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Na^+ CHANNELS IN CARDIAC AND NEURONAL CELLS DERIVED FROM A MOUSE EMBRYONAL CARCINOMA CELL LINE

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SUMMARY

1. Cells from a pluripotent murine embryonal carcinoma cell line (P19) were differentiated *in vitro* into cells with neurone- and cardiac-like phenotypes. Cells treated with 0·5 μM retinoic acid developed into neurone-like cells possessing extensive neurites. Dimethyl sulphoxide treatment (0·5%) produced large, spontaneously contracting cell aggregates with many properties of cardiac cells.

2. The neurone- and cardiac-like cells contained voltage-sensitive Na^+ channels with properties similar to those of native neuronal and cardiac cells.

3. We used whole-cell patch clamp techniques to measure inward currents from the neurone- and cardiac-like cells. Undifferentiated (untreated) cells had only small inward currents (peak of −0·15 nA in 150 mM external Na^+). The peak inward current in the neurone-like and cardiac-like cells was −1·2 nA (in 154 mM external Na^+) and −2·8 nA (in only 46 mM Na^+), respectively. These large currents were absent when the external solution contained no Na^+.

4. Tetrodotoxin (TTX) blocked the Na^+ currents in the neurone- and cardiac-like cells in a dose-dependent manner. The K_d for TTX block of the Na^+ current in the neurone-like cells was 6·7 nM. The Na^+ current in the cardiac-like cells was much more resistant to TTX; the half-blocking concentration was two orders of magnitude higher, 710 nM.

5. The kinetic properties of the Na^+ channel currents in the neurone- and cardiac-like cells were similar but developed over somewhat different voltage ranges. The voltage sensitivity of *activation* was similar in both cell types but the activation mid-point voltage was different: −12 mV in the neuronal cells and −34 mV for cardiac cells. *Inactivation* of the neuronal Na^+ channels had a mid-point near −47 mV and was more sensitive to the membrane voltage than inactivation of the cardiac channels. The mid-point of inactivation for the cardiac Na^+ channels was −80 mV.

INTRODUCTION

Immortal cell lines have been valuable as model systems for studies of development and differentiation of nerve and skeletal muscle. These include the phaeochromocytoma (PC12) cell line, various neuroblastoma cell lines, and the rodent

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muscle cell lines L6, C2 and BC_3H1 (see various chapters in Nelson & Lieberman, 1981; Prasad, 1991; and references in Florini, Ewton & Magri, 1991). The use of these and similar cell lines have contributed significantly to our understanding of neuronal and muscle development.

Progress on similar studies of cardiac cell development have been hampered by the lack of a suitably characterized cardiac cell line. There are, however, recent reports of three cardiac cell lines. Embryonic rat heart tissue was used to establish the H9c2 cell line (Kimes & Brandt, 1976). These cells have been used for morphological and biochemical studies (Hescheler, Meyer, Plant, Krautwurst, Rosenthal & Schultz, 1991). Patch clamp experiments demonstrated that these cells possess some of the characteristics expected of cardiac cells (Hescheler *et al.* 1991; Sipido & Marban, 1991). A second cell line (MCMI) was isolated from a mouse atrial tumour (Behringer *et al.* 1988) and a recent abstract describes some of the electrophysiological properties of these cells (Sculptoreanu, Scheuer & Catterall, 1991). A third line (W1) was produced by transforming human fetal myoblasts with the SV40 T antigen (Wang, Neckelmann, Mayne, Herskowitz, Srinivasan, Sell & Ahmed-Ansari, 1991).

These cardiac myocyte cell lines appear to be committed to a cardiac phenotype and so may be of limited utility as general models of differentiation. Of wider utility would be a pluripotent stem cell line which could be differentiated *in vitro* into several different phenotypes. Such a line is the murine embryonic carcinoma cell line, designated P19, obtained from a teratocarcinoma induced in C3H/He strain mice (McBurney, Jones-Villeneuve, Edwards & Anderson, 1982; Jones-Villeneuve, McBurney, Rogers & Kalnins, 1982). Depending on the culture conditions used, these cells can be differentiated into muscle (cardiac, skeletal or smooth), neurone, or epithelial cells (McBurney *et al.* 1982; Edwards & McBurney, 1983; McBurney, Reuhl, Ally, Nasipuri, Bell & Craig, 1988).

The classifications of the differentiated phenotypes of P19 cells have been made using mostly morphological criteria (McBurney *et al.* 1982; Edwards & McBurney, 1983) but included some histochemical techniques (Jones-Villeneuve, Rudnicki, Harris & McBurney, 1983; Edwards, Harris & McBurney, 1983; Cheun & Yeh, 1991). Only recently have the electrophysiological properties of P19 cells been examined and only for the neurone-like cells (Kubo, 1989; Cheun & Yeh, 1991).

