Sodium and Calcium Currents in Neuroblastoma × Glioma Hybrid Cells Before and After Morphological Differentiation by Dibutyryl Cyclic AMP

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Abstract. Sodium and calcium inward currents (I_{Na} and I_{Ca}) were measured in neuroblastoma × glioma hybrid cells of clones 108CC5 and 108CC15 by a single suction pipette method for internal perfusion and voltage clamp. Morphologically undifferentiated, exponentially growing cells were compared with cells differentiated by cultivation with 1 mmol/l dibutyryl cyclic AMP. Outward currents were eliminated by perfusing the cells with a K^+-free solution. Voltage dependence and ion selectivity as well as steady state inactivation characteristics of I_{Na} and I_{Ca} resembled those of differentiated mouse neuroblastoma cells, clone N1E-115 (Moolenaar and Spector 1978, 1979). These parameters were identical in undifferentiated and differentiated cells of both clones. After differentiation the average density of the peak sodium and calcium currents was increased two and four-fold, respectively, in both cell lines. Our data indicate that exponentially growing, morphologically undifferentiated 108CC5 and 108CC15 neuroblastoma × glioma hybrid cells possess functional Na^+ and Ca^{2+} channels undistinguishable from those of non-proliferating cells of these clones differentiated morphologically by treatment with dibutyryl cyclic AMP. That Na^+ and Ca^{2+} spikes were not detected by other authors in these cells prior to morphological differentiation by dibutyrylcyclic AMP may be attributed to the fact that at the low resting membrane potential measured the Na^+ and Ca^{2+} channels are inactivated.

Key words: Voltage clamp — Suction pipette method — Nerve cells — Differentiation — Sodium and calcium currents — Dibutyryl cyclic AMP.

Introduction

Clonal nerve cell lines, foremost among them the mouse neuroblastoma clone N1E-115 and the mouse neuroblastoma × rat glioma clones 108CC5 and

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108CC15 (the latter also known as NG108-15) have served as models in studies of the ionic events that underlay the action potential in the somatic membrane of neurons (Moolenaar and Spector 1978; Reiser et al. 1977; Fishman and Spector 1981). During prolonged treatment with agents elevating cellular adenosine-3′,5′-monophosphate (cyclic AMP) levels, such as N^6-2′-0-dibutyryl cyclic AMP or prostaglandin E_1 and theophylline, the hybrid cells undergo differentiation that is easily recognizable as changes in morphology (Daniels and Hamprecht 1974) and is associated with increases in voltage-sensitive Ca^{2+} channel activity, as determined by assays of Ca^{2+} influx and Ca^{2+} action potentials (Hamprecht 1974, 1977; Reiser et al. 1977; Nirenberg et al. 1983; Furuya et al. 1983). Differentiated 108CC5 and 108CC15 cells have also been reported to generate tetrodotoxin-sensitive Na^+ spikes upon depolarization (Reiser et al. 1977; Furuya et al. 1983).

We have examined a number of properties of the voltage-dependent Na^+ and Ca^{2+} inward currents responsible for these signs of electrical activity, using the suction pipette method for internal perfusion and voltage clamping. The present communication is mainly concerned with the extent of expression of some of these properties in morphologically undifferentiated and dibutyryl cyclic AMP-differentiated 108CC5 and 108CC15 cells. The kinetics of activation and inactivation of the Ca^{2+} current in these cells are described in the accompanying paper (Hering et al. 1985).

Materials and Methods

Cell culture and preliminaries to voltage clamping

Cells of the mouse neuroblastoma × rat glioma hybrid clones 108CC5 and 108CC15 were kindly donated by Prof. B. Hamprecht. They were subcultured to passage numbers P12 to P26. There were no major differences between cells of different passage numbers with respect to the parameters measured.

