BK-Type $K_{\text{Ca}}$ Channels in Two Parasympathetic Cell Types: Differences in Kinetic Properties and Developmental Expression

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Cameron, Jill S. and Stuart E. Dryer. BK-type $K_{\text{Ca}}$ channels in two parasympathetic cell types: differences in kinetic properties and developmental expression. J Neurophysiol 84: 2767–2776, 2000. The intrinsic electrical properties of identified choroid and ciliary neurons of the chick ciliary ganglion were examined by patch-clamp recording methods. These neurons are derived from a common pool of mesencephalic neural crest precursor cells but innervate different target tissues and have markedly different action potential waveforms and intrinsic patterns of repetitive spike discharge. Therefore it is important to determine whether these cell types express different types of plasma membrane ionic channels, and to ascertain the developmental stages at which these cell types begin to diverge. This study has focused on large-conductance $Ca^{2+}$-activated $K^+$ channels ($K_{\text{Ca}}$), which are known to regulate spike waveform and repetitive firing in many cell types. Both ciliary ganglion cell types, identified on the basis of size and somatostatin immunoreactivity, express a robust macroscopic $K_{\text{Ca}}$, carried by a kinetically homogeneous population of large-conductance (BK-type) $K_{\text{Ca}}$ channels. However, the kinetic properties of these channels are different in the two cell types. Steady-state fluctuation analyses of macroscopic $K_{\text{Ca}}$ produced power spectra that could be fitted with a single Lorentzian curve in both cell types. However, the resulting corner frequency was significantly lower in choroid neurons than in ciliary neurons, suggesting that the underlying $K_{\text{Ca}}$ channels have a longer mean open-time in choroid neurons. Consistent with fluctuation analyses, significantly slower gating of $K_{\text{Ca}}$ channels in choroid neurons was also observed during macroscopic activation and deactivation at membrane potentials positive to $-30$ mV. Differences in the kinetic properties of $K_{\text{Ca}}$ channels could also be observed directly in single-channel recordings of identified embryonic day 13 choroid and ciliary neurons. The mean open-time of large-conductance $K_{\text{Ca}}$ channels was significantly greater in choroid neurons than in ciliary neurons in excised inside-out patches. The developmental expression of functional $K_{\text{Ca}}$ channels appears to be regulated differently in the two cell types. Although both cell types acquire functional $K_{\text{Ca}}$ at the same developmental stages (embryonic days 9–13), functional expression of these channels in ciliary neurons requires target-derived trophic factors. In contrast, expression of functional $K_{\text{Ca}}$ channels proceeds normally in choroid neurons developing in vitro in the absence of target-derived trophic factors. Consistent with this, extracts of ciliary neuron target tissues (striated muscle of the iris/ciliary body) contain $K_{\text{Ca}}$ stimulatory activity. However, $K_{\text{Ca}}$ stimulatory activity cannot be detected in extracts of the smooth muscle targets of choroid neurons.

INTRODUCTION

The electrophysiological properties of neurons are typically tuned for a specific physiological function. However, the factors controlling the developmental expression of a specialized electrophysiological phenotype are not well understood. The chick ciliary ganglion (CG) is a useful model system in which to study this process, as it contains two populations of mesencephalic neural crest-derived neurons that innervate different target tissues in the eye (Marwitt et al. 1971). Mature ciliary neurons innervate striated muscle cells in the iris and ciliary body, whereas choroid neurons innervate smooth muscle cells in the choroid layer. Both cell types are cholinergic, but choroid neurons also express somatostatin whereas ciliary neurons do not, and choroid neurons tend to be smaller than ciliary neurons (Darland and Nishi 1998; De Stefano et al. 1993; Epstein et al. 1988). These differences provide convenient criteria for identification of these neurons in dissociated preparations.

Choroid and ciliary neurons in the intact CG have markedly different electrophysiological properties (Dryer 1994). Ciliary neurons fire spikes at a constant high frequency ($>150$ Hz) in response to sustained injection of depolarizing current. The intrinsic firing properties of ciliary neurons are well matched to those of their striated muscle targets, which exhibit tetanic contraction only when stimulated at 100–150 Hz (Pilar and Vaughan 1969). In contrast, choroid neurons exhibit a maximum firing frequency of $\sim 35$ Hz and show substantial spike-frequency adaptation. Equally notably, the spike duration at half-amplitude in choroid neurons is about twice that of ciliary neurons (Dryer 1994).

