Target Tissues and Innervation Regulate the Characteristics of K^+ Currents in Chick Ciliary Ganglion Neurons Developing *in situ*

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The expression of appropriate ensembles of ionic channels is necessary for the differentiation and normal function of vertebrate neurons. Cell–cell interactions may regulate the expression and properties of ionic channels in embryonic neurons. Previous studies have shown that the expression of A-type K^+ channels (I_A) and Ca^{2+} -activated K^+ channels ($I_{K(Ca)}$) is abnormal in chick ciliary ganglion neurons developing *in vitro* in the absence of normal cell–cell interactions. Other voltage-activated currents develop normally under these conditions. The present studies were designed to establish the role of the target tissues and the preganglionic innervation in regulating the expression of these currents in embryonic chick ciliary ganglion neurons developing *in situ*. Surgical manipulations were used to remove the developing optic vesicle, which contains the target tissues, the mid-dorsal region of the midbrain primordium, which contains the preganglionic nucleus, or both, all prior to the formation of the ciliary ganglion. I_A and $I_{K(Ca)}$ were then examined in acutely isolated neurons that developed *in ovo* in the presence (OV^+) or absence (OV^-) of the normal target tissues, in the presence (MB^+) or absence (MB^-) of preganglionic innervation, and in the absence of both preganglionic innervation and target tissues (OV^-/MB^-). The amplitude of I_A was unaffected by the operations. However, the activation and inactivation kinetics of I_A were two- to threefold faster in OV^- or OV^-/MB^- cells compared to neurons isolated from control OV^+ ganglia at embryonic days 11–14 (E11–E14). There were no changes in the voltage dependence of activation or steady-state inactivation, or in the time course of recovery from inactivation. By contrast, neurons isolated from MB^- ganglia expressed an I_A with amplitude, voltage dependence, and kinetics that were indistinguishable from those of control MB^+ and OV^+ ganglia. Therefore, interactions with target tissues in the eye play a role in determining the characteristics of I_A in developing ciliary ganglion neurons, whereas preganglionic innervation does not. Furthermore, the amplitude of $I_{K(Ca)}$ was reduced by 90–100% in OV^- , MB^- , and OV^-/MB^- neurons isolated at E12–E14 as compared to MB^+ and OV^+ controls. Voltage-activated Ca^{2+} currents were present at normal amplitudes in all of these neurons. Thus,

the expression of $I_{K(Ca)}$ in chick ciliary ganglion neurons is regulated by both target tissue interactions and preganglionic innervation. Therefore, cell–cell interactions are necessary for the expression of a normal ensemble of ionic channels in chick ciliary ganglion neurons developing *in situ*.

[Key words: ionic channels, A-currents, Ca^{2+} -activated K^+ currents, kinetics, neuronal development, ciliary ganglion, cell–cell interactions, *in situ*]

Different populations of neurons often exhibit different intrinsic electrophysiological properties that are important for the transmission of signals and the processing of information. These properties are due primarily to the expression of a particular ensemble of ionic channels, and different electrophysiological characteristics arise from the expression of a different complement of ionic channels. In order to obtain normal function in the nervous system, it is necessary to ensure the timely expression of an appropriate complement of channels in individual neurons. However, relatively little is known about the factors that regulate the developmental expression of either voltage- or Ca^{2+} -activated ionic channels in vertebrate neurons.

Previous studies have shown that the mature complement of ionic channels is not expressed at the time of terminal mitosis of vertebrate neurons from precursor cells (reviewed by Spitzer, 1991; Ribera and Spitzer, 1992). Instead, different types of neurons express these proteins at developmental stages, and in a sequence, that are specific for a given cell type. Recent studies have suggested that in some neurons, the expression of ionic channels is regulated primarily by intrinsic factors, while in other neurons, extrinsic factors appear to influence ionic channel expression and properties. For example, in developing amphibian spinal neurons, ionic channel expression appears to proceed according to a built-in developmental program. The same developmental sequence of ionic channel expression is seen *in vivo* (Spitzer and Baccaglini, 1976) and *in vitro* (Spitzer and Lomborghini, 1976; O'Dowd et al., 1988), even when the neurons are maintained as single-cell cultures (Henderson and Spitzer, 1986).

By contrast, in rat sympathetic and chick parasympathetic neurons, the expression of some ionic channels appears to be regulated by extrinsic influences. In rat sympathetic neurons, the developmental expression of I_A is dependent upon environmental factors (McFarlane and Cooper, 1992) possibly including soluble factors secreted from ganglionic non-neuronal cells (McFarlane and Cooper, 1993). In chick ciliary ganglion neurons developing *in situ*, nearly all voltage- and Ca^{2+} -activated ionic currents undergo an increase in amplitude and density between embryonic day 9 (E9) and E13 (Dourado and Dryer, 1992).

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Similarly, I_{Na} , I_{Ca} , and the delayed rectifier (I_{DR}) exhibit a normal developmental increase in amplitude when these cells are placed *in vitro* at E9 and examined 4 d later. This autonomy is similar to that of amphibian spinal neurons, and suggests that the developmental expression of these currents is not dependent on environmental factors. However, $I_{K[Ca]}$ and I_A are not expressed normally during development *in vitro* (Dourado and Dryer, 1992). Moreover, these currents first appear relatively late in development; they are not usually detectable in ciliary ganglion neurons before E9 (Dourado and Dryer, 1992), coinciding with the times when these neurons form synapses with target tissues, and preganglionic inputs mature. Together, these results suggest that in chick ciliary ganglion neurons, the developmental expression of I_A and $I_{K[Ca]}$ is regulated by extrinsic factors, possibly derived from preganglionic fibers, target tissues, or both.

