± 0.3 ms (n = 8) and 13.6 ± 1.9 ms (n = 8), respectively. Single-channel recordings from outside-out patches revealed A-type K⁺ channels with voltage-dependent activation, 4-aminopyridine (4-AP) sensitivity, and inactivation kinetics similar to those of Iₐ. The single-channel conductance obtained from cell-attached patches was 15.8 ± 1.3 pS (n = 4) in a physiological K⁺ gradient and 41.2 ± 3.7 pS (n = 5) in symmetrical 140 mM K⁺. Angiotensin II (Ang II, 100 nM) markedly reduced single A-type K⁺ channel activity by decreasing open probability (n = 4). The actions of Ang II on Iₐ and single A-type K⁺ channels were reversible either by addition of the selective angiotensin type 1 (AT₁) receptor antagonist losartan (1 μM) or on washout of the peptide. Thus, the activation of AT₁ receptors inhibits a tetraethylammonium-chloride-resistant, 4-AP-sensitive Iₐ, and single A-type K⁺ channels, and this may underlie some of the actions of Ang II on electrical activity of the brain.

INTRODUCTION

In the nervous system, A-type K⁺ current (Iₐ) plays an important role in the control of excitability, synaptic input, and neurotransmitter release (Cull-Candy et al. 1989; Dye 1991; Meir et al. 1995; Sheng et al. 1993). Iₐ may be responsible for the initial repolarization of the action potential, contribute to afterhyperpolarization, promote pacemaker activity, and facilitate neuronal firing (Bouskila and Dudek 1995; Chandler et al. 1994; Connors and Stevens 1971; Dekin 1993; Dureux and Puiziillout 1995; Grolleau and Lapied 1995; MacDermott and Weight 1982). Iₐ is known to be present in a variety of mammalian nervous tissues including sympathetic ganglia (Galvan and Sedlmeier 1984), hypothalamus, and hippocampal brain slices (Bouskila and Dudek 1995; Greene et al. 1990; Zbic and Weight 1985), neonatal supraoptic nucleus neurons (Cobett et al. 1989), and embryonic hypothalamus neurons (Muller et al. 1992). Electrophysiologically, Iₐ can be identified by its unique voltage-dependent activation, fast kinetics of inactivation, and steady-state inactivation. Pharmacologically, Iₐ is aminopyr-
Electrophysiological recordings

Results are expressed as means ± SEM. Statistical significance was evaluated with the use of paired t-test. Differences were considered significant at P < 0.05; n corresponds to the number of cells examined. Open probability (P_o) for single A-type K^+ channels was obtained during 100-ms depolarizing pulses.

Preparation of neuronal cultures

Neuronal cocultures were prepared from the brain stem and a hypothalamic block of 1-day-old Sprague-Dawley rats as described previously (Kang et al. 1992). Trypsin (375 U/ml) and DNase I (496 U/ml)-dissociated cells were resuspended in DMEM containing 10% PDHS and plated on poly-L-lysine precoated 35-mm Nunc plastic tissue culture dishes. After cells were grown for 3 days at 37°C in a humidified incubator with 95% air-5% CO2, they were exposed to 1 μM ARC for 2 days in fresh DMEM containing 10% PDHS. Then ARC was removed and the cells were incubated with DMEM (10% PDHS) for a further 9–12 days before use. At the time of use, cultures consisted of 90% neurons and 10% astrocyte glia, as determined by immunofluorescent staining with antibodies against neurofilament proteins and glial fibrillary acidic proteins (Summers et al. 1990).