We have previously shown that the differentiation of excitability in CG neurons is regulated by cell-cell interactions. Large CG neurons that develop in vivo in the absence of normal target tissues fail to express functional $Ca^{2+}$-activated $K^+$ channels ($K_{\text{Ca}}$) (Dourado et al. 1994). Moreover, large CG neurons developing in vitro fail to express $K_{\text{Ca}}$ unless iris extracts or iris-derived factors are included in the culture media (Cameron et al. 1998, 1999; Dourado and Dryer 1992; Subramony et al. 1996). The essential iris-derived factor appears to be an avian ortholog of TGF$\beta$1. Thus application of TGF$\beta$1 can markedly accelerate the functional expression of $K_{\text{Ca}}$ in vivo or in vitro, and a TGF$\beta$ neutralizing antiserum blocks the normal in vivo expression of $K_{\text{Ca}}$ as well as the in vitro actions of iris extracts (Cameron et al. 1998). Macroscopic $K_{\text{Ca}}$ in CG neurons is mediated by large-conductance (BK-type) channels (Cameron et al. 1998; Dryer et al. 1991; Lhuillier and Dryer 1999) encoded by the slo gene (Dryer 1998; Subramony et al. 1996). Although other types of $K_{\text{Ca}}$ channels with lower uni-
tary conductance can be detected in inside-out patches excised from CG neurons, these lower conductance KCa channels cannot be detected on depolarization of intact cells and do not appear to contribute to macroscopic currents (Lhuillier and Dryer 1999). Their functional significance, if any, remains unknown. However, macroscopic KCa contributes to spike repolarization and afterhyperpolarization in CG neurons (Dryer et al. 1991), and therefore differences in the properties of large-conductance KCa channels could contribute to the physiological differences between choroid and ciliary neurons.

Our previous studies on the developmental regulation of KCa channels and macroscopic KCa have focused on cells that, based on their size, are likely to consist primarily of ciliary neurons. Given the results in ciliary neurons, we hypothesized that KCa regulation in developing choroid neurons would follow a similar pattern, i.e., that expression of an identical population of KCa channels would require trophic factors secreted from target cells in the smooth muscle choroid layer. Here we show that both of these hypotheses are incorrect. Instead, developmental expression of macroscopic KCa in choroid neurons appears to be cell-autonomous, and certainly proceeds normally in the absence of target-derived factors. By contrast, functional expression of KCa in ciliary neurons is not cell-autonomous and requires exposure to trophic factors that are present in the iris, but that cannot be detected in the choroid smooth muscle layer. Moreover, the kinetic properties of large-conductance KCa channels, once they are present in the plasma membrane, are different in the two cell types. Specifically, choroid cells express large-conductance KCa channels with substantially slower kinetics than those of ciliary neurons. This conclusion is based on several independent kinetic methods, including analyses of macroscopic current fluctuation, activation, and deactivation, as well as more direct measurements in inside-out patches. These differences in macroscopic behavior can provide an explanation for at least some of the intrinsic differences in spike discharge between choroid and ciliary neurons. It is also possible that differences in gating properties and regulation of functional expression are consequences of a common molecular mechanism.

**METHODS**

**Cell isolation and culture**

Ciliary ganglion neurons were dissociated at embryonic day 9 (E9) or E13 as described in detail previously (Cameron et al. 1998, 1999; Dourado and Dryer 1992; Lhuillier and Dryer 2000; Subramony and Dryer 1997; Subramony et al. 1996). In most experiments, currents were evoked by depolarizing steps to 0 mV from a holding potential of −40 mV in normal and Ca2+-free external saline. Normal external saline consisted of 145 mM NaCl, 5.4 mM KCl, 5.4 mM CaCl2, 0.8 mM MgCl2, 5 mM d-glucose, 13 mM HEPES-NaOH, and 250 mM tetrodotoxin, pH 7.4. Nominally Ca2+-free external saline was the same except that it contained 5.8 mM MgCl2 and no added CaCl2. Net Ca2+-dependent outward currents (KCa) were then obtained by digital subtraction (control − Ca2+-free) and normalized for cell size by measuring soma surface area for each cell as described previously (Cameron et al. 1998, 1999; Dourado and Dryer 1992; Subramony et al. 1996). For steady-state fluctuation analysis, currents in each cell were evoked by a series of 10 depolarizing steps of 2.2 s duration to various command potentials in normal saline, followed by 10 identical steps after application of Ca2+-free saline. Data were digitized at 2 kHz, and the DC components of the evoked currents were removed. The steady-state portions of the evoked currents were cosine-tapered (w[n] = cos {π n/N}), and power spectra were computed from 4,096 digital samples using routines implemented in Pclamp software (Clampfit v. 8.0, Axon Instruments, Foster City, CA). Spectra obtained for currents evoked by each of the 10 depolarizing steps were averaged, and the resulting mean spectra were subtracted (control spectrum − Ca2+-free spectrum). This served to remove contributions from instrumentation noise and other voltage-evoked ionic currents (except for Ca2+- currents), and to thereby isolate the mean power spectrum of macroscopic KCa. The resulting KCa power spectra were smoothed by adjacent point averaging and then fitted with Lorentzian curves of the form \( S(f) = \frac{S(0)}{1 + (2\pi f)^2} \) as described in the text using a Levenburg-Marquardt least-squares algorithm (Microcal Origin, v 6.0, Northampton, MA). Single Lorentzian curves provided excellent fits to the KCa power spectra at all command potentials, a finding consistent with our previous studies with cell-attached patches in which we found that only a single population of large-conductance KCa channels became active on depolarization of intact cells, as well as single-exponential macroscopic tail currents over a wide range of test potentials (Lhuillier and Dryer 1999). This spectral subtraction procedure allows calculation of variance contributed by those macroscopic currents that depend on external Ca2+, which with these voltage-clamp protocols consist of voltage-activated Ca2+ currents and KCa. To determine whether Ca2+ currents make a significant contribution to the variance, we added to control media at a concentration of 3% (vol/vol). Choroid extracts were added to media at various concentrations as indicated.