The chick ciliary ganglion is an ideal model system for investigating the role of cell-cell interactions in regulating ionic channel expression in neurons developing *in situ*. The source of preganglionic input and the target tissues have been identified, and these interactions are amenable to manipulation *in ovo*. In addition, several studies have determined the developmental stages when ciliary ganglion neurons leave the cell cycle (D'A-mico-Martel, 1982; Rohrer and Thoenen, 1987), receive synaptic inputs from preganglionic cells (Landmesser and Pilar, 1972; Jacob, 1991), and form synapses with target tissues in the eye (Landmesser and Pilar, 1974a,b; Narayanan and Narayanan, 1981; Meriney and Pilar, 1987). It is clear that interactions with other cells, including the preganglionic fibers and the target tissues, regulate many important developmental processes in ciliary ganglion neurons. For example, there is considerable evidence for the existence of target-derived trophic factors that regulate the survival of these cells (Landmesser and Pilar, 1974a; Pilar et al., 1980) as well as the expression of neurotransmitters, their receptors, and their biosynthetic enzymes (reviewed by Tuttle, 1985; Berg et al., 1989; Dryer, 1994). Similarly, preganglionic innervation is known to play a role in regulating cell survival (Furber et al., 1987) as well as nicotinic ACh receptor protein and transcript levels in developing and mature chick ciliary ganglion neurons (Berg et al., 1989; Jacob, 1991; Schwartz-Levey et al., 1992; Arenella et al., 1993).

In the present study, we have examined the expression of I_A and $I_{K[Ca]}$ in chick ciliary ganglion neurons that have developed *in ovo* following surgical manipulations that prevent the formation of normal pre- and postganglionic interactions. To preclude the influence of target tissues in the eye, the optic vesicle was removed unilaterally at E2 (stages 10–13), prior to the formation of the ciliary ganglion. As a result of this procedure, neurons on the operated side developed entirely in the absence of direct interactions with their appropriate target tissues (OV⁻ neurons), and they were compared to neurons from the contralateral unoperated side (OV⁺ neurons). To prevent interactions with preganglionic fibers, the region of the midbrain primordium containing the preganglionic nucleus was ablated at E3.5–E4 (stages 20–23) (MB⁻ neurons). These stages precede the time of synapse formation with ciliary ganglion neurons (Landmesser and Pilar, 1972; Jacob, 1991); therefore, MB⁻ neurons developed entirely in the absence of innervation (Arenella et al., 1993). Sham-operated embryos were used as controls (MB⁺ neurons). In some embryos, these two surgical procedures were combined (OV⁻/MB⁻) to prevent both innervation and normal target tissue interactions throughout development. The results indicate that ciliary ganglion neurons that have developed *in*

situ in the absence of their postganglionic target tissues in the eye express an I_A of normal amplitude but with abnormal kinetic properties, whereas ganglionic neurons that have developed in the absence of innervation express a normal I_A that is indistinguishable from controls. In comparison, the amplitude of $I_{K[Ca]}$ was dramatically reduced in ganglionic neurons that had developed in the absence of innervation, target tissues, or both. Voltage-activated Ca^{2+} currents were not altered by these surgical procedures. Thus, cell-cell interactions are necessary for the normal expression of I_A and $I_{K[Ca]}$ in chick ciliary ganglion neurons developing *in situ*.

Materials and Methods

Surgical procedures. Fertilized White Leghorn chick eggs (Spafas, Norwich, CT) were maintained at 37°C in a forced-draft incubator. For OV⁻ ganglia, the developing optic vesicle was removed unilaterally at E2 (stages 10–13) (Hamburger and Hamilton, 1951), prior to the formation of the ciliary ganglion (Landmesser and Pilar, 1974a). OV⁺ ganglia from the contralateral unoperated side served as a control. Briefly, a small hole was made in the blunt end of the eggshell. Using a dissecting microscope with fiber-optic illumination and fine forceps, the amniotic fold was stretched open to reveal the head of the embryo. Using the forceps to maneuver and hold the embryo, the entire developing optic vesicle on one side was removed with a second pair of forceps. Great care was taken to avoid damaging the extraembryonic vascular plexus and the yolk sac. The opening in the shell was then sealed with a coverslip and melted wax, and the egg was returned to the incubator and allowed to develop until E12–E14. Survival rates varied from 30% to 50%.

For MB⁻ ganglia, the accessory oculomotor nucleus, the sole source of presynaptic inputs to the chick ciliary ganglion, was surgically ablated at E3.5–E4 (stages 20–23) as described elsewhere (Furber et al., 1987; Arenella et al., 1993). Briefly, access to the embryo was obtained as described above. A small flap of the amnion was pulled away to reveal the head of the embryo. Using fine forceps to hold the dorsal mesencephalic region in this opening, a small battery-operated electrocautery (Storz Instruments, St. Louis, MO) whose tip had been sharpened to a fine point was used to cauterize (for 2–4 sec) the entire mid-dorsal mesencephalic region containing the preganglionic nucleus. The head of the embryo was then recovered with the flap of amniotic membrane, and the egg was resealed and placed into the incubator to continue development until E11–E14. Survival rates were 40–80%. For MB⁺ ganglia, sham operations were performed on other E3.5–E4 embryos using the same procedures with the exception of the cauterization step.