In this study, we characterized some of the electrophysiological and pharmacological properties of the Na^+ channels in P19 cells differentiated into the cardiac phenotype and compared these with the properties of the Na^+ channels in the neuronal form. The activation and inactivation kinetics of the Na^+ channels in the cardiac and neuronal phenotypes were quite similar but the cardiac channels activated at somewhat more negative potentials. The cardiac Na^+ channels were much less sensitive than the neuronal Na^+ channels to block by tetrodotoxin (TTX). The voltage dependence of steady-state inactivation was shifted towards more depolarized potentials in the neuronal cells. All of these properties are consistent with those of Na^+ channels in native cells. We conclude that cells from the embryonal carcinoma cell line, P19, differentiate *in vitro* into cells that exhibit many of the properties appropriate for cardiac and neuronal phenotypes. Thus, this cell line may be a useful model system for studies of the development and differentiation of the neuronal and cardiac phenotypes and perhaps other phenotypes as well.

A preliminary report of this work has appeared in abstract form (Arreola, Spires & Begenisich, 1992).

METHODS

Undifferentiated mouse embryonal carcinoma P19 cells (American Type Culture Collection no. ATCC CRL 1825) were maintained at a relatively high density in the alpha modification of Eagle's minimal essential medium (αMEM; Gibco, 320-2571AG) supplemented with 10% fetal bovine serum. The cells were subcultured after they formed a confluent monolayer – approximately every 48 h. The cells were grown at 37 °C in 25 cm^2 tissue culture flasks in a 96% air–4% CO_2 humidified incubator.

In vitro differentiation of embryonal carcinoma cells

The general features of the methods we used for *in vitro* differentiation of P19 cells followed those described by Rudnicki & McBurney (1987) and Cheun & Yeh (1991). An important step in the induction of differentiation of the P19 cells is the formation of cell aggregates. In order to form aggregates, the cells must be plated onto a substrate to which they do not adhere. We tested many types and brands of dishes and substrates and found only the 100 mm FISHERbrand plastic Petri dish (catalogue no. 8-757-13) suitable for development of floating aggregates under all experimental conditions. When plated in these dishes, P19 cells form large, round floating aggregates with well-defined edges.

To induce the neuronal phenotype, P19 cells were grown for 48 h in tissue culture flasks with αMEM containing 0·5 μM retinoic acid. After this treatment, the monolayer of cells was dispersed and the cells were resuspended and transferred to the FISHERbrand 100 mm plastic Petri dishes. The cells were incubated in αMEM containing 0·5 μM retinoic acid for another 48 h, during which time aggregates formed. The aggregates were then transferred to culture dishes containing glass coverslips coated with CellTak (Collaborative Research Inc., Bedford, MA, USA). For this part of the procedure, the culture medium was αMEM containing 5 μg/ml cytosine β-D-arabino-furanoside HCl (Ara-C). The Ara-C inhibits propagation of the non-differentiated cells. Within 24 h the cells begin to produce neurites. The medium was replaced every 48 h. The neuronal P19 cells can be maintained for at least 2 weeks, the longest time we examined.

Edwards & McBurney (1983) reported that low (1 nM) concentrations of retinoic acid produced a large population of cardiac-like P19 cells. We were unable to reliably produce cardiac cells with retinoic acid. We used dimethyl sulphoxide (DMSO) and methods similar to those described by Edwards *et al.* (1983).

P19 cells were differentiated into a cardiac form by maintaining the floating aggregates in the presence of 0·5% DMSO for a total of 5 days. Every 24 h the media and aggregates were moved from the Petri dish to a 15 ml centrifuge tube. The aggregates settled at the bottom of the tube and the old medium was removed. The aggregates were resuspended in fresh αMEM with 0·5% DMSO and moved to a clean Petri dish.

After 5 days in αMEM + 0·5% DMSO the aggregates were resuspended in αMEM (no DMSO) with 5 μg/ml Ara-C and plated onto 35 mm tissue culture grade polystyrene dishes. The medium (with Ara-C) was replaced every 48 h for the next 14 days, then every third day. After 21 days Ara-C was no longer necessary.

The aggregates attached to the tissue culture dishes and cells migrated off the aggregates onto the plastic to form a monolayer of electrically coupled cells. Five to seven days after plating, regions of the monolayer began to exhibit spontaneous contractions. Beating cells were maintained in culture for as long as 2 months. McBurney *et al.* (1982) found that adrenaline increases the rate of spontaneous contractions and induces rhythmic beating in previously quiescent areas of the monolayer. We confirmed this finding but with isoprenaline as the agonist.

Dissociation of beating aggregates

Enzymatic treatment was used to dissociate single beating cardiac cells from the aggregates of beating cells. Dulbecco's phosphate-buffered (Ca^{2+} and Mg^{2+} free) saline with 1·5 mg/ml of collagenase Type I and 0·3 mg/ml of protease Type XXIV (both from Sigma Chemical Co., USA) was added to the tissue culture dish. The cells were incubated at 37 °C in the enzyme solution for 25 min and then swirled on a shaker plate for 20 min at room temperature. The enzyme solution and

cells were then transferred to a centrifuge tube and the enzyme activity was quenched with fetal bovine serum. After centrifugation the dissociated cells were resuspended in αMEM supplemented with 200 u/ml of penicillin G sodium and 0·2 mg/ml of streptomycin sulphate and plated onto glass coverslips coated with CellTak (Collaborative Research Inc.). The plated cells were maintained in the incubator undisturbed and used after attachment to the coverslips, approximately 1 h after dissociation. Only single beating cells were selected for electrophysiological recording.