**Immunocytochemistry**

Somatostatin staining was performed essentially as described by Darland and Nishi (1998). Briefly, CG neurons were dissociated at E13, fixed for 30 min in Zamboni’s solution (4% paraformaldehyde, 15% picric acid, and 0.1 M sodium phosphate buffer, pH 7.4), rinsed in PBS, and blocked overnight in 10% horse serum, 0.5% Triton X-100, and 0.2% sodium azide in PBS. Cells were then incubated overnight at room temperature (22°C) with monoclonal rat antisomatostatin (Accurate Chemical and Scientific, Westbury, NY, cell line YC7; 1:100 dilution in blocking solution without detergent). Cells were rinsed and incubated with PBS containing 10% H2O2 and 30% ethanol to inactivate endogenous peroxidase, and staining was revealed by the ABC method (Vectastain kit, Vector Laboratories Burlingame, CA) using diaminobenzidine as a chromogen according to the manufacturer’s directions. No signal was obtained from control CG cultures treated with preabsorbed primary antibody or with no primary antibody.

**Electrophysiology and data analysis**

Whole cell recordings were made using standard methods as described in detail previously (Cameron et al. 1998, 1999; Dourado and Dryer 1992; Lhuillier and Dryer 2000; Subramony and Dryer 1997; Subramony et al. 1996). In most experiments, currents were evoked by depolarizing steps to 0 mV from a holding potential of −40 mV in normal and Ca2+-free external saline. Normal external saline consisted of 145 mM NaCl, 5.4 mM KCl, 5.4 mM CaCl2, 0.8 mM MgCl2, 5 mM d-glucose, 13 mM HEPES-NaOH, and 250 mM tetrodotoxin, pH 7.4. Nominally Ca2+-free external saline was the same except that it contained 5.8 mM MgCl2 and no added CaCl2. Net Ca2+-dependent outward currents (KCa) were then obtained by digital subtraction (control − Ca2+-free) and normalized for cell size by measuring soma surface area for each cell as described previously (Cameron et al. 1998, 1999; Dourado and Dryer 1992; Subramony et al. 1996). For steady-state fluctuation analysis, currents in each cell were evoked by a series of 10 depolarizing steps of 2.2 s duration to various command potentials in normal saline, followed by 10 identical steps after application of Ca2+-free saline. Data were digitized at 2 kHz, and the DC components of the evoked currents were removed. The steady-state portions of the evoked currents were cosine-tapered (w[n] = cos {π n/N}), and power spectra were computed from 4,096 digital samples using routines implemented in Pclamp software (Clampfit v. 8.0, Axon Instruments, Foster City, CA). Spectra obtained for currents evoked by each of the 10 depolarizing steps were averaged, and the resulting mean spectra were subtracted (control spectrum − Ca2+-free spectrum). This served to remove contributions from instrumentation noise and other voltage-evoked ionic currents (except for Ca2+- currents), and to thereby isolate the mean power spectrum of macroscopic KCa. The resulting KCa power spectra were smoothed by adjacent point averaging and then fitted with Lorentzian curves of the form \( S(f) = \frac{S(0)}{1 + (2\pi f)^2} \) as described in the text using a Levenburg-Marquardt least-squares algorithm (Microcal Origin, v 6.0, Northampton, MA). Single Lorentzian curves provided excellent fits to the KCa power spectra at all command potentials, a finding consistent with our previous studies with cell-attached patches in which we found that only a single population of large-conductance KCa channels became active on depolarization of intact cells, as well as single-exponential macroscopic tail currents over a wide range of test potentials (Lhuillier and Dryer 1999). This spectral subtraction procedure allows calculation of variance contributed by those macroscopic currents that depend on external Ca2+, which with these voltage-clamp protocols consist of voltage-activated Ca2+ currents and KCa. To determine whether Ca2+ currents make a significant contribution...
Identification of isolated choroid and ciliary neurons