Electrophysiological recordings

Macroscopic and single-channel currents were recorded with the use of the whole cell, outside-out, and cell-attached patch-clamp configurations of the patch-clamp technique (Hamill et al. 1981). Experiments were performed at room temperature (22–23°C) with the use of an Axopatch-200A amplifier and Digidata 1200A interface (Axon Instruments, Burlingame, CA). Cells were bathed in Tyrode’s solution containing (in mM) 140 NaCl, 5.4 KCl, 2.0 CaCl2, 2.0 MgCl2, 0.3 NaH2PO4, 10 HEPEs, and 10 dextrose, pH adjusted to 7.4 with NaOH. Neurons in the culture dish (volume 1.5 ml) were superfused at a rate of 2–4 ml/min. The patch electrodes (Kimax-5.1, Kimble Glass, Toledo, OH) had resistances of 3–4 MΩ when filled with an internal pipette solution containing (in mM) 140 KCl, 2 MgCl2, 5 ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 4 ATP, 0.1 guanosine 5'-triphosphate, 10 dextrose, and 10 HEPEs, pH adjusted to 7.2 with KOH. Cell-attached patches were formed by sealing the patch electrode to the cell body. Single-channel activity was recorded from patches with a seal resistance >5 GΩ. The whole cell configuration was formed by applying negative pressure to the patch electrode. For whole cell recordings, cell capacitance was canceled electronically and the series resistance (<10 MΩ) was compensated by 75–80%. Outside-out patches were formed by slowly elevating the patch electrode away from the cell body after formation of the whole cell configuration. A junction potential of −10 mV was corrected for all command potentials.

Sodium current (I_{Na}) was blocked by TTX (100 nM). Calcium current (I_{Ca}) was blocked by CdCl2 (100 μM). Ca2+-activated K+ current (I_{KCa}) was reduced to insignificant levels by omitting CaCl2 from the pipette solution, blocking I_{Ca} with CdCl2, and buffering intracellular free Ca2+ with EGTA. In most of the experiments, I_{KCa} was blocked by extracellular TEA (140 mM), which replaced the NaCl (140 mM).

To evaluate the steady-state inactivation of I_{Na}, a depolarizing test pulse to +42.5 mV (200 ms in duration) was preceded by conditioning pre pulses from −102.5 to −5 mV in 7.5-mV steps (1 s in duration). I_{Na} was measured as the peak current amplitude elicited by the depolarizing test pulses and expressed as the membrane conductance (g_{Na} = I_{Na}/(V_{M} − V_{E})), where V_{M} is membrane potential and V_{E} is the K* equilibrium potential (−82 mV) determined from the concentration gradient with the assumption that intracellular K* was equal to pipette potassium (140 mM). In some cases, I_{Na} was converted to conductance values (nS).

In outside-out patch-clamp experiments, single A-type K+ channel activity was elicited with the use of pulses from −100 mV to various test potentials (0 to +40 mV). In cell-attached patch-clamp experiments, A-type K+ channel activity was elicited either with the use of voltage steps from +40 mV to various test potentials (−100 to 0 mV) when patch pipettes were filled with Tyrode’s solution (5.4 mM KCl) or by pulses from −100 mV to various test potentials (−20 to +100 mV) when patch pipettes were filled with 140 mM KCl. For convenience, the current–voltage relationship for single-channel currents obtained from cell-attached patches was constructed by plotting single-channel current amplitude as a function of driving force (the pipette potential minus the average resting potential, −60 mV when the bath contained normal Tyrode’s solution and −20 mV when the bath contained 140 mM TEA).

Data acquisition and analysis were performed with the use of pCLAMP 6.03. Whole cell currents were filtered at 1 kHz (frequency filter −3 dB) and digitized at 2 kHz. Single-channel currents were filtered at 2 kHz (frequency filter −3 dB) and digitized at 10 kHz. By convention, outward current is depicted as upward deflections of current.

Data analysis

Results are expressed as means ± SE. Statistical significance was evaluated with the use of paired t-test. Differences were considered significant at P < 0.05; n corresponds to the number of cells used.

RESULTS

Neurons used for the experiments had an oval- or triangular-shaped cell body with two or three small and short processes that did not form a network with adjacent cells. The maximum diameter of the neurons was ~15–25 μm. The passive membrane input resistance of the cells was 301.4 ± 21.7 MΩ (n = 45). The average cell capacitance was 51.15 ± 21.15 μF (n = 73). Typical neuronal action potentials, either spontaneous or stimulated, were present in these cells (see companion paper). There were at least four transmembrane currents that underlie the depolarization and repolarization of the action potential. These are defined as 1) TTX-sensitive I_{Na}, 2) Cd2+-sensitive I_{Ca}, 3) TEA-sensitive I_{K}, and 4) 4-AP-sensitive transient K* current (I_{A}). The present work focuses on the 4-AP-sensitive I_{A}.