Cells grown without chemical treatment

In the procedures described above, cell differentiation was induced by cell aggregation and chemical treatment. To investigate the importance of the chemical reagents, we subjected embryonic carcinoma cells to the *in vitro* differentiation procedure, but without DMSO or retinoic acid. The cells formed aggregates without spontaneous contractions or neurite formation. Single cells could be dissociated from these aggregates with the enzyme procedure described above.

Electrophysiological recordings

The whole-cell configuration of the patch clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) was used. For most experiments, macroscopic currents were recorded with an Axopatch 1D amplifier (Axon Instruments, USA). Electrode resistance was between 1 and 2 MΩ when filled with the internal solution (see below). In experiments with the Axopatch clamp, 70–80% of the measured series resistance was compensated. A few experiments were performed with a custom-built circuit designed to clamp large cells with large currents and allowing 92–95% series resistance compensation (Spires & Begenisich, 1992). Linear capacitative and leakage currents were subtracted using the $-P/4$ protocol (Bezanilla & Armstrong, 1977). Ionic currents were filtered at 5 kHz using an 80 dB/decade low-pass Bessel filter. Electrical signals were sampled at 28·5 kHz with a 12 bit analog-to-digital converter using custom-designed software and hardware. In some experiments, an exponential time function was fitted to the capacitative current transient and the fitted parameters used to estimate the cell capacitance. For the round, cardiac-like cells, the capacity transient was well fitted by a single exponential function. There were two components in the neurone-like cells, no doubt due to charging the membrane in the neurites. The second component was of small amplitude and slow duration. For these cells, the estimates of cell capacitance were made from the large, fast transient.

The solutions used were designed to minimize currents through K^+ and Ca^{2+} channels. The external solution used for the P19 cardiac-like and guinea-pig cells contained a reduced Na^+ concentration in order to reduce series resistance errors. The composition was (mM): NaCl, 46; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 1; $CoCl_2$, 4; Hepes, 10; TEA (tetraethylammonium)-Cl, 98; glucose, 10; pH adjusted to 7·3 with TEA-OH. For experiments with neuronal and undifferentiated cells, Na^+ replaced TEA to bring the total Na^+ concentration to 154 mM. The low Ca^{2+} concentration and the addition of the Co^{2+} served to reduce Ca^{2+} channel currents. The internal solution used for all experiments was (mM): CsF, 105; CsCl, 20; $CaCl_2$; 1; $MgCl_2$, 2; EGTA, 11; Hepes, 10; the pH was adjusted to 7·3 with CsOH. All the experiments were done at room temperature (20–24 °C).

In order to provide a quantitative assessment of the voltage dependence of activation and inactivation, the data were fitted by a form of the Boltzmann equation:

$$F(E_m) = \frac{1}{1+e^{(E_m-E_{\frac{1}{2}})/s}}, \quad (1)$$

where E_m is the membrane voltage and F represents the appropriate activation or inactivation parameter. In this form, the voltage at which the relevant process reaches half its maximal value is given by $E_{\frac{1}{2}}$. The parameter s (in mV) reflects the voltage sensitivity of the process; the smaller the value of s, the more voltage dependent the process.

RESULTS

Whole-cell ionic currents were recorded from undifferentiated control cells and from cells differentiated into neurone- and cardiac-like phenotypes. The photographs of Fig. 1 illustrate the morphology of the three cell types. This figure also contains

superimposed currents from these cell types recorded during voltage clamp steps to various potentials.

Undifferentiated P19 cells were generally round (upper panel) with diameters near 15 μm and expressed rather little inward current. Cheun & Yeh (1991) found that only 12% of undifferentiated cells exhibited inward currents and the level was small, less than 100 pA. Our results were consistent with these observations.

Cells treated with retinoic acid as described in Methods acquired a neuronal morphology (Fig. 1, middle panel). These cultures contained large numbers of round or triangular soma of 10–15 μm in width with very long neurites. Cheun & Yeh (1991) found that these types of cells invariably express neurone-specific enolase-like immunoreactivity. Such neurone-like cells also expressed large, voltage- and time-dependent inward currents.

The elongated morphology of these neurone-like cells might present problems for the spatial uniformity of membrane voltage. Hallmarks of such problems include bizarre current records, an unusually steep current–voltage relation, and channel kinetics that depend on current magnitude. Even with the 'normal' external Na^+ concentration of 154 mM, we did not observe unusual currents. The magnitude of peak inward Na^+ current in these neuronal cells ranged from $-0{\cdot}22$ to $-3{\cdot}5$ nA. In spite of this large variation, the voltage dependence of activation and inactivation were quite consistent from cell to cell (see below) with no obvious correlation with current magnitude. This observation and the data in Figs 2*B*, 3 and the inset of Fig. 4 suggested that, if they were present, spatial control problems were not of sufficient magnitude to invalidate the basic observations in this study. These same considerations indicate that series resistance errors were not significant, consistent with the relatively small current magnitudes and the small residual, uncompensated series resistance values (see Methods).