For growth the cells were seeded at a density of 3 to 4×10^4 cells/cm^2 in 25 cm^2 3013 Falcon flasks and cultured as monolayers at 37 °C in 5 ml of Dulbecco's modified Eagle medium containing 10 % fetal bovine serum, 0.1 mmol/l hypoxanthine, 1.0 μmol/l aminopterin, and 16 μmol/l thymidine in an atmosphere of 90 % air/10 % CO_2 saturated with water vapor. The culture medium was replaced with fresh medium every day. For differentiation, which was monitored by phase contrast microscopy as changes in morphology, in the first place outgrowth of neurites (Fig. 1; Hamprecht 1977), the cells were seeded at a density of 4 to 8×10^3 cells/cm^2 and cultured as above, except that the medium was supplemented with 1 mmol/l dibutyryl cyclic AMP and 1 % instead of 10 % fetal bovine serum. Not long after instituting the dibutyryl cyclic AMP treatment the cells stopped proliferating and started sending out neurites. Proliferation in the growth medium was determined as increases in DNA content per flask and day, using the ethidium bromide method of Karsten and Wollenberger (1972) in its modified version (Karsten and Wollenberger 1977). Doubling time during exponential growth of the 108CC15 cells was found to be 25.5 hours.

Ionic currents in the undifferentiated hybrid cells were studied between days 3 and 4 after plating.

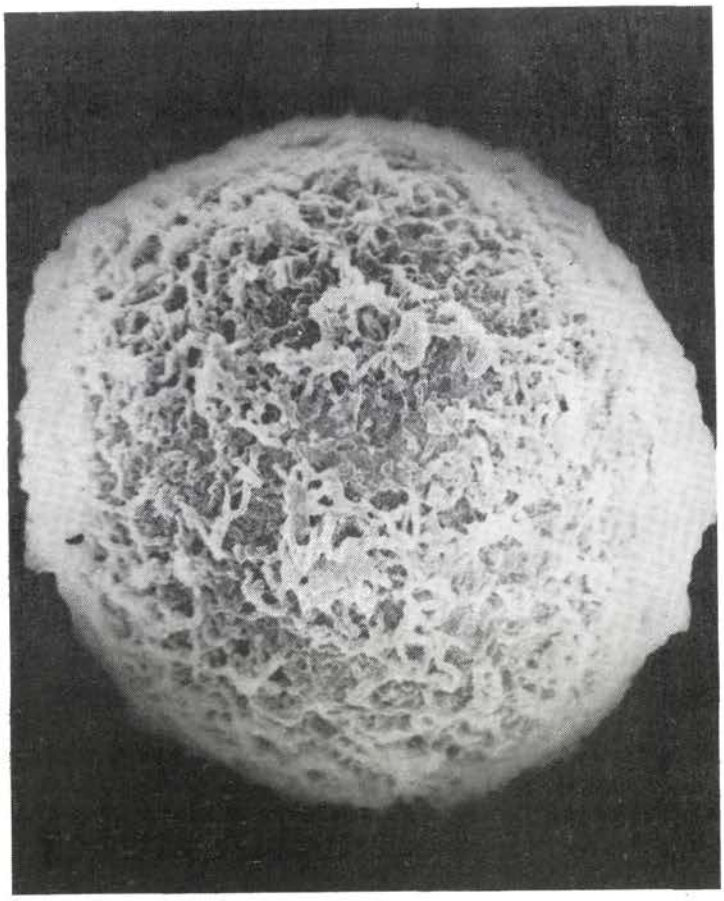

Fig. 1. Clonal neuroblastoma × glioma hybrid cells, line 108CC15, after 5 days of cultivation with 1 mmol/l dibutyryl cyclic AMP and 1 % fetal calf serum. Phase contrast, magnification ×400.

At this time the cells were in the exponential growth phase and had not yet reached confluency. In the cells cultured with dibutyryl cyclic AMP the ionic currents were studied between the 5th and 8th day after plating, when morphological differentiation was fully developed and proliferation had come to a complete standstill, as shown by a steady DNA content in the cultures of 6.5—6.9 μg per flask.