Isolation of I_{A}

In normal Tyrode’s solution, when I_{Na} and I_{Ca} were blocked by TTX (100 nM) and CdCl2 (100 μM), respectively, a voltage-dependent total outward current was recorded (Fig. 1A). The total outward current could be pharmacologically dissected into at least two components. When TEA (140 mM) was bath applied, significant inhibition of a delayed-rectifier-like K* current was observed (Fig. 1B). Subtracting the current traces in Fig. 1B from those in Fig. 1A reveals an outward K* current that is defined as a volt-
age-dependent, TEA-sensitive $I_K$ similar to that of the Kv class of K$^+$ channel (Fig. 1C). The second component of outward current was sensitive to 4-AP. Bath application of 4-AP (5 mM) significantly reduced the transient portion of the outward current (Fig. 1D). Subtracting the current traces in Fig. 1D from those in Fig. 1B reveals a voltage-dependent, transient outward K$^+$ current that was defined as a TEA-resistant and 4-AP-sensitive $I_A$ (Fig. 1E). $I_A$ activated rapidly and inactivated within 40 ms. The conductance-voltage relationship for $I_A$ is presented in Fig. 1F ($n = 14$).

**Biophysical properties of $I_A$**

Steady-state inactivation of $I_A$ was studied in a total of 14 neurons. The steady-state inactivation current traces of $I_A$ (Fig. 2) were obtained with the use of the voltage paradigms described in the METHODS section. The peak amplitude of $I_A$ elicited by repetitive depolarizing pulses (+42.5 mV) began to decrease at conditioning potentials of −80 to −70 mV (Fig. 3, A and C). When the conditioning potential was more positive than −65 mV, steady-state inactivation became accelerated and complete inactivation occurred at a conditioning potential of −27.5 mV. The averaged steady-state inactivation data were fit with the following Boltzmann function

$$g_A/g_{A\text{(max)}} = [1 + \exp((V - V_{1/2})/k)]^{-1}$$

where $g_A/g_{A\text{(max)}}$ is the conductance normalized to its maximum value, $V$ is the membrane potential, $V_{1/2}$ is the mem-

**Fig. 1.** Isolation of neuronal A-type K$^+$ current ($I_A$). Sodium current ($I_{Na}$) and calcium current ($I_{Ca}$) were blocked by tetrodotoxin (TTX, 100 nM) and Cd$^{2+}$ (100 μM), respectively. Cell membrane potential was held at −90 mV. A: total outward current traces elicited by 200-ms depolarizing pulse from −35 to +42.5 mV in 7.5-mV steps following 1-s prehyperpolarizing pulse to −110 mV. B: current traces recorded in presence of external tetraethylammonium chloride (TEA, 140 mM). C: TEA-sensitive difference current [$A - B$, mainly delayed rectifier K$^+$ current ($I_K$)]. D: current traces recorded in presence of TEA and 4-aminopyridine (4-AP, 5 mM). E: TEA-resistant, 4-AP-sensitive transient difference current ($I_A$) obtained by subtracting currents recorded in presence of 4-AP from currents recorded in absence of 4-AP (i.e., $B - D$). F: conductance-voltage relationship (current amplitude was measured as peak current during depolarizing pulse) of $I_A$. These results are representative of 14 cells.