As described in Methods, the beating aggregates of the cardiac-like phenotype were dissociated into single cells. A photograph of these single cardiac-like cells is shown in the lower panel of Fig. 1. Almost all of the single cells were round with a diameter of 20 to more than 40 μm. We selected cells of approximately 20–30 μm for our electrophysiological experiments. Even these smaller cells expressed such large inward currents in full Na^+ that possible series resistance errors were a concern. Consequently, we used solutions with a reduced Na^+ concentration of 46 mM (see Methods). Even in reduced Na^+, these cells expressed large voltage- and time-dependent inward currents as illustrated in the figure.

The time and voltage dependences of the inward currents expressed in the neuronal and cardiac forms of the P19 cells (Fig. 1) appear to be similar to those of Na^+ channel currents in native neuronal and cardiac cells. These currents disappear from the neuronal (Cheun & Yeh, 1991) and from the cardiac cells (data not shown) when Na^+ is removed from the bathing solution. These results and the actions of tetrodotoxin (see below) identify these inward currents with voltage-gated Na^+ channels.

The voltage dependence of the peak currents of Fig. 1 can be seen more clearly in Fig. 2. The currents from undifferentiated cells (Fig. 2*A*) activated near -20 mV and peaked at rather positive potentials (about $+15$ mV in the example illustrated). These currents were small and the mean maximum peak amplitude was 150 ± 20 pA (S.E.M.; $n = 11$).

The Na^+ channel current from neurone-like cells (Fig. 2*B*) activated at potentials more positive than about -40 mV and reached maximum amplitude near 0 mV. In

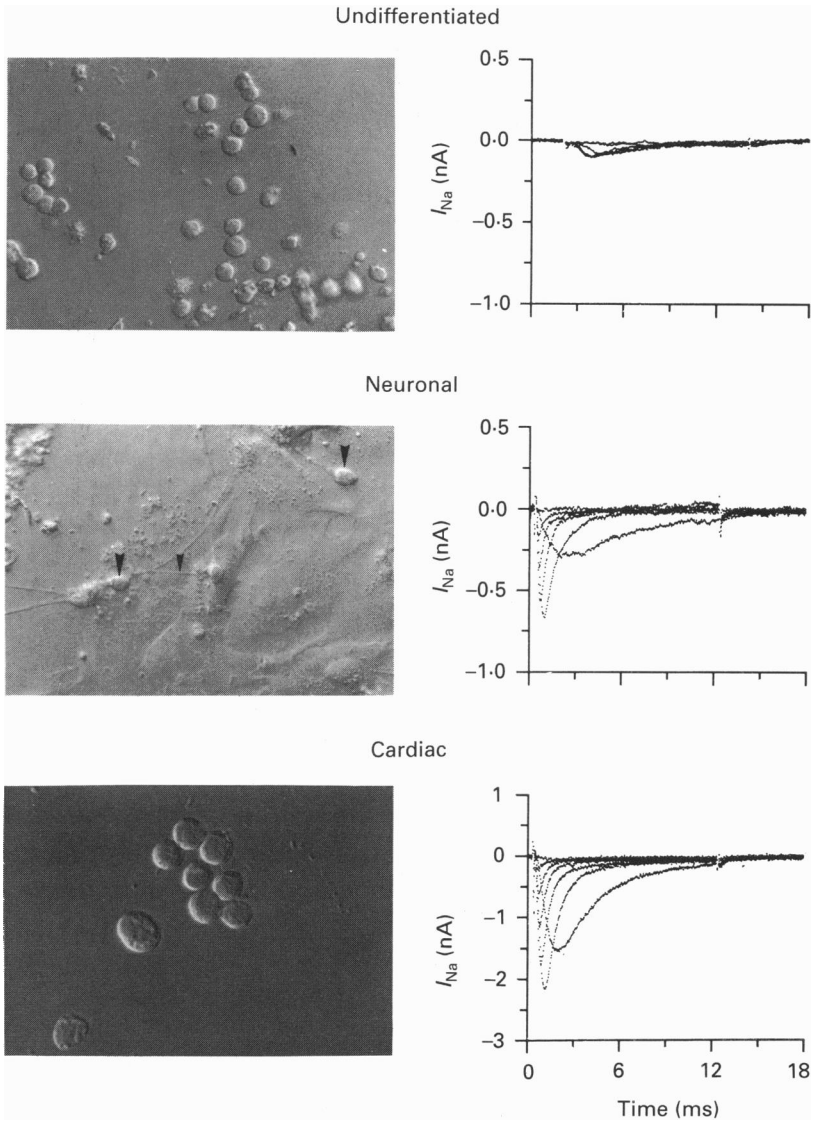

Fig. 1. Morphology and ionic currents of different phenotypes of P19 cells. Upper panel, photograph (left) and ionic currents (right) of undifferentiated P19 cells. The superimposed ionic current records were obtained at voltages of −40, −20, 0, 20 and 40 mV from a holding potential of −100 mV. Middle panel, photograph and ionic currents of P19 cells differentiated into a neuronal phenotype. Large arrowheads mark neuronal cell bodies; the small arrowhead marks a small neurite. Other larger neurites and axons are also visible. The superimposed ionic current records were obtained at voltages of −40, −20, 0, 20, 40 and 60 mV from a holding potential of −100 mV. Lower panel, photograph and ionic currents of dissociated cardiac-like P19 cells. The superimposed ionic current records were obtained at voltages of −50, −30, −10, 10, 30, 50 and 70 mV. All photographs were taken with the experimental microscope using interference contrast optics.