To obtain single cells in suspension for voltage clamping by the suction pipette method, the cultured cells were detached from the substratum by a gentle stream of culture medium delivered by a pipette, centrifuged at $168 \times g$ for 2 minutes, and resuspended in physiological saline containing 130 mmol/l NaCl, 10 mmol/l Tris-HCl, 1.8 mmol/l $CaCl_2$, 4 mmol/l KCl, and 5 mmol/l glucose, pH 7.4. The suspension of the cells, which had assumed a spherical shape, was then transferred to a Petri dish and a suitable cell, with a diameter between 20 and 40 μm, was selected with the aid of a phase contrast microscope for the voltage clamp experiments.

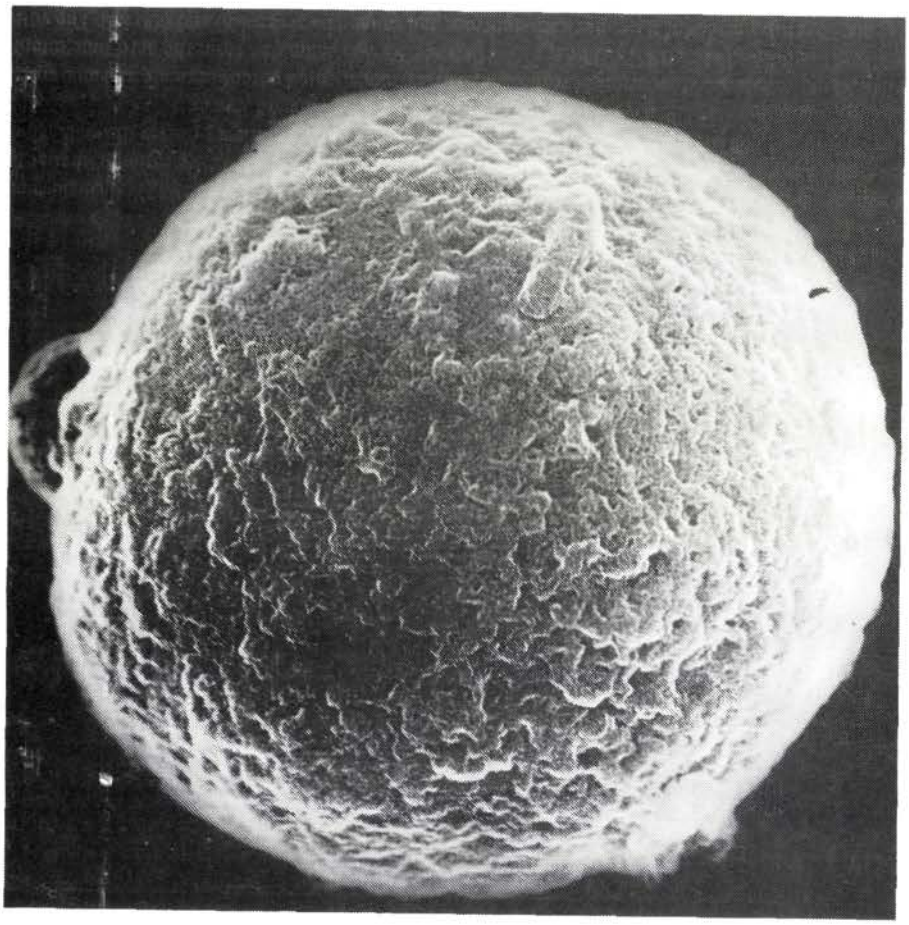

Fig. 2. A scanning electron micrograph of a differentiated neuroblastoma × glioma hybrid cell, line 108CC15, in suspension. Cell diameter is 30 μm. Note the ruffled surface due to microvilli-like projections. Courtesy of Dr. C.-U. von Mickwitz.

