IONIC CHANNELS IN A LINE OF EMBRYONAL CARCINOMA CELLS INDUCED TO UNDERGO NEURONAL DIFFERENTIATION

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ABSTRACT The gigaseal patch clamp technique was used to investigate the electrophysiological properties of a line of embryonal carcinoma cells (PCC4) that were induced to undergo neuronal differentiation. A large increase in number of voltage-dependent potassium and sodium channels was observed during differentiation. The pharmacology and kinetics of the macroscopic sodium and potassium currents in the differentiated cells closely resembled those of the rapid inward sodium current and the delayed rectifier, respectively. The kinetic behavior of single-channel potassium currents was consistent with the properties of hte macroscopic delayed rectifier current.

INTRODUCTION

Mouse embryonal carcinoma (EC) cells have been used to study early mammalian development (Jacob, 1978). In tissue culture, some lines of these pluripotential cells will form embryoid bodies that morphologically resemble the normal blastocyst with inner cells and a layer of outer cells. If the EC cells are injected into a blastocyst which is then implanted into a pseudopregnant mouse, they will integrate with the inner cell mass of the host and contribute to the formation of almost all the tissues in the resulting chimeric mice.

In addition to providing information about early embryogenesis, EC cells can be used for studying terminal differentiation into specific tissues. Some lines of EC cells can be induced by physical or chemical means to form neurons as judged by a number of biochemical and morphological criteria (Paulin et al., 1982; Pfeiffer et al., 1981; Liesi et al., 1983). However, these cells have never been shown to behave functionally like neurons. In the present study the gigaseal patch clamp technique was used to investigate the electrophysiological properties of a line of EC cells (PCC4) that were induced to undergo neuronal differentiation. The results demonstrate the development of electrical excitability during differentiation. Furthermore, the relatively low density of delayed rectifier channels in the differentiated cells permitted us to study the single-channel kinetics of this channel.

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METHODS

Cell Culture

The EC line used for the experiments was a subclone of the azaguanine resistant line, PCC4, cultured as described by Speers et al., 1979. The cells were induced to undergo neuronal differentiation by growing them in suspension in the presence of 10 mM *N,N*-dimethylacetamide (DMA).

Electrophysiology

Cells attached to glass coverslips were placed in a chamber mounted on the stage of an inverted microscope. The cells were bathed by a saline solution containing (millimolars) 140 NaCl, 2.8 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 Hepes-NaOH. In some experiments 25 μg/ml tetrodotoxin (TTX) was added to the bathing solution to block sodium currents. All experiments were performed at 22–24°C.

A patch clamp (model 8900; Dagan Corp., Minneapolis, MN) was used for single channel and whole cell experiments. The pipettes were made from borosilicate glass and coated with Sylgard (Dow Corning, Midland, MI) according to Hamil et al. (1981). The recorded current was filtered by a four-pole Bessel filter. An on-line computer (Minc 11/23; Digital Equipment Corp., Maynard, MA) was used to generate command pulse signals and collect data. Sample intervals ranged between 75 μs and 5 ms. The data were analyzed off-line by the computer, with operator inspection and approval of each transition. A simple threshold-detection method was used to measure open and closed time durations with thresholds of both open and closed times set at 50% of the channel amplitude. Only patches that contained a single channel and whose seal resistances were >80 GΩ were used in analysis of open-, closed-, and burst-time durations. The histograms presented below are representative of data obtained from four experiments.

Histology and Electron Microscopy

Cells were fixed overnight in 10% formaldehyde. Silver impregnation was carried out as described (Bodian, 1937). Preparation of samples for

transmission electron microscopy has been described previously (Theofilopoulos et al., 1980). Areas to be thin sectioned were carefully selected to exclude extraneous cell types in mixed populations.

RESULTS

Morphological Differentiation of EC Cells

The different cell types were defined by their morphological characteristics at the level of the light microscope. The EC stem cells tended to grow as sheets of cells. They had a high nuclear-to-cytoplasmic ratio, a single prominent nucleolus, and indistinct cell borders. In contrast, the differentiated cells formed small colonies of closely packed, round cells with numerous long processes. The processes were invariably stained by silver impregnation. These colonies were examined by electron microscopy that showed abundant microtubules and occasional dense core vesicles consistent with neuronal differentiation. No synapse formation was noted.