nine experiments, the maximum peak amplitude was $-1{\cdot}2 \pm 0{\cdot}25$ nA ($n = 9$). The maximum inward Na^+ current density of the neurone-like cells was 150 pA/pF when normalized by the mean measured cell capacitance of $8{\cdot}2 \pm 1{\cdot}6$ pF ($n = 9$).

The Na^+ channel current in the cardiac-like cells (Fig. 2*C*) activated at potentials more negative (-40 to -50 mV) than did the currents from the neuronal cells. These currents were maximal at -10 to 0 mV with peak amplitudes ranging between $-0{\cdot}46$ and $-10{\cdot}79$ nA with a mean amplitude of $-2{\cdot}8 \pm 0{\cdot}37$ nA ($n = 44$) (in reduced extracellular Na^+, see Methods). The mean measured capacitance of the cardiac-like

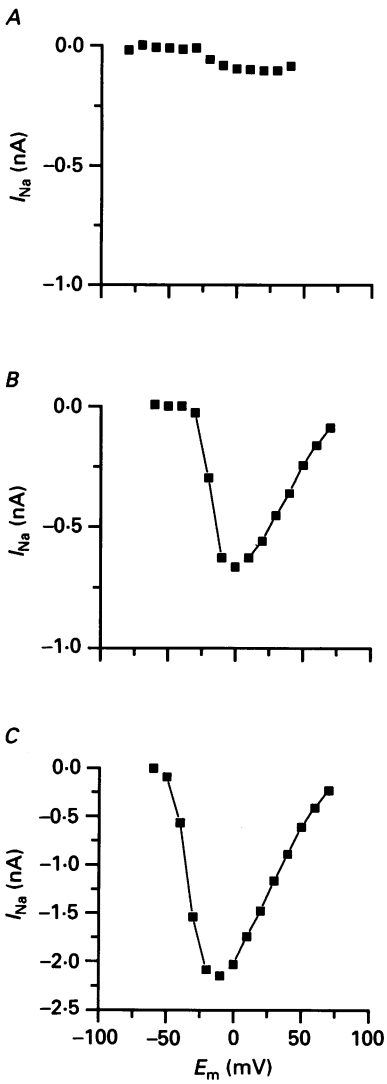

Fig. 2. Voltage dependence of peak sodium current from different phenotypes of P19 cells. All peak current values were obtained from the data of Fig. 1. *A*, undifferentiated cell; *B*, neurone-like cell; *C*, cardiac-like cell.

cells was 29·5 ± 3·2 pF (n = 28). Thus, the maximum inward Na^+ current density of the cardiac-like cells was 95 pA/pF.

In order to provide a quantitative assessment of the voltage dependence of activation, we converted peak current to chord conductance:

$$g_{Na} = \frac{I_{Na}}{E_m - E_{Na}}, \quad (2)$$

where E_{Na} is the measured Na^+ current reversal potential. Such data (normalized by the maximum conductance) from both a cardiac-like and a neurone-like cell are shown in Fig. 3. These data were fitted by the Boltzmann equation (see Methods) and values of $E_{\frac{1}{2}}$ for the cardiac- and neurone-like cells of −33 and −11 mV, respectively, were obtained. This approximately 20 mV difference in the activation of the Na^+ channels in the cardiac and neuronal cells was a consistent finding and was reflected in the mean $E_{\frac{1}{2}}$ values of −34 ± 1·6 mV (14 cardiac cells) and −12 ± 3·3 mV (8 neuronal cells). This difference is significant at the P = 0·001 level (independent t test).

The voltage sensitivity of Na^+ channel activation appears to be similar in both types of cells. In the examples in Fig. 3, the s parameters (see Methods) for the cardiac and neuronal cells were 5·6 and 7·0 mV, respectively. The mean values of this parameter were 6·1 ± 0·4 (14 cardiac cells) and 6·0 ± 0·5 mV (8 neuronal cells).