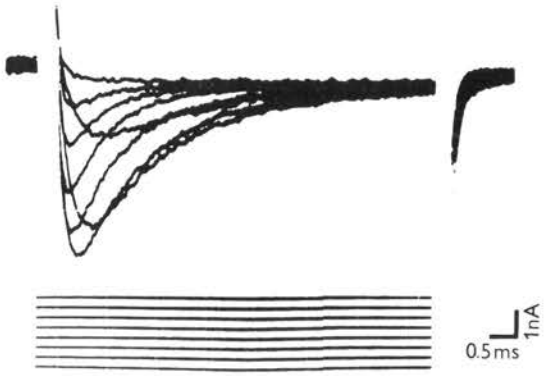

Fig. 3. Sodium currents in a differentiated 108CC5 neuroblastoma × glioma hybrid cell. Currents were recorded with the cell perfused with a K^+-free internal solution and bathed in external solution containing 130 mmol/l Na^+ and 1.8 mmol/l Ca^{2+}. Holding potential (V_H) was −90 mV and voltage traces represent clamped potentials from −60 to +10 mV in 10 mV steps.

Voltage clamping and data recording

The voltage clamp set-up was that described in an earlier report (Bodewei et al. 1982). A single suction pipette with a pore diameter of 10—20 μm (Veselovski et al. 1977) was used. We estimate that at least 90 per cent of the cell membrane was clamped. On application of depolarizing pulses the cells selected for voltage clamping displayed without exception Na^+ and Ca^{2+} inward currents.

The shunt resistance R_L was linear with voltage in the investigated potential range and amounted to 30—50 MΩ (Bodewei et al. 1982). The series resistance R_S was estimated from the zero time intercept of the semilogarithmic plot of the capacitative current and ranged between 400 and 600 kΩ. The voltage clamp capacitative current transient, measured during application of 10-mV voltage steps, had a single exponential decay with time constants of 150—600 μs, dependent on cell size, and was as a rule about 250 μs. The clamped membrane area was estimated from the measured membrane capacitance C_M (between 200 and 800 pF), assuming a specific C_M of 1 μF/cm^2. The above physiological saline was used as extracellular solution when the sodium current was to be measured. For the measurement of the calcium current NaCl in the saline was replaced by 3 to 15 mmol/l $CaCl_2$ and Tris-HCl in a concentration intended to bring the osmolarity of the solution to that of physiological saline. The intracellular (internal perfusion) solution used for the measurement of the sodium current contained 110 mmol/l Tris F — 30 mmol/l NaF, pH 7.2, that used for the calcium current measurements contained 140 mmol/l Tris phosphate, pH 7.2. All experiments were done at room temperature (20—22 °C).

Voltage homogeneity during current measurements

Criteria for adequate voltage control were the same as those specified previously (Hering et al. 1983): 1. Absence of delayed activation kinetics of I_{Na} in the negative resistance range. 2. Only preparations with R_L values of 30—50 MΩ were selected for the voltage clamp analysis. The estimated R_S/ /R_L-relationship guaranteed a satisfactory temporal resolution for the measurements to be performed (Bodewei et al. 1982). 3. A good voltage control of the I-V curve for peak I_{Na} in the negative resistance

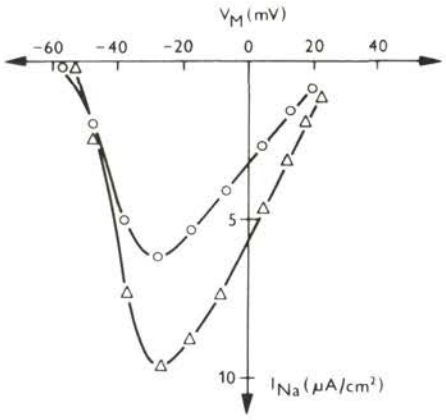

Fig. 4. Current-voltage relationship of peak I_{Na} in undifferentiated (○) and differentiated (△) hybrid cells of line 108CC5.

range; the voltage for maximum current had to be about 30 mV more positive than threshold (see Fig. 3). The adequacy of the voltage control during the measurements of I_{Na} was ascertained by deactivating the current at its peak and observing the speed of its decay following repolarization. 4. As an additional criterion of adequate voltage control, we compared the graphically determined and the calculated reversal potential of the sodium inward current E_{Na}. The difference between calculated and measured values was not greater than ±4 mV in the selected preparations.