Electrophysiological Changes During Differentiation

Fig. 1 *A* and *B* shows current traces recorded from an undifferentiated and a differentiated EC cell in the whole cell configuration, respectively. In undifferentiated cells, application of depolarizing steps from a holding potential of −60 mV failed to elicit any significant inward or outward currents. In contrast, in differentiated cells similar depolarizing steps produced rapid, transient inward currents followed by a delayed outward current. The inward current (not shown) could be blocked by TTX and corresponded to the rapid inward sodium current seen in other excitable cells. The delayed outward current activated at potentials positive to −30 mV. It consisted of two phases: a rapid activation phase followed by a slow phase of inactivation. The time course of inactivation could be reasonably well described by a single exponential process with a time constant of 2.4–3.2 s at potentials between −20 and 20 mV. At long times inactivation was 80–90% complete. Application of 15 mM external tetraethylammonium caused block of the outward current. The current did not require internal calcium for activation since the free calcium concentration in the pipette was only 2 nM. The pharmacology and kinetics of this current most closely resemble that of the delayed rectifier (Moolenaar and Spector, 1978).

Single-Channel Properties of the Delayed Rectifier

The most common channel in the membrane of the differentiated cells was a small, outward potassium channel that will from now on be referred to as the delayed rectifier channel as it will be shown to possess many of the properties of the delayed rectifier channel in other cells. A similar channel was occasionally observed in the undifferentiated cells. Fig. 2 *A* shows records from a single delayed rectifier channel recorded in a cell-attached patch with normal saline solution in the pipette. All potentials in this figure as well as in the remainder of the paper were measured with respect to the cell's resting potential. The holding potential was −20 mV relative to the resting potential. When depolarizing pulses to potentials ≥ 20 mV were applied, a small outward channel turned on. The probability of channel opening increased with potential. At 80 mV the channel was open almost all of the time.

The open channel current-voltage relationship was linear between 20 and 70 mV with a slope conductance of 8.1 pS. At higher potentials the I-V relationship showed a negative curvature. In five other experiments the average slope conductance was 10.3 ± 3.2 pS. Since current flowing through the channel was outward, it must be carried by either potassium or chloride ions. In order to

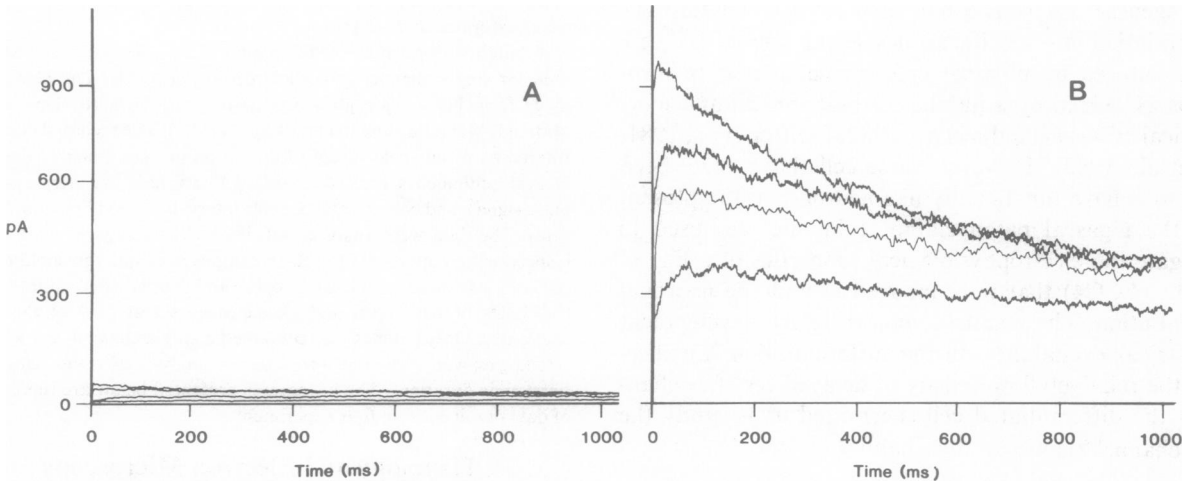

FIGURE 1 Comparison of whole cell currents recorded from EC stem cells and differentiated neuronal cells. (*A*) EC cell. V_H = −60 mV. Voltage steps to 0, 10, 15, and 40 mV. (*B*) Differentiated neuronal cell after exposure to TTX to block the fast inward current. V_H = −60 mV. Voltage steps to −40, −20, 0, and 20 mV. Pipette solution: 140 mM KF, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 11 mM EGTA, and 10 mM Hepes.