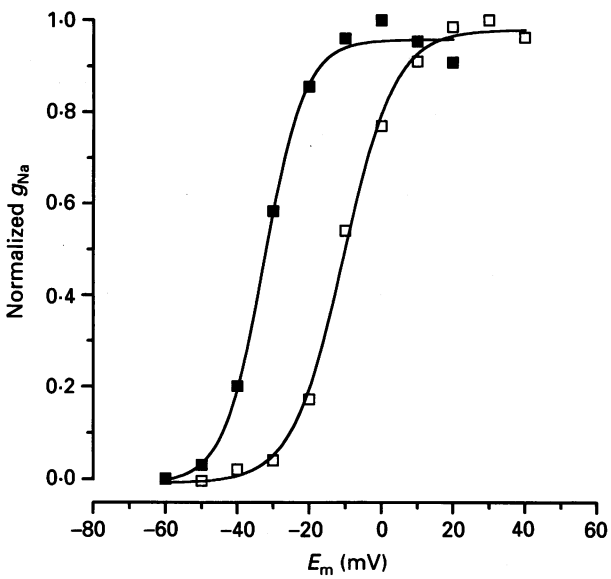

Fig. 3. Voltage dependence of activation of Na^+ channels in neuronal and cardiac phenotypes of P19 cells. Normalized peak Na^+ channel conductance for a cardiac (■) and a neuronal cell (□) as a function of membrane voltage. The continuous lines are Boltzmann functions (eqn (1), see Methods) with mid-points ($E_{\frac{1}{2}}$) of −33 and −11 mV for the cardiac and neuronal cells, respectively. The voltage sensitivity of activation for the cardiac and neuronal cells was similar and reflected in the fitted s parameters of 5·6 and 7·0 mV, respectively.

Na^+ channel inactivation in cardiac- and neurone-like P19 cells

We studied the inactivation of Na^+ channels from the neuronal and cardiac form of P19 cells with a double-pulse protocol. Channels were inactivated by a 120 ms prepulse of variable amplitude. The fraction of Na^+ channels available to be

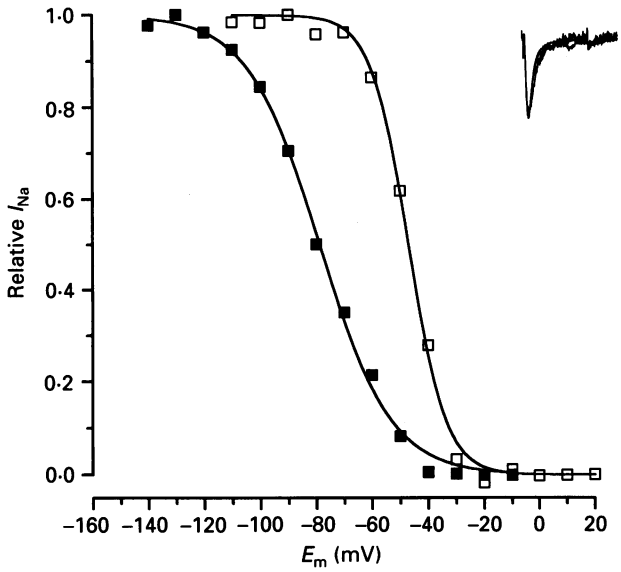

Fig. 4. Voltage dependence of steady-state inactivation of Na^+ channels. Sodium currents from cardiac- and neurone-like cells were recorded with a double-pulse protocol. Vertical scale, peak inward currents activated by a test pulse to −10 mV in cardiac-like and to 0 mV in neurone-like cells normalized to the maximum peak current recorded with the most negative prepulse. Horizontal scale, prepulse voltage. Control experiments (not shown) established a prepulse duration of 120 ms as sufficiently long to approach steady-state 'fast' inactivation but short enough to avoid contamination by slower inactivation processes. The continuous lines are the best fits of the Boltzmann function (eqn (1), see Methods) to normalized sodium currents from a cardiac (■) and a neuronal (□) cell. These fits yielded $E_{\frac{1}{2}}$ values for cardiac- and neurone-like cells of −79 and −47 mV, respectively, and s values of 12·7 (cardiac) and 6·5 mV (neuronal). Inset, see text.

activated was determined by a test pulse to −10 and 0 mV in cardiac and neuronal cells, respectively. Na^+ channel currents recorded at the test voltage with different prepulse amplitudes were normalized to the maximum amplitude recorded with the most negative prepulse. Figure 4 shows an example of the voltage dependence of steady-state inactivation obtained with this procedure for a neurone (□) and a cardiac cell (■).

The data for each type of cell were fitted by the Boltzmann equation (see Methods) shown as the continuous lines in Fig. 4. In the example of Fig. 4, the cardiac Na^+ channels were half-inactivated at −79 mV with a voltage sensitivity (s) of 12·7 mV. Inactivation of the channels in the neuronal cell occurred at more positive potentials ($E_{\frac{1}{2}}$ of −47 mV) and with a greater sensitivity to voltage (s = 6·5 mV). We obtained mean $E_{\frac{1}{2}}$ values of $-80 \pm 3{\cdot}7$ mV for eleven cardiac cells and $-46 \pm 0{\cdot}7$ mV for six

neuronal cells. The difference in these mean values is significant at the $P = 0{\cdot}001$ level. There was also a consistent difference in the voltage sensitivity of inactivation of the Na^+ channels reflected in the mean values of s of $12 \pm 0{\cdot}9$ mV ($n = 11$) and $7{\cdot}3 \pm 0{\cdot}5$ mV ($n = 6$) for cardiac and neuronal cells, respectively. This difference in voltage sensitivity was statistically significant, but only at a $P = 0{\cdot}01$ level.