Materials

Dibutyryl cyclic AMP and tetrodotoxin were kindly donated by Boehringer Mannheim GmbH (FRG). Dulbecco's modified Eagle medium was purchased from Gibco BioCult (UK; Catalogue No. 430—1600) and fetal bovine serum from Flow Laboratories GmbH (FRG). Other materials, obtained from commercial sources, were of reagent grade purity.

Statistics

Average values and their variations listed are the means ±S.D.'s.

Results

The inward sodium current

A record of the inward sodium current I_{Na} of a differentiated 108CC5 hybrid cell in response to various depolarization levels is shown in Fig. 3. This rapid inward current, which reached its peak at −30 mV in 0.62 ms, could be completely blocked in both undifferentiated and differentiated hybrid cells by 5 μmol/l

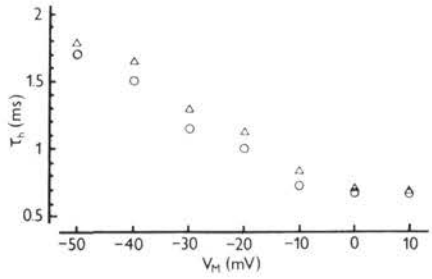

Fig. 5. Inactivation time constant τ_h of the sodium current in undifferentiated (○) and differentiated (△) 108CC5 hybrid cells as a function of the membrane potential.

tetrodotoxin. Outward currents were eliminated by internal perfusion with K^+-free intracellular Tris fluoride solution. Wash-out of K^+ was completed in this experiment after 5 minutes of internal perfusion.

Fig. 4 depicts the peak current-voltage relation of the transmembrane I_{Na} for an undifferentiated and a differentiated cell. In both cases the curves show a threshold at around −55 mV and a minimum at around −30 mV. The voltage axis was approached at a potential estimated to be +28 to 30 mV with 30 mmol/l NaF in the intracellular solution, a value very close to the Nernst potential E_{Na} of +32 mV.

The average peak sodium current density in 11 undifferentiated 108CC15 cells was calculated to be 4.3 ± 1.63 μA cm^{-2}. It increased to 8.47 ± 2.1 μA cm^{-2} ($n = 17$) after differentiation. The corresponding values for 108CC5 line cells were 3.14 ± 1.8 ($n = 15$) and 6.41 ± 1.72 ($n = 13$). The clamped membrane area as estimated from the capacitative current flow greatly exceed the area calculated on the assumption that the cells had the shape of spheres with a smooth surface and that 90 per cent of the membrane was clamped. The excess was 9.7 ± 2.7-fold ($n = 8$) in undifferentiated and 11.3 ± 2.9-fold ($n = 8$) in differentiated 108CC15 hybrid cells. The surface of neuroblastoma × glioma hybrid cells is studded with a plethora of microvilli that evidently form a major part of the functional cell surface area (see also Fig. 2). This fact was previously pointed out by Huang et al. (1982) and it applies to both undifferentiated and differentiated neuroblastoma × glioma cell hybrids, since transmission electron microscopy has revealed no obvious differences between undifferentiated and differentiated NG108-15 cells with respect to the number of their microvilli (M. Daniels, personal communication 1984).

The inactivation time constant τ_h of I_{Na} averaged 1.7 ± 0.3 ms and 1.8 ± 0.2 ms, respectively,in undifferentiated and differentiated cells at a membrane potential of −50 mV and 0.7 ± 0.2 ms in both cell populations at +10 mV (Fig. 5).

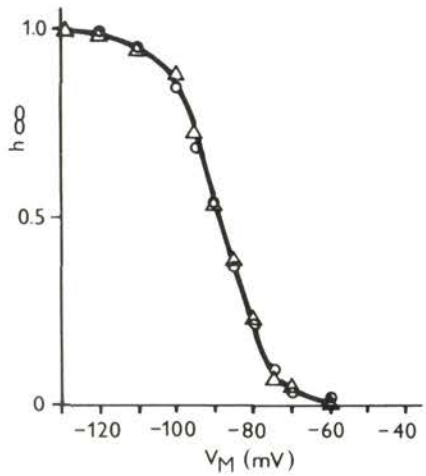

Fig. 6. Relationship of steady-state inactivation h_{∞} of I_{Na} and membrane potential in undifferentiated (○) and differentiated (△) hybrid cells 108CC5. Abscissa: conditioning potential step (500 ms); interpulse interval 0.5 ms. Ordinate: amplitude of I_{Na} after test pulse, normalized in correspondence to the maximum I_{Na}. The curve fits the function

$$h_{\infty} = \frac{1}{1 + \exp((V - V_h)/K)}$$

(Hodgkin and Huxley 1952), where $V_h = -88$ mV and $K = 7.5 \pm 0.6$ mV.