**Fig. 2.** Voltage-dependent steady-state inactivation of $I_A$. Cell membrane potential was held at −90 mV. Experiments were performed in presence of TTX (100 nM), Cd$^{2+}$ (100 μM), and TEA (140 mM) in bath solution. A: representative current traces were elicited by 200-ms repetitive depolarizing pulses to +42.5 mV, which were preceded by 1-s conditioning prepulses from −102.5 to 5 mV in 7.5-mV steps in absence of 4-AP. B: current traces recorded in presence of 4-AP (5 mM). C: TEA-resistant, 4-AP-sensitive $I_A$ ($A - B$). D: steady-state inactivation curve of $I_A$ for 14 neurons. Points: normalized mean peak conductance of $I_A$ at each test potential. Solid line is obtained by fitting mean data with Boltzmann function.
brane voltage at which $I_A$ is half-maximum, and $k$ is the Boltzmann slope factor. The averaged data, when fit with a Boltzmann function, revealed a half-steady-state inactivation potential ($V_{1/2}$) of $-52.2$ mV and a voltage-sensitive slope factor ($k$) of $-6.5$ mV.

The activation and inactivation (decay during a voltage step) time constants of the 4-AP-sensitive $I_A$ were studied in eight neurons. Figure 3A shows a representative trace of $I_A$ during a 200-ms depolarizing voltage step from $-110$ to $+42.5$ mV. The activation (rising) and the inactivation (decaying) phases of the current were fit best by a single-exponential equation

$$I = I_{\text{max}}[1 - \exp(-t/\tau)]$$

where $I$ is the amplitude of $I_A$ at time $t$, $I_{\text{max}}$ is the peak $I_A$ at time $0$, and $\tau$ is the time constant for activation or inactivation. In this experiment, activation and decay time constants were 1.4 and 14.3 ms, respectively. The mean values of activation and inactivation time constants for a depolarizing pulse to $+20$ mV were $2.1 \pm 0.3$ ms and $13.6 \pm 1.9$ ms, respectively.

**FIG. 3.** Activation and inactivation time constants of $I_A$. A: representative $I_A$ trace (---) elicited by depolarizing pulse from $-110$ to $+42.5$ mV. Dashed lines are obtained by fitting rising (activation) and decay phases (inactivation) of current trace with 1st-order exponential equation. B: mean data showing voltage dependence for activation time constant ($n = 8$). C: mean data showing voltage dependence for inactivation time constant ($n = 8$).

The time to reach peak activation of $I_A$ was voltage dependent (Fig. 3B). The greater the depolarization, the faster $I_A$ activated. For example, $\tau$ was equal to $4.0 \pm 0.6$ ms during a depolarizing pulse to $+5$ mV but was only $1.1 \pm 0.1$ ms during a voltage step to $+42.5$ mV. In comparison, the inactivation (decay) time constant of $I_A$ was voltage dependent only when the depolarizing potential was more negative than 0 mV. When the depolarizing test potential was more positive than 0 mV, the decay time constant showed little voltage dependence (Fig. 3C).

**Single-channel characteristics of 4-AP-sensitive $I_A$**

Single neuronal A-type K$^+$ channel currents were recorded from both cell-attached and outside-out patches. In a physiological external K$^+$ solution [K$^+$ concentration in the pipette ($[K^+]_{\text{pip}}$) = extracellular K$^+$ concentration ($[K^+]_o$) = 5.4 mM], A-type K$^+$ channel activity occurred
when the patch of membrane was depolarized to a potential more positive than −40 mV (Fig. 4A). Under these conditions, the single-channel conductance was 15.8 ± 1.3 pS (n = 4) and the extrapolated reversal potential was −85.2 ± 1.7 mV (n = 4, Fig. 4). This is close to the predicted equilibrium potential of K+ calculated with the use of the Nernst equation. Figure 5 illustrates biophysical and pharmacological fingerprints of single A-type K+ channel currents recorded from outside-out patches. During a voltage step from −100 to +40 mV, rapidly activating and inactivating channels were observed. The current trace illustrated in Fig. 5A is an ensemble average of 30 traces. The inactivation time constant of the ensemble averaged single-channel currents in this experiment was 16.7 ms and the average was 16.1 ± 4.2 ms (n = 4), which was similar to the decay time constant of I_A at +42.5 mV (15.3 ± 3.4 ms, Fig. 3). To pharmacologically fingerprint these inactivating channels, 4-AP (5 mM) was added to the bathing solution. 4-AP significantly decreased the activity of A-type K+ channels without affecting the single-channel current amplitude (Fig. 5B). On the basis of the above biophysical and pharmacological data, it is concluded that the activity of these 4-AP-sensitive, rapidly activating and inactivating single K+ channels underlies I_A.