In addition, OV⁻/MB⁻ and OV⁺/MB⁻ ganglia were obtained by removal of the optic vesicle unilaterally at E2 (stages 10–13), followed by bilateral ablation of the preganglionic nucleus at E3.5–E4 (stages 20–23) as described above. Embryos were then returned to the incubator and allowed to develop until E11–E14. Survival rates varied from 15% to 20%.

Acute isolation of neurons. Chick ciliary ganglion neurons were isolated acutely as described previously (Dryer et al., 1991a,b; Dourado and Dryer, 1992; Wisgirda and Dryer, 1993a). Briefly, ciliary ganglia were removed from operated and sham-operated E11–E14 embryos (stages 38–40) and incubated at 37°C in a saline free of divalent cations containing 0.5 mg ml⁻¹ collagenase (Sigma type II) for 20 min. Ganglia were transferred to cell culture medium and dissociated by trituration, and the cell suspension was plated onto poly-D-lysine-coated glass coverslips. Neurons were allowed to settle for 30 min to 4 hr at 37°C prior to recording to ensure sufficient adhesion to the substrate.

Electrophysiology. Whole-cell recordings were made at room temperature (21–23°C) using standard techniques as described previously (Dryer et al., 1991a,b; Dourado and Dryer, 1992, 1994; Wisgirda and Dryer, 1993a,b). Normal external saline consisted of (in mM) 145 NaCl, 5.4 KCl, 5.4 CaCl₂, 0.8 MgCl₂, 5 D-glucose, 13 HEPES/NaOH, and 500 nM tetrodotoxin at a pH of 7.4. For Ca^{2+} -free saline, the CaCl₂ was replaced on an equimolar basis by MgCl₂. For analysis of I_A , Ca^{2+} -free saline was supplemented with 10 mM tetraethylammonium (TEA). Pipette saline consisted of (in mM) 120 KCl, 2 MgCl₂, 10 HEPES/NaOH, 10 EGTA, 5 ATP, 2 GTP, and 0.1 leupeptin at a pH of 7.4. For experiments on voltage-activated Ca^{2+} currents (I_{Ca}), the pipette salines contained 120 mM CsCl (instead of KCl) and all external salines contained 10 mM TEA in order to reduce outward currents (Dryer et al.,

Figure 1. A-currents in acutely isolated chick ciliary ganglion neurons that have developed *in ovo* in the presence (OV⁺) or absence (OV⁻) of target tissue. *A*, Whole-cell currents in OV⁺ and OV⁻ neurons evoked by depolarizing steps to -40 , -20 , 0 , and $+20$ mV from a holding potential of -120 mV. Currents were automatically leak subtracted. *B*, A-currents evoked in an OV⁺ neuron (*top left*) and an OV⁻ neuron (*bottom left*) by a depolarizing step to -20 mV from a holding potential of -120 mV. The time course of inactivation of the currents are shown to the *right*. Arrows indicate portions of the currents used for analyses. Data points are shown along with superimposed fitted double-exponential curves with weights and time constants as indicated. *C*, Mean amplitude of I_A evoked by a depolarizing step to -20 mV from a holding potential of -120 mV in OV⁺ and OV⁻ neurons, as indicated. Error bars are SEM, with the number of cells studied shown in parentheses.

1991b). Pipette resistance was 2–6 M Ω . It was generally possible to compensate 90% of this resistance without introducing oscillations into the current output of the clamp amplifier (Axopatch 1D, Axon Instruments, Foster City, CA). Voltage commands and data analysis were performed using a personal computer equipped with a Tecmar LAB-MASTER 125 kHz A/D board and commercially available software (pCLAMP version 5.1, Axon Instruments). The characteristics of I_A were determined by application of a series of depolarizing voltage steps from a holding potential of -120 mV as described previously (Dourado and Dryer, 1992, 1994; Wisgirda and Dryer, 1993a,b). For quantitation of current amplitude, I_A was measured as the difference between peak and steady-state currents evoked by a depolarizing step to -20 mV from a holding potential of -120 mV. With this protocol and in the presence of 10 mM TEA, contributions from residual unblocked I_{DR} are mini-

mized (Wisgirda and Dryer, 1993a). Analyses of I_A inactivation kinetics were performed using pCLAMP software. The characteristics of $I_{K[Ca]}$ were determined by application of 50 msec depolarizing voltage steps to 0 mV from a holding potential of -40 mV. These steps were made in normal and Ca²⁺-free salines, and the net Ca²⁺-dependent currents obtained by digital subtraction as described elsewhere (Dryer et al., 1991a; Dourado and Dryer, 1992). The amplitude of $I_{K[Ca]}$ was then taken as the amplitude of net Ca²⁺-dependent current at the end of the depolarizing step. Similar procedures were used to determine the amplitude of voltage-activated Ca²⁺ currents as described previously (Dryer et al., 1991b; Dourado and Dryer, 1992). Statistical tests of differences in current amplitude were performed using Student's unpaired *t* test with $p < 0.05$ regarded as significant.