The dependence of the steady state inactivation of I_{Na} on the membrane potential in an undifferentiated and a differentiated cell is illustrated in Fig. 6.

The inward calcium current

Calcium currents were recorded in Na^+-free extracellular solution containing 10 mmol/l Ca^{2+}. Ca^{2+} currents stable for 30 to 60 min could be recorded in both undifferentiated and differentiated hybrid cells. Fig. 7 shows tracings of the slow inward current being overlapped by outward currents recorded in 10 mmol/l Ca^{2+} solution at different times of internal perfusion with 140 mmol/l Tris phosphate buffer. The time required to eliminate the outward currents in this hybrid cell, which had a diameter of 40 μm, was 5 min. The current was activated at −58 mV by a depolarizing step from a holding level of −80 mV (Fig. 8). Fig. 9 shows the current-voltage relationship of I_{Ca} in an undifferentiated and a differentiated hybrid cell of the line 108CC5. As in the case of the sodium current, the voltage dependence of the calcium current in the 108CC15 cells was similar to that found in 108CC5 cells.

The average peak Ca^{2+} current density in 10 mmol/l extracellular Ca^{2+} was 2.80 ± 1.52 μA cm^{-2} in 16 undiffferentiated 108CC5 cells and $10.17 \pm$

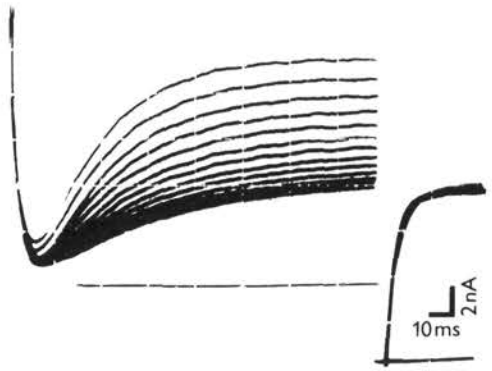

Fig. 7. Diminution of the outward current during internal perfusion of a hybrid cell 40 μm in diameter. The washout of K^+ was completed after 5 min of perfusion with intracellular solution. Recordings were made every 20 s.

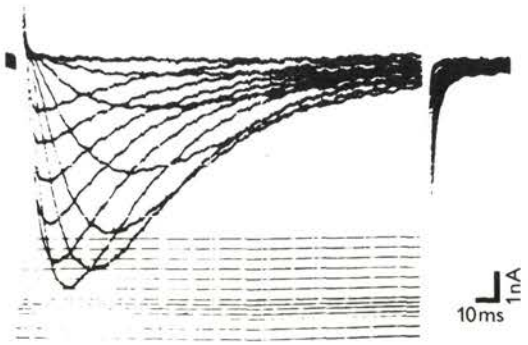

Fig. 8. Calcium currents I_{Ca} in a differentiated 108CC5 neuroblastoma × glioma hybrid cell after suppression of Na^+ and K^+ currents. Holding potential, V_H, was −80 mV and voltage traces represent clamped potentials from −60 mV to +40 mV in 5 mV or 10 mV steps.

3.6 μA cm^{-2} in 22 differentiated cells of this line. The corresponding values for 108CC15 line cells were 2.68 ± 1.44 ($n = 11$) and 9.87 ± 2.57 μA cm^{-2} ($n = 17$). The current was time and voltage-dependent and reached peak amplitude at a potential between −30 and −20 mV. Brief treatment (30 min) of perfused undifferentiated cells with extracellular 1 mmol/l dibutyryl cyclic AMP or intracellular perfusion of these cells for 20 min with 2 mmol/l Mg ATP plus 50 μmol/l cyclic AMP or with highly purified catalytic subunit of cyclic AMP-dependent protein kinase (kindly supplied by Dr. H. Will, final concentration 0.5 μmol/l) plus 2 mmol/l Mg ATP had no effect on current densities.