Ang II regulation of I_A

We next examined the pharmacological regulation of I_A by Ang II in the presence of the AT_2 receptor antagonist PD 123319 (1 μM). PD 123319 had no effect on baseline K+ current or channel activity. Ang II, when superfused into the recording chamber, significantly reduced I_A in a dose-dependent manner (Fig. 6, A and B, asterisk: \( P < 0.01, n = 12 \) for Ang II (100 nM), n = 5 for the other concentrations). Therefore we chose to use a maximum effective dose of Ang II, 100 nM, in the rest of our experiments. Ang II (100 nM) reduced peak I_A and shifted the activation curve to the right in eight neurons tested (Fig. 7). Figure 7A shows a
were reversed either on washout (Fig. 8, right) or by addition of losartan (1 μM) to the superfusate solution (data not shown). The ensemble averaged current is illustrated below the single-channel current traces and reflects control, application of Ang II, and washout, respectively. The ensemble averaged data are markedly similar to those data for Ang II regulation of \(I_A\) illustrated in Fig. 7. Similar results were observed in four other cell-attached patches. Figure 9 illustrates the effects of Ang II on single-channel \(N_P_o\). The data in Fig. 9 were used to generate amplitude histograms and calculate \(N_P_o\). Under control conditions, in the presence of Ang II (100 nM), and after washout, \(N_P_o\) was 0.21, 0.10, and 0.16, respectively. The mean values for \(N_P_o\), under these conditions were 0.22 ± 0.04, 0.10 ± 0.08, and 0.17 ± 0.11, respectively (\(n = 4\)).

**DISCUSSION**

The transient outward \(K^+\) current reported here was defined as a TEA-resistant, 4-AP-sensitive A current (\(I_A\)). This conclusion was based on the following criteria. First, under our experimental conditions, \(I_{SA}, I_{Ca}, I_K\), and \(I_{KCa}\) were blocked by TTX, \(Ca^{2+}\), TEA, and internal EGTA, (5 mM) respectively. Second, the voltage dependence and the kinetics of activation and inactivation of \(I_A\) were similar to previously published results (Figs. 1–3) (Bouskila and Dudek 1995; Mei et al. 1995). Finally, the single-channel conductance demonstrated for channels that underlie \(I_A\) was similar to that reported for other neuronal preparations (Figs. 4 and 5) (Cooper and Shrier 1985; Kasai et al. 1986).

This study also demonstrated that Ang II, via activation of \(AT_1\) receptors, reduced \(I_A\) and decreased the activity of a 15-pS, rapidly inactivating, 4-AP-sensitive \(K^+\) channel (Figs. 8 and 9). Our previous studies showed that hypothalamus and brain stem neuronal cocultures contained \(\sim 70\% AT_2\) and \(\sim 30\% AT_1\) receptors (Sumners and Raizada 1993; Sumners et al. 1991, 1994). We previously determined that activation of \(AT_1\) receptors by Ang II decreases net outward current via inhibiting \(I_K\) and stimulating \(I_{Ca}\), which was dependent on protein kinase C activation (Summers et al. 1996). Our recent study also showed that intracellular injection of a 25-amino-acid peptide corresponding to the third intracellular loop of the cloned \(AT_1a\) receptor (\(AT_{1a/c}\)) elicited changes in \(I_K\) and \(I_{Ca}\) that were similar to those obtained with application of Ang II via \(AT_1\) receptors. By contrast, injection of a 19-amino-acid peptide corresponding to the second intracellular loop did not modulate \(I_K\) or \(I_{Ca}\). Importantly, our data elucidated that the modulation of neuronal \(I_K\) and \(I_{Ca}\) by \(AT_{1a/c}\) involves protein kinase C, inositol-(1,4,5)-trisphosphate (\(IP_3\)), and intracellular \(Ca^{2+}\), similar to the \(AT_1\) receptor modulation of \(I_K\) and \(I_{Ca}\) by Ang II (Zhu et al. 1997). These effects are consistent with the increases in neuronal activity observed following \(AT_1\) receptor activation (Ambuh et al. 1992; Yang et al. 1992). Combined with our present findings, we suggest that by potentiating \(I_{Ca}\) and diminishing \(I_K\) and \(I_A\), Ang II increases the excitability of neurons from the hypothalamus and brain stem. This too is consistent with our recent observation that Ang II increases the spontaneous firing rate of the cultured neurons (see companion paper).