Results

A-currents in ciliary ganglion neurons developing in the absence of normal target tissues

Unilateral extirpation of the optic vesicle at E2 caused ciliary ganglion neurons on the operated side to develop in the absence of their normal target tissues. When this procedure was performed successfully, ocular tissue could not be discerned on the operated side at the time of dissection, whereas the contralateral side contained a normal eye. If any traces of ocular tissue were present on the operated side, the embryo was not used. In the absence of the developing optic vesicle, ciliary ganglion cell migration was altered, and several small ganglia formed (Landmesser and Pilar, 1974). It is possible that neurons from OV⁻ ganglia form synaptic connections with abnormal target tissues, such as residual extraocular muscles or blood vessels, but they clearly cannot interact with the normal target tissues, that is, iris, ciliary body, or choroid.

Neurons isolated acutely from OV⁺ and OV⁻ ganglia at E11–E14 expressed TEA-resistant transient outward currents (I_A) evoked by depolarizing voltage steps from a holding potential of -120 mV. Although the amplitude of I_A was similar, the kinetic properties of I_A were very different in OV⁻ neurons compared to OV⁺ controls (Fig. 1, Table 1). Neurons isolated from OV⁻ ganglia expressed an I_A that activated faster, and subsequently exhibited much faster inactivation kinetics, than the currents evoked in OV⁺ neurons examined at the same developmental stages under identical conditions (Fig. 1*A,B*). The time course of I_A inactivation in normal chick ciliary ganglion neurons can usually be fitted only as the sum of at least two exponential components (Wisgirda and Dryer, 1993b). As expected, this was also true in the majority of OV⁺ neurons, where the fast and slow components of inactivation made approximately equal contributions to the overall process. But in the case of OV⁻ neurons, the fast component of inactivation made a much larger contribution to the overall process. In fact, the slow component of I_A inactivation could not be detected in 21 of 33 cells examined from 11 different OV⁻ ganglia. By contrast, the slow component of I_A inactivation was missing in only 5 of 25 cells examined from nine different OV⁺ ganglia. In addition, the mean fast time constant in OV⁻ neurons was shorter by approximately 50% compared to OV⁺ controls (Table 1). The kinetics of I_A in OV⁻ neurons were similar to those of ciliary ganglion neurons that develop *in vitro* in the presence of pectoral muscle striated myotubes (Dourado and Dryer, 1994). These kinetic changes caused the overall inactivation time course to be twice as fast in OV⁻ neurons. Nevertheless, the peak amplitude of I_A was similar in OV⁺ and OV⁻ neurons (Fig. 1*C*). For these graphs, I_A amplitude was measured as the difference between peak and steady-state currents evoked by voltage steps to -20 mV from a holding potential of -120 mV. This pro-

Table 1. Kinetic properties of A-currents in acutely isolated chick ciliary ganglion neurons developing under different experimental conditions *in ovo*

Cell type	Time to peak current (msec)	% Fast ^a	T_f (msec)	% Slow ^b	T_s (msec)
OV ⁺ (25)	5.3 ± 0.2 (23)	39 ± 8.4 (23)	16.3 ± 1.4 (14)	61 ± 8.4 (23)	48 ± 3.7 (18)
OV ⁻ (33)	3.5 ± 0.3* (28)	83 ± 5.5* (28)	7.4 ± 0.5* (26)	17 ± 5.5* (28)	53 ± 4.9 (12)
MB ⁺ (18)	5.6 ± 0.3 (15)	39 ± 6.3 (15)	16.7 ± 0.9 (13)	61 ± 6.3 (15)	53 ± 3.3 (15)
MB ⁻ (25)	5.5 ± 0.3 (21)	40 ± 7.3 (21)	15.7 ± 1.2 (16)	60 ± 7.3 (21)	57 ± 4.0 (19)
OV ⁻ /MB ⁻ (10)	2.9 ± 0.2* (8)	71 ± 5.7* (8)	7.2 ± 0.5* (8)	29 ± 5.7* (8)	50 ± 4.7 (8)

Currents were evoked by a depolarizing voltage step to -20 mV from a holding potential of -120 mV. The time course of the inactivation of the currents was fitted with single- or double-exponential curves of the form $I(t) = A \exp(-t/T_f) + B \exp(-t/T_s)$, where T_f and T_s are time constants, and A and B are weighting factors measured in pA. Numbers in parentheses are the number of cells in which it was possible to make the determination. All values are mean ± SEM.

^a % Fast = 100 $A/(A + B)$.

^b % Slow = 100 $B/(A + B)$.

* $p < 0.05$ compared to OV⁺ controls.

cedure minimized contamination from residual unblocked I_{DR} , which, in the presence of 10 mM TEA, is very small at this command potential (Dourado and Dryer, 1992; Wisgirda and Dryer, 1993b).