In intact ganglia, the two populations of CG neurons can be distinguished on the basis of size, ultrastructure, and somatostatin immunoreactivity. Choroid neurons express somatostatin, whereas ciliary neurons do not, and the majority of choroid neurons are smaller than ciliary neurons, although there is an overlap in their size distributions (Darland and Nishi 1998; De Stefano et al. 1993; Epstein et al. 1988). Here we have observed that the current density of macroscopic KCa in choroid and ciliary neurons isolated acutely at embryonic day E13 is significantly different in choroid and ciliary neurons isolated acutely at embryonic day E13. In subsequent experiments, neurons with diameters ≤12 μm were considered to be choroid neurons, whereas neurons with diameters ≥20 μm were considered to be ciliary neurons.

Differences in macroscopic KCa kinetics in choroid and ciliary neurons

We have previously shown that macroscopic KCa in CG neurons reaches maximum current density by E13 (Dourado and Dryer 1992). Here we have observed that the current density of macroscopic KCa is not significantly different in choroid and ciliary neurons isolated acutely at this developmental stage (Fig. 2). However, the kinetic properties of the underlying channels are different in the two cell types. This was established in part by three different types of macroscopic measurements. Whole cell recordings have the advantage of allowing measurements from a unimodal Gaussian distribution. Throughout, $P < 0.05$ was regarded as significant.

FIG. 1. Criteria for identification of choroid and ciliary neurons. A: distribution of neuronal diameters of ciliary ganglion (CG) neurons isolated acutely at embryonic day E13 (E13). Adequate fit of the distribution requires the sum of 2 Gaussian curves (shown superimposed) suggesting 2 size classes of CG neurons. These data are significantly different from a unimodal normal distribution (Kolmogorov-Smirnov 1-sample test). B: immunocytochemical staining for somatostatin in acutely dissociated E13 CG neurons. Note robust staining of the small CG neurons (choroid cell) but not the large CG neuron (ciliary neuron). Calibration bar indicates 20 μm. C: size distribution of somatostatin-positive choroid cells (left) and somatostatin-negative ciliary cells (right) in a preparation of CG neurons isolated acutely at E13. In subsequent experiments, neurons with diameters ≤12 μm were considered to be choroid neurons, whereas neurons with diameters ≥20 μm were considered to be ciliary neurons.
steps (shown above traces) in normal and Ca\(^{2+}\)-free salines as indicated. Typical traces are shown for a ciliary neuron (left) and a choroid neuron (right). Mean KCa current densities and SE from a large number of choroid and ciliary neurons are shown below, and are not significantly different in the 2 cell types. Numbers above bars denote number of cells tested.