As discussed above, the presence of neurites on the neuronal cells could compromise the spatial uniformity of membrane voltage. This non-uniformity could invalidate the analysis of the voltage dependence of inactivation in these cells. One test for the lack of significant voltage control problems is illustrated in the inset of Fig. 4. The time course of Na^+ currents (at 0 mV) from the neuronal cell is shown with three different prepulse potentials: -110, -50 and -40 mV. The peak currents associated with these three potentials were $-1{\cdot}65$, $-1{\cdot}04$ and $-0{\cdot}47$ nA, respectively. The two small current records were scaled to match their peak currents with that of the largest (inset). The kinetics of the scaled records are quite similar to those of the record with the largest current. Thus, the current kinetics appear to be independent of current magnitude indicating that there were no significant problems in estimating the voltage dependence of channel inactivation.

As described in Methods, both aggregation and chemical treatment were required to form cells with neuronal or cardiac morphology. We examined the inward currents in cells that had been treated to produce aggregation but without retinoic acid or DMSO. We could not distinguish these currents from those of undifferentiated (untreated) cells.

Sensitivity to tetrodotoxin of cardiac- and neurone-like cell Na^+ channels

Most types of neuronal Na^+ channels are sensitive to nanomolar concentrations of the neurotoxin, tetrodotoxin (TTX). One hallmark of cardiac Na^+ channels is their resistance to block by TTX. Consequently, we examined the TTX sensitivity of the neuronal and cardiac forms of differentiated P19 cells.

The upper left panel of Fig. 5 shows Na^+ channel currents recorded at 0 mV from a neuronal P19 cell. The record labelled 'C' was recorded before application of 60 nM TTX. As seen in this figure, this concentration of TTX eliminated almost all of the inward current. The upper right panel illustrates a similar experiment with the cardiac form of P19 cells. A much larger concentration of TTX (1 μM) blocked only about half of the Na^+ channel current.

The lower panel of Fig. 5 shows the effects of different doses of TTX on the peak sodium current of neuronal and cardiac P19 cells. The data represented by the open squares were obtained from a single cardiac cell. Each filled square represents a single concentration of TTX on a single cardiac cell. The open symbols (○, △, ▽) represent data from three neuronal cells which were treated with several TTX concentrations. The remaining symbols (●) represent data from single TTX concentrations on single cells.

The pooled data from both the cardiac and neuronal cells were fitted with the following function:

$$\text{fraction of } I_{Na} = \frac{1}{1 + \dfrac{[TTX]}{K_d}}, \tag{3}$$

where the left-hand side represents the fraction of Na^+ channel current not blocked by TTX and K_d is the dissociation constant for TTX.

The concentration dependence of block of Na^+ channels in both the neuronal and

cardiac cells was reasonably well fitted (continuous lines) by the equation with K_d values of 6·7 and 714 nM, respectively. This result shows that sodium channels from cardiac-like cells are more than 100 times less sensitive to TTX than sodium channels from the neuronal cells.

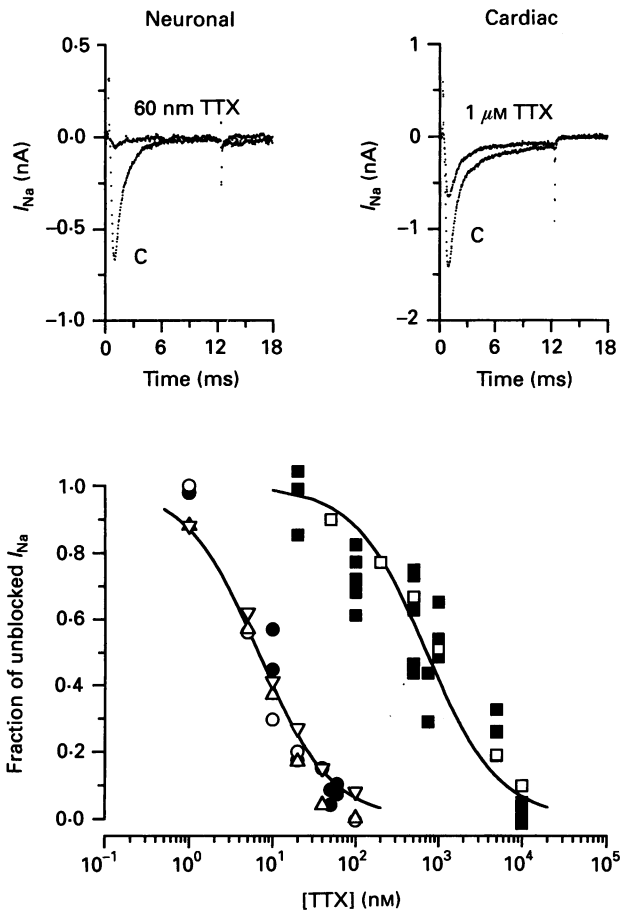

Fig. 5. Tetrodotoxin block of Na^+ channels in cardiac- and neurone-like cells. Left upper panel, Na^+ channel currents from a neurone-like cell activated by a voltage step to 0 mV in the absence (C) and in the presence of TTX (60 nM TTX). Right upper panel, Na^+ channel currents from a cardiac-like cell activated by a voltage step to 0 mV in the absence (C) and in the presence of TTX (1 μM TTX). Block by TTX was entirely reversible (data not shown) although recovery was slow at high (μM) TTX concentrations. Lower panel, concentration-dependent block of Na^+ channels by TTX. The ordinate was obtained from the maximum peak inward current in the presence and absence of TTX (usually near 0 mV). The symbols are as described in the text. The continuous lines are the best fits of eqn (3) to pooled data from cardiac and neuronal cells which yielded K_d values of 6·7 and 714 nM, respectively.