In spite of their markedly different kinetics, the I_A in OV⁺ and OV⁻ neurons exhibited a similar voltage dependence of activation (Fig. 2A) and inactivation (Fig. 2B). In Figure 2A, normalized peak conductance is plotted against command potential for the records shown in Figure 1A. These plots are fitted with superimposed Boltzmann curves of the form $G/G_{\max} = 1/1 + \exp(V_h - V/k)$, where G is the peak conductance at command potential V , $G_{\max}$ is the maximum peak conductance, V_h is the voltage at which conductance is half-maximal, and k is a constant related to the slope of the activation curve. Conductance was calculated assuming a reversal potential of -85 mV. I_A conductance in the OV⁺ neuron was half-maximal at -27 mV with $k = 16$ mV. I_A conductance in the OV⁻ neuron was half-maximal at -29 mV with $k = 12$ mV. Similar results were obtained in 10 other OV⁻ neurons and six other OV⁺ neurons representing experiments performed on several different ganglia, and there were no systematic differences between the two groups. Further, the voltage dependence of steady-state inactivation was examined in OV⁺ and OV⁻ neurons by application of a series of 500 msec conditioning prepulses to various test potentials followed by a depolarizing step to 0 mV (Fig. 2B). In both cell types, as with previous studies of normal ciliary ganglion neurons (Wisgirda and Dryer, 1993a,b), the amplitude of I_A increased when it was evoked from more negative prepotentials. In Figure 2B (bottom) normalized current amplitudes are shown plotted against prepulse potential for the records shown above. These data are fitted with superimposed Boltzmann curves of the form $I/I_{\max} = 1/1 + \exp(V - V_h/k)$ where I is the peak A-current evoked from a prepotential V , $I_{\max}$ is the maximum current, V_h is the prepotential at which I_A is half-inactivated, and k is a constant related to the slope of the inactivation curve. In the OV⁺ neuron, I_A was half-inactivated at -94 mV, with $k = -9.0$ mV. In the OV⁻ neuron, I_A was half-inactivated at -95 mV with $k = -7.6$ mV. Similar results were obtained in four

other OV⁺ and five other OV⁻ neurons with no systematic differences between the two groups. In other experiments we also found that the time course of recovery from inactivation was not different in OV⁺ and OV⁻ neurons, with maximum recovery observed within 20 msec in both cell types (not shown).

A-currents in ciliary ganglion neurons developing in the absence of innervation

Bilateral ablation of the mid-dorsal region of the mesencephalon at E3.5-E4 results in the complete destruction of the preganglionic accessory oculomotor nucleus prior to the time of synapse formation in the ciliary ganglion. Ganglia from these embryos do not have a preganglionic nerve determined at the time of dissection and histological studies confirm the absence of synapses within these ganglia (Arenella et al., 1993). By contrast, the midbrain region of sham-operated embryos is normal, and the ciliary ganglia are innervated by preganglionic fibers coursing through an intact oculomotor nerve.

Neurons isolated acutely from MB⁺ and MB⁻ ganglia at E11-E13 express qualitatively and quantitatively similar A-currents as illustrated in Figure 3 and in Table 1. For example, the kinetics, voltage dependence, and amplitudes of I_A were not significantly different in the two groups of cells and were not significantly different from OV⁺ ganglionic neurons.

A-currents in ciliary ganglion neurons developing in the absence of both innervation and target tissues

The expression of I_A was examined in neurons isolated from ciliary ganglion neurons after ablation of the midbrain primordium and unilateral removal of the optic vesicle (Fig. 4). As described above for MB⁻ cells, the amplitude and kinetics of I_A were normal in OV⁺/MB⁻ cells (Fig. 4A). The amplitude of I_A was also normal in 10 E13 ciliary ganglion neurons isolated from OV⁻/MB⁻ ganglia. Mean I_A amplitude in these cells was 655 ± 163 pA (mean ± SEM), similar to that of neurons isolated from control OV⁺ and MB⁺ ganglia, as well as from OV⁻ and MB⁻ ganglia (Figs. 1, 3). However, the kinetic properties of I_A in OV⁻/MB⁻ neurons were abnormal and were essentially iden-

Figure 2. Voltage dependence of A-currents in acutely isolated chick ciliary ganglion neurons that have developed *in situ* in the presence or absence of target tissue. **A**, Plot of normalized I_A conductance versus command potential for the two neurons shown in Figure 1, **A** (OV^+ , ●) and **B** (OV^- , ▲). Points are fitted with superimposed Boltzmann curves as described in Results. **B**, Steady-state inactivation of I_A in an OV^+ and an OV^- neuron as indicated. A series of 500 msec conditioning prepulses to various potentials were given prior to application of a test pulse to 0 mV. Data are shown for conditioning prepulses to -70 mV, -90 mV, -100 mV, and -120 mV. A plot of normalized amplitude of currents versus prepulse potential for the same OV^+ (●) and OV^- (▲) neurons is shown below. Data points are superimposed on fitted Boltzmann curves as described in Results.

tical to those of neurons isolated from OV^- ganglia (Fig. 4B, Table 1). For example, the I_A in OV^-/MB^- cells peaked less than 3 msec after the onset of the depolarizing voltage step, and subsequently inactivated very quickly. By contrast, the I_A in OV^+/MB^- cells typically required more than 5 msec to peak, and subsequently inactivated slowly. As with OV^- neurons, the abnormally fast inactivation in OV^-/MB^- cells was due to a larger contribution of the fast time constant to the overall decay process, as well as a shorter fast time constant (Table 1).