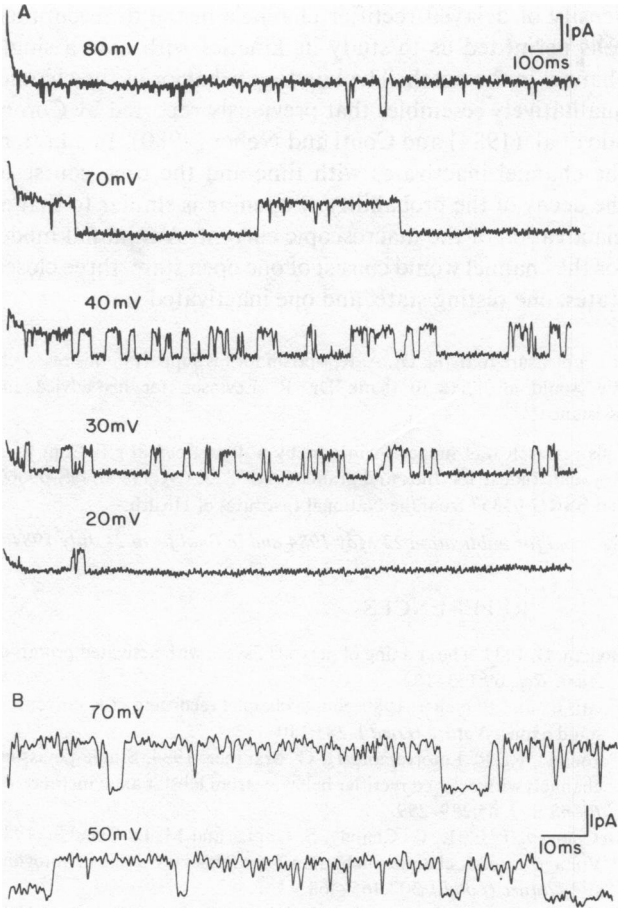

FIGURE 2 (*A*) Single-channel currents recorded from a patch of plasma membrane (cell-attached patch) from a differentiated neuronal cell. Membrane potentials are expressed with respect to the cell's resting potential. The membrane potential was held at −20 mV and then stepped to the indicated voltages at time zero. The small declining current observed during the first 20 ms of the voltage-clamp step is a capacitive artifact. The records were low-pass filtered at 200 Hz. (*B*) Single-channel data selected to show fast closings. Low-pass filtered at 1 KHz. Pipette solution: 140 mM NaCl, 2.8 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, and 10 mM Hepes.

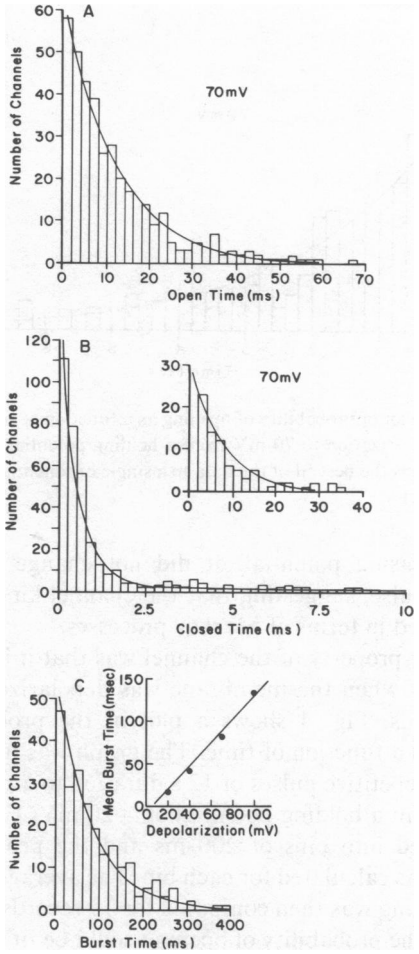

FIGURE 3 (*A*) Distribution of open times at 70 mV. Data have been fit to a single exponential with a time constant of 11.8 ms (solid line). Bin width is 2 ms. (*B*) Distribution of closed times at 70 mV. The data have been fit by eye with the sum of two exponentials with time constants of 0.46 and 7.35 ms (solid line). Bin width is 0.2 ms. The inset shows the closed-time distribution at a slower time scale to demonstrate the distribution of the long closed intervals. Both *A* and *B* were filtered at 1 KHz. The sampling rate was 13.3 KHz. (*C*) Burst-time histogram at 70 mV. Bin width is 20 ms. The solid line is the best fit of the data to a single exponential with a time constant of 80.5 ms. The inset shows a plot of the mean burst time as a function of membrane potential. The straight line was drawn by eye. Filtered at 200 Hz.

discriminate between these two possibilities, isethionate, which does not permeate any known Cl^- channel, was substituted for chloride in the pipette. No change in channel amplitude was observed, indicating that the channel was primarily selective for potassium ions.