Ang II produced three important biophysical, physiologi-
Fig. 8. Effects of Ang II on single $I_A$ recorded from cell-attached patches. Experiments were performed under same conditions as Fig. 4. Currents were activated by driving force of $+100 \text{mV} (V_{\text{pip}} = \text{membrane potential} = +100 \text{mV})$. Left: control single-channel currents and ensemble average current. Middle: effects of bath application of Ang II (100 nM) on single-channel activity and ensemble average current. Right: recovery on washout of Ang II. This is representative of 4 experiments.

Biophysically and pharmacologically, we have also characterized $I_A$ and single A-type $K^+$ channels from these neurons. $I_A$ and single A-type $K^+$ channels showed voltage-dependent activation and steady-state inactivation parameters similar to those reported previously (Bouskila and Duduk 1995; Cooper and Shrier 1985; Kasai et al. 1986; Mei et al. 1995). However, $I_A$ and single A-type $K^+$ channels recorded in this study displayed different inactivation kinetics when compared with the results of Cooper and Shrier (1985) and Kasai et al. (1986). Our present results show that $I_A$ and single A-type $K^+$ channels had time constants of inactivation of 14 ms (Fig. 3) and 15 ms (Fig. 5), respectively. Data from Cooper and Shrier (1985) and Kasai et al. (1986) show that $I_A$ and single A-type $K^+$ channels had inactivation time constants of 30 and 100 ms, respectively.
The similarity in steady-state inactivation and differences in inactivation kinetics during a voltage step in different neuronal preparations may be due to specific molecular mechanisms involving the coexistence of N-type and C-type inactivation mechanisms in A-type K⁺ channels (Armstrong and Bezanilla 1977; Baukrowitz and Yellen 1995; Choi et al. 1991; Grissmer and Cahalan 1989; Yellen et al. 1994; Zagotta et al. 1990).

The conductance of single A-type K⁺ channels recorded under the cell-attached configuration is related to the transmembrane potassium gradient. It is well accepted that the A-type K⁺ channels have a small conductance (~15–20 pS, Fig. 4 (Rudy 1988) in a physiological K⁺ gradient ([K⁺]o = 5.4 mM). Finally, pharmacologically Iₖ and single A-type K⁺ channels from neurons of the hypothalamus and brain stem resemble other neurons in their 4-AP sensitivity. Kasai et al. (1986) reported that 4-AP blocked A-type K⁺ channels from the inside of the cell by diffusing through the membrane. We also observed that bath application of 4-AP could inhibit Iₖ (Fig. 1) and single A-type K⁺ channel activity recorded in outside-out patches (Fig. 5).

In summary, we have characterized a TEA-resistant, 4-AP-sensitive Iₖ and the single channel underlying it in rat cultured neurons with the use of whole cell, outside-out patch, and cell-attached patch configurations. We have also shown that Ang II, through activation of AT₁ receptors, inhibits Iₖ and single A-type K⁺ channels. The Ang II regulation of neuronal Iₖ is one of the bases for its physiological actions through changes in action potential firing patterns in the brain. Future experiments will include the elucidation of the signal transduction mechanism(s) involved in Ang II regulation of Iₖ.

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ANGIOTENSIN II REGULATION OF NEURONAL A CURRENT