Ca²⁺-activated K⁺ currents in ciliary ganglion neurons developing in the absence of target tissues, innervation, or both

In all OV^+ neurons, switching from normal to Ca^{2+} -free external saline caused a large decrease in total voltage-evoked outward current, as described previously (Dryer et al., 1991; Dourado and Dryer, 1992). In contrast, changing from normal saline to Ca^{2+} -free external saline did not cause any decrease in outward current in most OV^- neurons, indicating much reduced levels of $I_{K[Ca]}$. Indeed, in many OV^- cells, the resulting inhibition of voltage-activated Ca^{2+} currents caused a net increase in total

voltage-evoked outward current, due to the fact that the amplitude of $I_{K[Ca]}$ was less than that of I_{Ca} . This phenomenon is also observed in ciliary ganglion neurons developing *in vitro* (Dourado and Dryer, 1992), which also express much reduced $I_{K[Ca]}$ even under culture conditions where all of the dissociated neurons survive. In fact, the mean amplitude of $I_{K[Ca]}$ was reduced by 90–100% in OV^- neurons compared to OV^+ controls (Fig. 5). Similar reductions in $I_{K[Ca]}$ were observed in MB^- neurons (Fig. 6) and in OV^-/MB^- neurons (not shown) compared to sham-operated MB^+ controls. The mean amplitude of net steady-state Ca^{2+} -dependent current in OV^-/MB^- cells was -17 ± 61 pA (mean $\pm$ SEM), much below the level of MB^+ and OV^+ controls (Figs. 5, 6). These data were obtained from neurons isolated from at least five different ganglia in each group. It should be noted that voltage-activated Ca^{2+} currents (I_{Ca}) of normal amplitude were present in OV^- , MB^- , and OV^-/MB^- neurons. Examples from typical OV^- and MB^- neurons are shown in Figure 7. In these experiments, I_{Ca} was evoked by depolarizing steps to 0 mV from a holding potential of -40 mV in the presence of 10 mM external TEA and 500 nM external

Figure 3. A-currents in acutely isolated ciliary ganglion neurons that have developed *in situ* in the presence (MB^+) or absence (MB^-) of preganglionic innervation. *A*, Whole-cell currents in an MB^+ neuron evoked by depolarizing steps to -40 , -20 , 0 , and $+20$ mV from a holding potential of -120 mV. *B*, Whole-cell currents evoked using the same protocol in an MB^- neuron. *C*, Mean amplitude of I_A evoked by a depolarizing step to -20 mV from a holding potential of -120 mV in MB^+ and MB^- neurons as indicated. Error bars are SEM, with the number of cells studied shown in parentheses.

TTX using CsCl pipettes. Sustained inward currents dependent upon external Ca^{2+} ions were present in all cells tested. In six OV neurons, the mean amplitude of I_{Ca} was $441 \text{ pA} \pm 76 \text{ pA}$ (mean $\pm$ SEM), while in five MB^- cells mean I_{Ca} amplitude was $412 \text{ pA} \pm 95 \text{ pA}$. These amplitudes are similar to values previously reported for normal ciliary ganglion neurons isolated acutely at similar developmental stages (Dryer et al., 1991b; Dourado and Dryer, 1992). I_{Ca} could also be seen as inward phase of current observed in normal external saline in many cells examined with KCl recording pipettes, especially during the first 5 msec of the depolarizing voltage step. Indeed, these inward currents were more apparent in MB^- and OV neurons (such as those shown in Figs. 5, 6) due to the reduced expression of $I_{K[Ca]}$, which in control neurons tends to obscure the inward Ca^{2+} currents. It should also be noted that the MB^- cell illustrated in Figure 6 expressed an I_A with completely normal amplitude and kinetics (not shown for this cell, but see Fig. 3, Table 1). In summary, dramatic declines in $I_{K[Ca]}$ in all operated neu-

rons indicate that preganglionic innervation and postganglionic target tissues in the eye are both necessary for the normal functional expression of this current during development *in situ*.

Discussion

In this study, we have examined the expression of I_A and $I_{K[Ca]}$ in chick ciliary ganglion neurons that have developed *in situ* in the presence or absence of normal target tissues, innervation, or both. The results indicate that target tissue interactions, but not innervation, regulate the characteristics of I_A . In contrast, normal target tissue interactions and preganglionic innervation are *both* required for the functional expression of macroscopic $I_{K[Ca]}$ but not I_{Ca} . Thus, cell-cell interactions regulate the expression and properties of specific ionic channels on neurons developing *in situ*, and the preganglionic fibers and target tissues appear to provide different regulatory signals.

Previous studies of normal developing ciliary ganglion neurons have also suggested that the expression of I_A and $I_{K[Ca]}$ is

Figure 4. A-currents in acutely isolated ciliary ganglion neurons that have developed in the absence of normal innervation and target tissues (OV^-/MB^-). Currents were evoked by a depolarizing voltage step to -20 mV from a holding potential of -120 mV. *A*, Current in a neuron isolated from a ganglion that developed in the absence of preganglionic innervation on the side containing an intact eye (OV^+/MB^-). Arrows indicate portion of current used for kinetic analysis (top). The time course of I_A inactivation is shown as points with a superimposed double-exponential curve with weights and time constants as indicated (bottom). *B*, Current in a neuron isolated from a ganglion that developed in the absence of both innervation and target tissue (OV^-/MB^-). Time course of I_A inactivation is shown as in *A*.