Fig. 2 *B* shows similar data recorded at a higher bandwidth and time resolution. It is evident that the openings occurred in bursts within which there were numerous fast closings, which were poorly resolved in Fig. 2 *A*.

Fig. 3 *A* and *B* shows the open- and closed-time histograms filtered at 1 KHz. The open-time histogram could be described by a single exponential process with a time constant of 11.8 ms. The distributions of closed-times could be described by two exponentials with time constants of 0.46 and 7.35 ms. In some of the experiments a third exponential term with a time constant of several hundred milliseconds was required to describe the data completely. Filtering similar data from the same cell at 2 KHz did not significantly change our findings, although it did cause a substantial increase in baseline noise, making automatic detection of opening and closing events difficult. Comparison of measurements at 50 and 70 mV shows that the mean open time increased with increasing depolarization. The mean burst length was also examined, where a burst is defined as a group of openings and fast closings separated from the next such group by a closed time of >2 ms. Fig. 3 *C* shows a burst-time distribution computed from the same set of data shown in Fig. 2 *A*. The burst time could be described by a single exponential with a time constant of 80.5 ms. The inset shows the mean burst time as a function of potential. The mean burst duration increased linearly

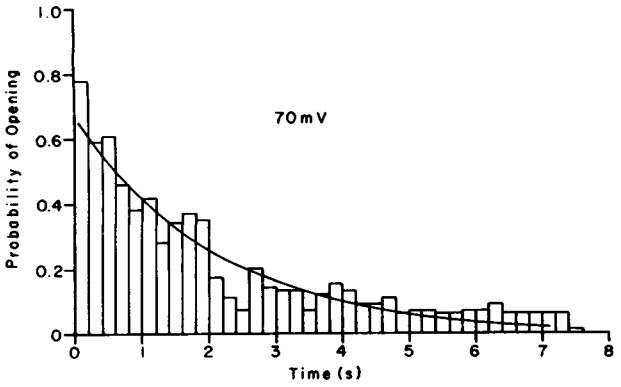

FIGURE 4 Plot of probability of opening as a function of time following onset of depolarization to 70 mV from a holding potential of −20 mV. The solid line is the best fit of the data to a single exponential with a time constant of 2.1 s.

with increasing potential. It did not change with time during a pulse, suggesting that the channel kinetics could be described in terms of Markov processes.

Another property of the channel was that it inactivated completely when the membrane was depolarized for several seconds. Fig. 4 shows a plot of the probability of opening as a function of time. The graph was obtained by applying repetitive pulses of 12 s duration to a potential of 70 mV from a holding potential of −20 mV. Each record was divided into bins of 200 ms and the probability of opening was calculated for each bin. The average probability of opening was then computed for 15 records. The time course of the probability of opening could be fit by a single exponential with a time constant of 2.1 s. This value was similar to that determined for inactivation of the macroscopic current.

DISCUSSION

There is a large increase in the number of potassium and sodium channels in EC cells induced to undergo neuronal differentiation. The pharmacology and kinetics of the macroscopic sodium and potassium current in the differentiated cells closely resemble those of the rapid inward sodium current and the delayed rectifier, respectively. No calcium current was observed in the undifferentiated or differentiated cells but this may be due to our recording solution, which contained KF in the pipette and 1 mM calcium in the bathing solution. Kostyuk et al. (1975) reported that calcium currents in snail neurons are eliminated by fluoride-containing solutions.

The delayed rectifier was also studied at the level of the single channel. The conductance of the channel under physiological conditions was 10.3 pS, which is similar to that reported in mouse neuroblastoma cells (Quandt, 1984) and squid axons (Conti and Neher, 1980; Llano and Bezanilla, 1983) when corrections are made for differences in temperature. This value is somewhat smaller than that reported in T-lymphocytes (DeCoursey et al., 1984) or lobster axons (Coronado et al., 1984). The relatively low density of delayed rectifier channels in the differentiated cells permitted us to study its kinetics with only a single channel in the patch. The bursting behavior of the channel qualitatively resembles that previously reported by Coronado et al. (1984) and Conti and Neher (1980). In addition, the channel inactivates with time and the time course of the decay of the probability of opening is similar to that of inactivation of the macroscopic current. A minimal model for the channel would consist of one open state, three closed states, one resting state, and one inactivated state.

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