In the first approach, the kinetic properties of whole cell KCa were determined by steady-state fluctuation analysis. If one assumes that channels gate independently, then it is possible to extract information about the underlying kinetics from spectral density analysis of macroscopic current fluctuations (Anderson and Stevens 1973). Although fine details of the kinetics are masked by bandwidth limitations inherent in whole cell recording, this type of analysis has been successfully used to resolve kinetic differences associated with developmental changes in the subunit stoichiometry of ligand-gated ionic channels (e.g., Fischbach and Schuetze 1980; Vicini and Schuetze 1985). In our experiments, currents in each CG cell were evoked by a series of 10 depolarizing step pulses of 2.2-s duration to various holding potentials from a holding potential of \(-40\) mV in the presence of Ca\(^{2+}\). These protocols were then repeated in the absence of external Ca\(^{2+}\) (Fig. 3, A and B) as described previously (Cameron et al. 1998, 1999; Dourado and Dryer 1992; Dourado et al. 1994; Subramony et al. 1996). The mean power spectra of macroscopic current fluctuations were calculated for currents evoked in the presence and absence of Ca\(^{2+}\), and the resulting spectra were subtracted digitally to determine the Ca\(^{2+}\)-dependent component of the variance associated with evoked steady-state outward currents (Fig. 3, C and D). The subtracted power spectra were fitted with Lorentzian curves of the form \(S(f) = S(0) / [1 + (2\pi f \tau)^2]\), where \(S(f)\) is power as a function of frequency, \(S(0)\) is the extrapolated power at frequency \(= 0\) Hz, \(f_c\) is the frequency at which power is \(S(0)/2\), and \(\tau\) is a time constant defined as \(1/2\pi f_c\) and related but not precisely equal to the mean open-time. In both populations of neurons, single Lorentzian curves provided excellent fits to the subtracted power spectra, suggesting that a kinetically homogeneous population of channels underlies macroscopic KCa (Fig. 3, C and D). These panels show spectra calculated for currents evoked by a depolarizing step to 0 mV, and they indicate that KCa channels have different kinetic properties in the two populations of cells. The mean time constant of fitted Lorentzian curves calculated from pulses to 0 mV in a large number of choroid cells was more than twice that of ciliary cells (Fig. 3E). These differences are statistically significant and are not a consequence of different probabilities of channel opening (\(P_o\)). It is possible to determine \(P_o\) during a depolarizing step by means of binomial statistics (Anderson and Stevens 1973). Thus \(i = (1 - P_o) \sigma^2 / \mu_i\), where \(i\) is the unitary current flowing through a single open KCa channel, \(\sigma^2\) is the variance of the macroscopic fluctuations associated with KCa gating, and \(\mu_i\) is the mean Ca\(^{2+}\)-dependent outward current. Independent measurements with cell-attached patches indicate that \(i = 6.5\) pA at 0 mV with close to physiological ionic gradients (Lhuillier and Dryer 1999). Current variance associated with KCa gating (\(\sigma^2\)) is simply the integral of the fitted Lorentzian curve, which is given by \(S(0)/2\). The mean current (\(\mu_i\)) is the difference in net outward current evoked in the presence and absence of Ca\(^{2+}\). From these parameters, the mean \(P_o\) during a step pulse to 0 mV was calculated to be 0.92 \pm 0.06 in ciliary cells, and 0.95 \pm 0.07 in choroid cells. These results are not
significantly different and indicate that step pulses to 0 mV evoke comparable $K_{Ca}$ activation in the two cell types. In a separate set of experiments, similar analyses were carried out on both cell types using a range of depolarizing steps (to $-25$ mV, 0 mV, and $+25$ mV) from a holding potential of $-40$ mV. The resulting time constants derived from power spectral analysis were significantly ($P < 0.05$) longer in choroid cells than in ciliary cells at all three membrane potentials (Fig. 3F). However, these differences were more apparent at more depolarized potentials, i.e., the voltage dependence of $K_{Ca}$ gating appears greater in choroid neurons than in ciliary neurons.

This pattern can also be seen in analyses of $K_{Ca}$ deactivation kinetics in the two cell types. For these experiments, $K_{Ca}$ was activated by a 25-ms step to 0 mV from a holding potential of $-40$ mV, at which time the membrane was stepped through a series of test potentials between $-70$ and $-20$ mV. This protocol was repeated in normal and Ca$^{2+}$-free salines, and the resulting Ca$^{2+}$-dependent currents were obtained by digital subtraction (Fig. 4A). The decay phases of the $K_{Ca}$ tail currents were fitted with single-exponential curves, which provided good fits to the data in both cell types as described previously for ciliary neurons (Dryer et al. 1991; Lhuillier and Dryer 1999). As with fluctuation analysis, the resulting deactivation time constants were significantly longer in choroid neurons than in ciliary neurons, but only at $-30$ and $-20$ mV. Tail-current decay time constants were not significantly different in the two cell types at more negative membrane potentials, an observation consistent with the trends noted above in fluctuation analysis.

Significant differences in macroscopic $K_{Ca}$ behavior in the two cell types also appeared in analyses of activation kinetics derived from test pulses to 0 mV from a holding potential of $-40$ mV (Fig. 4B). The time course of activation was fitted with a single-exponential. Activation of $K_{Ca}$ was several-fold slower in choroid neurons than in ciliary neurons, and the differences in the resulting time constants are statistically significant ($P < 0.01$). Macroscopic $K_{Ca}$ is the largest outward current that can be evoked from normal resting potential in both cell types, and these data therefore provide a mechanism for the different spike durations in the two cell populations.