Figure 5. Expression of Ca^{2+} -activated K^{+} currents in ciliary ganglion neurons that developed *in situ* in the presence or absence of target tissues. The currents were evoked by depolarizing steps to 0 mV from a holding potential of -40 mV in normal and Ca^{2+} -free salines as indicated. Note that a substantial Ca^{2+} -dependent outward current ($I_{K[Ca]}$) can be detected in the OV^{+} but not the OV^{-} neuron. Bar graph indicates a significant decrease in $I_{K[Ca]}$ amplitude in OV^{-} neurons compared to OV^{+} controls. Error bars are SEM, with number of cells indicated in parentheses. Asterisk indicates significant difference between groups ($p < 0.05$).

dependent on environmental factors (Dourado and Dryer, 1992). I_A and $I_{K[Ca]}$ are expressed at low levels on ciliary ganglion neurons at or before E9, but undergo a sharp increase in amplitude between E9 and E13 (Dourado and Dryer, 1992), coinciding with the period of target tissue innervation and preganglionic synapse maturation. Developmental changes in the kinetics of I_A were not observed at these times. Moreover, this increase in I_A and $I_{K[Ca]}$ amplitude does not occur when ciliary ganglion neurons are placed *in vitro* at E9 and examined 4 d later (Dourado and Dryer, 1992). However, in recent cell culture studies, we have found that coculture of ciliary ganglion neurons with intact pectoral myotubes, or acidic fibroblast growth factor (FGF),

can cause the neurons to express I_A at normal or near normal levels (Dourado and Dryer, 1994). However, the resulting kinetics were abnormally fast, and closely resembled those of the OV^{-} neurons described in the present study. Coculture with fibroblasts, application of basic FGF or ciliary neurotrophic factor (CNTF), and growing cells in explant cultures did not induce I_A expression during development *in vitro*.

These results clearly demonstrate that cell-cell interactions, in particular the target tissues, and at least some soluble trophic factors, are capable of regulating I_A expression during development *in vitro*. On the other hand, there are apparent differences between the results obtained in simplified cell culture

Figure 6. Expression of Ca^{2+} -activated K^{+} currents in ciliary ganglion neurons that developed *in situ* in the presence or absence of preganglionic innervation. The currents were evoked by depolarizing steps to 0 mV from a holding potential of -40 mV in normal and Ca^{2+} -free salines as indicated. Note that $I_{K[Ca]}$ is present in the MB^{+} but not the MB^{-} neuron. Bar graph indicates significant decrease in $I_{K[Ca]}$ amplitude in MB^{-} neurons compared to MB^{+} controls. Error bars are SEM, with number of cells indicated in parentheses. Asterisk indicates significant difference between groups ($p < 0.05$).

Figure 7. Expression of voltage-activated Ca^{2+} currents in ciliary ganglion neurons developing in the absence of the postganglionic target tissues or preganglionic innervation. Currents were evoked by a depolarizing step to 0 mV from a holding potential of -40 mV in normal and Ca^{2+} -free salines as indicated (*left*). Currents dependent upon external Ca^{2+} ions were obtained by digital subtraction (*right*). All recordings were made with CsCl saline in the recording pipettes and in the presence of 10 mM external TEA and 500 nM TTX. *A*, Typical records from an OV⁻ ciliary ganglion neuron. *B*, Typical records from an MB⁻ neuron.

systems and those obtained in the present *in situ* study. For example, ciliary ganglion neurons developing in the absence of both innervation and the appropriate target tissues express an I_A of normal amplitude but abnormal kinetics, while ciliary ganglion neurons grown alone in culture express an I_A of much reduced amplitude. One possible explanation is that the amplitude of I_A is regulated by interactions with certain tissues, such as striated or smooth muscle, but that the kinetics of I_A are dependent upon interactions with the appropriate target tissue or the intact eye. Thus, I_A expression in OV⁻ or OV⁻/MB⁻ neurons may be induced by interactions with tissues other than the appropriate target, such as residual extraocular muscles or blood vessels. Similarly, the recent study of McFarlane and Cooper (1993) suggests that ganglionic non-neuronal cells provide soluble factors, such as CNTF, that influence I_A expression in postnatal rat sympathetic neurons developing *in vitro*. By contrast, ganglionic non-neuronal cells and CNTF do not appear to influence I_A expression in chick ciliary ganglion neurons developing *in vitro* (Dourado and Dryer, 1994).

How do environmental factors such as target tissue interactions regulate the kinetics of I_A ? Changes in I_A kinetics could occur by at least two different mechanisms: changes in the subtype of I_A channel expressed or posttranslational modification of existing channels. For example, channels with a different subunit stoichiometry may be inserted into the plasma membrane in response to target tissue interactions (Christie et al., 1990; Isacoff et al., 1990; Ruppersberg et al., 1990). Altered I_A subunit stoichiometry could result from changes in the pattern of expression of genes encoding K^+ channel subunits. These changes could involve the activation of new genes or alternative splicing at the gene or mRNA level (Luneau et al., 1991). Alternatively, changes in K^+ channel expression may be due to changes in the rate of synthesis and/or the stability of the various channel subunits, or their mRNAs, or from differences in the rate at which different channel subunits are inserted into the plasma membrane. Finally, target tissue interactions, or other extrinsic factors, could cause posttranslational modifications, such as phosphorylation or glycosylation, that lead to changes

in the kinetics of activation and inactivation (Kalman et al., 1990). The present data cannot distinguish between these possibilities, but it should be emphasized that the effects of target tissues on the kinetics of I_A could be caused by relatively minor modifications of the channel proteins.