DISCUSSION

Na^+ currents in cardiac- and neurone-like cells

In general, the Na^+ channel currents recorded from P19 cells differentiated into neurone- and cardiac-like cells were similar. This is seen in the general time course of the currents illustrated in Fig. 1. However, we found several differences in detail: (1) Na^+ channel currents from the cardiac form activated at more negative voltages than did those from the neuronal form; (2) currents from cardiac cells inactivated at potentials more than 30 mV more negative than did currents from neuronal cells; (3) the inactivation process in channels of neuronal cells was much more voltage dependent than inactivation of channels in cardiac cells; (4) the Na^+ channels in the cardiac-like cells were more than 100-fold less sensitive to TTX than the neuronal phenotype. These results suggest that the *in vitro* differentiation of P19 cells into cardiac- and neurone-like phenotypes includes the expression of different types of Na^+ channels.

Comparisons to native channels

In general, cardiac Na^+ channels appear to operate over a more negative range of voltages than neuronal channels, consistent with the maximum diastolic potentials or resting potentials of these cell types. For example, steady-state half-inactivation of macroscopic Na^+ currents from rat myocytes (Brown, Lee & Powell, 1981), canine cardiac Purkinje cells (Fozzard & Hanck, 1992), intact rat papillary muscles (Antoni, Böcker & Eickhorn, 1988), and segments of human heart cells (Bustamante & McDonald, 1983) occurs at -60, -97, -78 and -75 mV, respectively. Even more negative values were found in studies of the inactivation properties of single channels (Lawrence, Yue, Rose & Marban, 1991; Scanley, Hanck, Chay & Fozzard, 1990).

While there is a similar variation in Na^+ channels in neuronal cells, inactivation appears to occur at relatively more depolarized potentials. For example, macroscopic Na^+ currents recorded from *Loligo* giant axons are 50% inactivated at -48 mV (Hodgkin & Huxley, 1952). Whole-cell Na^+ current half-inactivation in retinal ganglion neurones (Barres, Chun & Corey, 1989) and guinea-pig hippocampal CA1 neurones (Sah, Gibb & Gage, 1988) occurs at -55 and -75 mV, respectively.

The half-inactivation voltages of -80 and -46 mV found for cardiac and neuronal P19 cells certainly are consistent with the range of values found in native cardiac and neuronal cells. Indeed, the large variation found in different preparations illustrates one of the advantages of the P19 cell system: different phenotypes from the same stem cell population can be directly compared.

It should be noted that the spherical shape of the dissociated P19 cardiac cells is very different from the rod-shaped atrial and ventricular cells acutely dissociated from adult rats or guinea-pigs. However, after 12–24 h in culture, atrial guinea-pig cells assume a spherical shape (Bechem, Pott & Rennebaum, 1983). So even the morphology (or, more likely, the cytoskeleton) of these cells may not be too different from native, adult cardiac cells.

Tetrodotoxin sensitivity

While there are isolated exceptions, neuronal Na^+ channels are blocked by TTX with K_d values of a few nanomolar. This is also true for cloned Na^+ channels. For example, the rat brain II Na^+ channel isoform expressed in *Xenopus* oocytes is

blocked by TTX with a K_d value of 18 nM (Noda, Suzuki, Numa & Stuhmer, 1989; Terlau *et al.* 1991).

In contrast, block of cardiac Na^+ channels requires TTX concentrations near 1 μM. Na^+ channels in rat cardiac myocytes are blocked with a K_d of 5–6 μM (Brown *et al.* 1981). The rat heart I Na^+ channel isoform (Cribbs, Satin, Fozzard & Rogart, 1990) and human cardiac Na^+ channels (Gellens, George, Chen, Chahine, Horn, Barchi & Kallen, 1992) expressed in *Xenopus* oocytes are blocked by TTX with K_d values of 1·5 and 5·7 μM, respectively. The K_d values of 6·7 and 714 nM that we obtained from the neuronal and cardiac phenotypes of the P19 cells appear to be appropriate for neuronal and cardiac Na^+ channels.

Taken all together, our results suggest that P19 embryonal carcinoma cells differentiated *in vitro* into cardiac- and neurone-like cells express a form of the Na^+ channel with properties appropriate for the differentiated phenotype. Thus, embryonal carcinoma P19 cells may be a useful model system for studies of differentiation and development. These cells can be grown in culture and reliably differentiated into several phenotypes including cardiac and neuronal cells. Standard electrophysiological methods can be used for studying the ion channels in these cells. It should be possible to use these P19 stem cells with the entire repertoire of modern cellular, molecular, and electrophysiological techniques to address specific questions of the mechanism of cellular differentiation.

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