**Differences in single-channel $K_{Ca}$ kinetics in choroid and ciliary neurons**

These analyses were carried out using two different single-channel recording configurations. In one set of experiments, the kinetic properties of $K_{Ca}$ channels were determined in inside-out patches excised from choroid and ciliary neurons. This method has the advantage of examining $K_{Ca}$ channel gating more directly under controlled membrane potential and [Ca$^{2+}$], but has the disadvantage of sampling all $K_{Ca}$ channels in the patch membrane, whether or not they would normally contribute to macroscopic currents. In these experiments, patches were excised into a Ca$^{2+}$-free bath saline (containing 10 mM EGTA) and held at $+25$ mV. $K_{Ca}$ channels were quiescent in Ca$^{2+}$-free bath saline but became active on bath application of salines containing 5 μM free Ca$^{2+}$ (Fig. 5). These conditions were chosen because they yielded a mean $P_{o} > 0.90$, comparable to the gating conditions of the macroscopic measurements described above (Fig. 6, A and B). Kinetic analyses were performed on patches that had one, or at most two, large-conductance $K_{Ca}$ channels, and that did not contain other detectable channel types. In particular, patches that contained intermediate-conductance $K_{Ca}$ channels were excluded, as these channels do not appear to contribute to macroscopic currents and are not activated by depolarization of intact CG neurons (Lhuillier and Dryer 1999). The unitary conductance of single large-conductance $K_{Ca}$ channels, derived from all-points histograms, was indistinguishable in ciliary and choroid neurons. In both cell types, this was 120 pS with $[K]_{o} = 37.5$ mM and $[K^+]_i = 150$ mM (data not shown). Open-time distributions were constructed from idealized data ignoring transitions of <0.5 ms duration. Single exponential curves provided excellent fits to the resulting distributions in both cell types. The open-time histograms shown in Fig. 6, A and B, were constructed from patches that contained only one $K_{Ca}$ channel based on maximum current amplitudes observed at high $P_{o}$. It should be noted that the time constants of the fitted exponential curves were significantly longer in choroid cells than in ciliary cells (Fig. 6, A and B). Mean time constants pooled from several excised patches were in good agreement with mean time constants obtained from fluctuation analysis (Fig. 6C).

**Different modes of $K_{Ca}$ regulation in developing choroid and ciliary neurons**

We have previously shown that large CG neurons isolated at E9 and allowed to develop in culture for 4 days do not express
macroscopic $K_	ext{Ca}$ at significant levels, in distinct contrast to CG neurons isolated acutely at $E13$ (Cameron et al. 1998, 1999; Dourado and Dryer 1992; Subramony and Dryer 1997; Subramony et al. 1996). Application of iris extracts (Subramony et al. 1996) or target-derived differentiation factors (Cameron et al. 1998, 1999) restores normal expression of $K_	ext{Ca}$ in CG neurons developing in vitro. The majority of those recordings were made from cells that, based on their size, were likely to be ciliary neurons. To determine whether the two cell types achieve their mature ($E13$) current density by different developmental mechanisms, we initially determined whether the two cell types acquire robust macroscopic $K_	ext{Ca}$ at the same developmental stages. Approximately half of the CG neurons at $E9$ are comprised of choroid cells (Landmesser and Pilar 1974). Therefore robust expression of $K_	ext{Ca}$ in $E9$ choroid neurons would predict a distinctly bimodal distribution of current densities and an inverse correlation between current density and cell size in the entire population of CG neurons. This was not observed in a large number of CG neurons isolated acutely at $E9$ ($E9 + 0$ in vitro cells), with care taken to sample neurons spanning the full range of neuronal diameters apparent in these preparations (Fig. 7A). Instead, the distribution of current densities was unimodal, and there was no correlation between current density and cell size either within a group or when the entire population of CG neurons was considered. These data indicate that very few cells of either type express $K_	ext{Ca}$ at significant densities by $E9$, in contrast to $E13$, when both cells express robust $K_	ext{Ca}$ at a comparable current density (Fig. 2). Does the developmental expression of $K_	ext{Ca}$ in choroid neurons require inductive interactions, as observed previously for ciliary neurons? To test this hypothesis, CG cells were isolated at $E9$, a stage prior to significant synapse formation with target tissues in either cell type. Neurons were then allowed to

**FIG. 5.** Examples of large-conductance $K_	ext{Ca}$ unitary currents in inside-out patches excised from identified CG neurons isolated at $E13$. Representative 2-s traces from a ciliary neuron (A) and a choroid neuron (B) before and after bath application of 5 μM Ca$^{2+}$ as indicated. Closed and open states are indicated to the left of the traces. Patches were held at $+25$ mV. Note that these patches were quiescent in Ca$^{2+}$-free bath salines, and robust activation of a single $K_	ext{Ca}$ channel after Ca$^{2+}$ application. Note also the greater frequency of channel closure in the presence of Ca$^{2+}$ in the patch excised from the ciliary neuron.