Although the molecular mechanisms that cause the change in I_A kinetics in OV⁻ and OV⁻/MB⁻ neurons are not known, we favor the idea that it is due to a change in a single I_A channel type expressed on ciliary ganglion neurons. In normal ciliary ganglion neurons, the time course of the inactivation of I_A is best fit by double-exponential kinetics (Wisgirda and Dryer, 1993b), whereas I_A inactivates with single-exponential kinetics in most OV⁻ and OV⁻/MB⁻ neurons. In previous studies, double-exponential decay kinetics were observed in homomultimeric I_A channels expressed by injection of *Kv4.2* (RK5) mRNA into *Xenopus* oocytes (Blair et al., 1991). A-currents of the *Kv4.2* type are pharmacologically and biophysically similar to those expressed in normal ciliary ganglion neurons (Wisgirda and Dryer, 1993b). These results suggest that individual I_A channels have complex inactivation kinetics characterized by multiple inactivated states, or multiple pathways leaving a single inactivated state. Moreover, Cooper and Shrier (1989) have demonstrated direct transitions between closed and inactivated states in single I_A channels of rat sensory ganglion neurons, a finding that also predicts complex multiexponential inactivation kinetics in macroscopic measurements made from a homogeneous population of channels. Thus, although the presence of two different populations of I_A channels with different kinetic properties on ciliary ganglion neurons cannot be ruled out, the available evidence argues against the idea of multiple independently regulated I_A channels.

The present study has also demonstrated that the developmental expression of $I_{K(\text{Ca})}$ is dramatically reduced in chick ciliary ganglion neurons developing *in situ* in the absence of either appropriate target tissues or preganglionic innervation. In contrast, voltage-activated Ca^{2+} currents of normal amplitude were present in all operated and control neurons. Similarly, ciliary ganglion neurons developing *in vitro* in the absence of other cell

types or exogenous trophic factors express normal levels of I_{Ca} but not $I_{K[Ca]}$, suggesting that intrinsic influences regulate I_{Ca} expression while extrinsic factors control $I_{K[Ca]}$. These results also suggest that I_A , $I_{K[Ca]}$, and I_{Ca} are regulated independently, and that the effects of disruptions of normal cell–cell interactions are specific in the sense that not all ionic channels are affected. During normal development, these extrinsic regulatory factors cause $I_{K[Ca]}$ to become the largest macroscopic outward current that can be evoked from normal resting membrane potentials in ciliary ganglion neurons (Dryer et al., 1991; Dourado and Dryer, 1992). In these cells, $I_{K[Ca]}$ channels control the late phases of spike repolarization and the majority of the spike afterhyperpolarization and are therefore critical for normal electrical behavior (Dryer et al., 1991). Thus, interactions with target tissues and the afferent innervation are essential for the expression of a normal electrophysiological phenotype in developing chick ciliary ganglion neurons.

There is strong precedence for cell–cell interactions regulating many aspects of the development and differentiation of ciliary ganglion neurons *in situ* (Cowan and Wenger, 1968; Landmesser and Pilar, 1974a,b; Narayanan and Narayanan, 1978; Pilar et al., 1980; Furber et al., 1987; Engisch and Fischbach, 1990; Arenella et al., 1993) and *in vitro* (Nishi and Berg, 1981; Coulombe and Nishi, 1991; Coulombe et al., 1993). In particular, many studies have demonstrated a role for cell–cell interactions in regulating functional nicotinic ACh receptor expression in developing ciliary ganglion neurons. For example, electrophysiological responses to ACh are reduced in OV^- ganglionic neurons (Engisch and Fischbach, 1990) as well as in ciliary ganglion neurons maintained *in vitro* for several days in the absence of other cell types (Crean et al., 1982). But coculture with pectoral myotubes, with which ciliary ganglion neurons form functional synapses *in vitro*, restores normal ACh sensitivity (Crean et al., 1982). Moreover, it has recently been demonstrated that a 50 kDa fraction derived from the chick eye can regulate the function and levels of ACh receptors on these neurons (Halvorsen et al., 1991). On the other hand, preganglionic innervation does not affect the whole-cell response to ACh (Engisch and Fischbach, 1992), although it should be noted that ACh receptor protein and transcript levels are reduced in input-deprived ciliary ganglion neurons (Schwartz-Levey et al., 1992; Arenella et al., 1993). In contrast, soluble factors derived from preganglionic tissue have been shown to increase the ACh sensitivity of chick sympathetic neurons developing *in vitro* (Role, 1988). It is possible that the regulation of ACh sensitivity, I_A kinetics, and $I_{K[Ca]}$ amplitude by cell–cell interactions may involve at least some common mechanisms.

In summary, we have examined the expression of I_A and $I_{K[Ca]}$ in chick ciliary ganglion neurons developing *in situ* in the absence of innervation, target tissues, or both. Both preganglionic innervation and target tissues are necessary for the normal developmental expression of $I_{K[Ca]}$, while neurons developing in the absence of normal target tissues express an I_A of normal amplitude but with abnormal kinetic properties. These results indicate that interactions with target tissues and the afferent innervation specifically regulate the expression of particular ionic channels, that is, I_A and $I_{K[Ca]}$, on the surface of developing vertebrate neurons. These ionic channels typically modulate the action potential waveform and the repetitive firing characteristics of vertebrate neurons, but they are not essential for the minimum manifestation of excitability. It is possible that the expression of ionic channels that confer a unique or specific

electrophysiological phenotype will prove to be dependent upon extrinsic factors during the normal embryonic development of many vertebrate neurons.

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