**FIG. 6.** Different mean open-times of large-conductance $K_	ext{Ca}$ channels in inside-out patches excised from $E13$ ciliary and choroid neurons. A: properties of $K_	ext{Ca}$ unitary currents in a ciliary neuron. Trace to the left shows 200-ms inset of data from a patch recorded in the presence of 5 μM free Ca$^{2+}$ at $+25$ mV. Middle graph shows probability of $K_	ext{Ca}$ opening ($P_o$) obtained from 10 s of continuous data in the presence of 5 μM free Ca$^{2+}$. Graph to the right shows open-time histogram constructed from this patch, with superimposed fitted single-exponential curve with a time constant of 6.0 ms. B: properties of $K_	ext{Ca}$ unitary currents in a choroid neuron. Note substantially longer time constant (18.7 ms) of single-exponential fit to the open-time histogram. Data in A and B are from patches that contained only 1 $K_	ext{Ca}$ channel. C: mean time constants of open-time histograms and SE obtained from several ciliary and choroid neurons as indicated. These means are significantly different and indicate substantially longer mean $K_	ext{Ca}$ open-time in choroid neurons.
develop in vitro for 4 days in standard culture medium (E9 + 4 in vitro cells), at which time KCa expression was determined by means of whole cell recordings. As with our previous studies, we found that KCa was either undetectable or expressed at very low density in E9 + 4 in vitro ciliary neurons. In contrast, macroscopic KCa expression was robust in E9 + 4 in vitro choroid neurons and the current density was comparable to that observed in CG neurons isolated acutely at E13 (Fig. 7B). These differences are statistically significant. As expected from these data, the distribution of KCa densities in the entire sample of E9 + 4 in vitro CG neurons was clearly bimodal with a statistically significant inverse correlation between cell size and KCa current density when the entire population of CG cells was considered (Fig. 7C). There was no correlation between cell size and current density within a group. In other words, qualitatively different distributions of current density were observed in E9 + 4 in vitro CG neurons compared with cells isolated acutely at either E9 or E13. These results support the hypothesis that KCa is regulated cell-autonomously in small choroid neurons, and clearly indicate different KCa regulatory mechanisms in developing choroid and ciliary neurons.

Differences in KCa stimulating activity of choroid coat and iris/ciliary body extracts

Given the differences in the functional regulation of KCa expression in choroid and ciliary neurons, it was of interest to determine whether choroid neuron target tissues in the eye express KCa stimulatory factors. We have previously shown that ciliary neurons that develop in vitro can be induced to express macroscopic KCa by iris extracts or by target-derived factors such as TGFβ1/4 (Cameron et al. 1998, 1999; Subramony et al. 1996). In the present experiments, we prepared extracts of the choroid smooth muscle layer and the iris/ciliary body in parallel, using procedures described previously (Cameron et al. 1998, 1999; Subramony et al. 1996). CG neurons were isolated at E9 and cultured for 12 h in the presence or absence of choroid extract and/or iris extract. Recordings were then made from CG neurons, at least one population of which express functional KCa in response to iris extract (Cameron et al. 1998, 1999; Subramony et al. 1996). As described previously, iris extracts evoked robust stimulation of KCa expression in CG neurons, probably owing to the ciliary neurons present in these cultures (Fig. 8). In contrast, application of choroid
extracts at several concentrations did not evoke \( K_{Ca} \) expression in CG neurons.

**DISCUSSION**

Choroid and ciliary neurons of the chick CG are both derived from cranial neural crest and undergo key developmental events at the same embryonic stages (reviewed in Dryer 1994). However, the two cell populations of cells differ in several mature phenotypic traits (De Stefano et al. 1993; Dryer 1994; Epstein et al. 1988; Marwitt et al. 1971; Ullian et al. 1997). Some of these, such as differences in neuropeptide expression, are regulated by cell-cell interactions during development (Coulombe and Nishi 1991; Coulombe et al. 1993; Darland and Nishi 1998; Darland et al. 1995). In this study, we have shown that the biophysical properties of large-conductance (BK-type) \( K_{Ca} \) channels, and the mechanisms controlling their developmental expression, are different in choroid and ciliary neurons of the chick CG. The unitary conductance of \( K_{Ca} \) channels is the same in both cell types. However, ciliary neurons express \( K_{Ca} \) channels with a relatively short open-time, which is associated with more rapid macroscopic activation and deactivation kinetics in ciliary neurons. Moreover, functional expression of \( K_{Ca} \) channels in ciliary neurons is dependent on cell-cell interactions during development, whereas choroid neurons regulate these channels by an apparently cell-autonomous mechanism. Consistent with this, \( K_{Ca} \) stimulatory factors are present in the iris, one of the target tissues of ciliary neurons, but are not expressed in the choroid smooth muscle layer, the target tissue of the choroid neurons.

This pattern may not be unique to the chick CG. Multiple cell populations with distinct electrophysiological properties are present in many autonomic ganglia and central nuclei. To cite just one example, B-cells and C-cells within bullfrog paravertebral sympathetic ganglia receive afferents from different spinal segments and innervate different target tissues (Dodd and Horn 1983; Horn et al. 1988; Smith 1994). These cells also have different conduction velocities, action potential waveforms, and intrinsic patterns of repetitive spike discharge (Smith 1994). Differences in the developmental expression of ionic channels may often occur in neighboring neurons that project to physiologically distinct target cells. In such cases, differential regulation of ion channel expression by inductive cell-cell interactions may provide a mechanism to ensure that the intrinsic properties of pre- and postsynaptic elements are appropriately matched. In this regard, we have observed that expression of macroscopic \( K_{Ca} \) is regulated by soluble trophic factors in essentially all chick lumbar paravertebral sympathetic neurons, but that only a subpopulation of these respond to nerve growth factor (Raucher and Dryer 1995).

The intrinsic properties of ciliary and choroid neurons are well matched to those of their respective target tissues. Specifically, ciliary neurons need to be able to discharge spikes at a sustained high frequency (>100 Hz) because tetanic contractions of their striated muscle targets in iris require stimulation at this range of frequencies (Pilar and Vaughan 1969). The relationship between stimulus frequency and contraction has not been studied in choroid smooth muscle, but ocular smooth muscles in other species exhibit maximum contractions at stimulus frequencies as low as 10 Hz (Suzuki 1983). It is not known whether \( K_{Ca} \) kinetics can account for these differences in CG neuron repetitive firing. However, the slower macroscopic kinetics of \( K_{Ca} \) in choroid neurons would predict a longer duration spike duration, as is observed (Dryer 1994). Other aspects of the behavior of choroid and ciliary neurons are quite similar under voltage clamp, and this is the first consistent difference that we have observed over many years of investigation. In this regard, it bears noting that neurons whose dominant outward currents exhibit more rapid activation and deactivation kinetics tend to have shorter duration action potentials and are able to follow high frequencies of synaptic stimulation (reviewed in Gan and Kaczmarek 1998; Rudy et al. 1999).

The molecular basis for the different developmental and kinetic properties of \( K_{Ca} \) channels in the two CG cell types is not known, but it is very plausible that these observations share a common mechanistic basis. One possibility is that the two cell types express different \( slo \) splice variants. Cell-specific expression of different \( slo \) variants has been observed in cochlear hair cells of the chick (Navaratnam et al. 1997; Rosenblatt et al. 1997) and turtle (Jones et al. 1999), as well as in the mammalian CNS (Tseng-Crank et al. 1994) and in *Drosophila* (Becker et al. 1995). Differences in \( K_{Ca} \) kinetics in hair cells are known to have large effects on oscillatory membrane behavior (reviewed in Fettiplace and Fuchs 1999). We have previously detected two different \( slo \) partial cDNAs in the chick CG. These cDNAs correspond to transcripts that differ by the presence or absence of a single 28 amino acid exon present in one isoform but not the other (Subramony et al. 1996). This alternative exon is located close to the C-terminus, and variations in this portion of the molecule do not produce particularly large differences in the kinetics of SLO channel alpha subunits expressed in heterologous systems (Ramanathan et al. 1999, 2000). However, it is certainly possible that variability in this portion of the channel molecule could result in differences in plasma membrane targeting. Preliminary data from single-cell RT-PCR indicate that both cell types express the smaller of these two \( slo \) splice variants, but to date these experiments have been inconclusive as to possible differential expression of the larger variant (data not shown).

It is also possible that the two populations of CG neurons differentially express auxiliary (beta) subunits that result in different \( K_{Ca} \) kinetics (Ramanathan et al. 1999, 2000) and/or
developmental regulatory mechanisms. This hypothesis is especially attractive because avian beta subunits produce more substantial effects on KCa kinetics (Ramanathan et al. 1998, 2000), and could also play a role in membrane targeting. In this regard, we have recently obtained evidence that a significant component of the trophic regulation of KCa expression in ciliary neurons entails trafficking of preexisting intracellular KCa channels to the plasma membrane. Moreover, target-derived factors such as TGFβ1, which cause robust stimulation of functional KCa expression in ciliary neurons, do not affect expression of slo transcripts in those cells (Lhuillier and Dryer 2000). Therefore it is possible that auxiliary subunits selectively expressed in one CG cell type regulate plasma membrane insertion and the kinetics of KCa channels. There is precedent in the literature for these types of mechanisms. For example, two different proteins isolated from Drosophila have been shown to interact with SLO channels and inhibit their insertion into the plasma membrane (Schopperle et al. 1998; Xia et al. 1998). One of these, known as dSLIP1, can also insertion into the plasma membrane (Schopperle et al. 1998; Xia et al. 1998). Vertebrate homologues of dSLIP1 have not been identified, but it is certainly possible that multiple SLO interacting proteins exist, some of which could act selectively on specific SLO isoforms. These types of interactions are not unique to KCa channels. For example, auxiliary subunits of various voltage-activated K+ channels appear to regulate both the gating kinetics and the efficiency of plasma membrane insertion in heterologous systems (e.g., Fink et al. 1996; Salinas et al. 1997; Shi et al. 1996).

In summary, we have shown that two closely related populations of parasympathetic neurons that innervate different target tissues express large conductance KCa channels with different gating properties. These neurons regulate the functional expression of KCa channels by different mechanisms, which may allow for appropriate matching between the intrinsic firing properties of the neurons and the physiological properties of their target tissues.

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