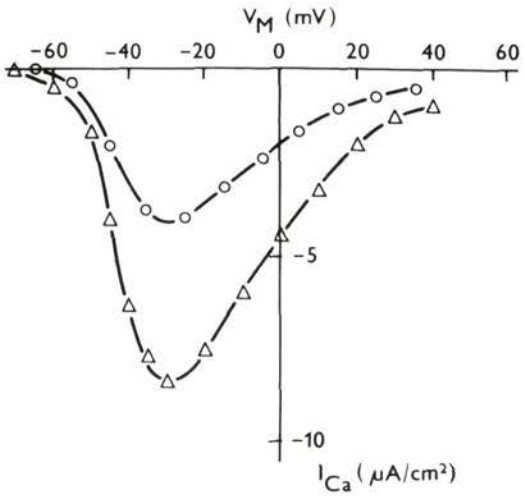

Fig. 9. Current-voltage relationship of peak I_{Ca} in undifferentiated (○) and differentiated (△) 108CC15 neuroblastoma × glioma hybrid cells. Abscissa: membrane potential; ordinate: maximum value of I_{Ca} at the corresponding membrane potential.

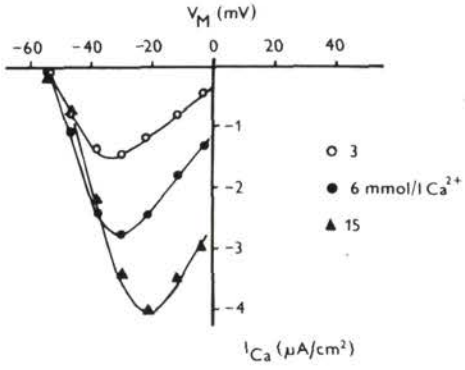

Fig. 10. Current-voltage relationship in the membrane of an undifferentiated 108CC15 hybrid cell in solutions of different Ca^{2+} concentrations.

Elevation of the extracellular Ca^{2+} concentration enhanced current peak values of I_{Ca}. The maximum of the I—V curve was shifted by 10 mV (mean value, $n = 4$) in the depolarizing direction after increasing $[Ca^{2+}_{out}]$ from 3 to 15 mmol/l (Fig. 10). I_{Ca} was completely blocked by 2 mmol/l Ni^{2+}, a half-maximal block being obtained with 0.5 mmol/l Ni^{2+}. As shown in Fig. 11, there was no significant difference between undifferentiated and differentiated hybrid cells with respect to the steady state inactivation of I_{Ca}. Neither did the undifferentiated and differen-

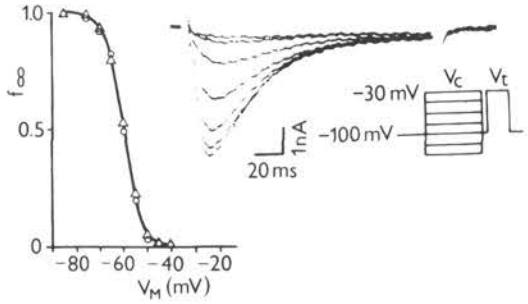

Fig. 11. Relationship of steady-state inactivation f_{∞} of I_{Ca} and membrane potential. Inset is the prepulse test pulse program in which the prepulse potential (1 s) was changed from −100 to −85, −75, −65, −60, −55, and −50 mV, with the test pulse voltage at −30 mV; interpulse interval 1.5 ms. The values are averages from 4 experiments. Half-inactivation potential, V_h, was about −60 mV and the curve was fitted to

$$f_{\infty} = 1/(1 + (\exp(V - V_h)/K),$$

where K, the slope factor, was calculated to be 3.4 ± 0.4 mV.

tiated cells differ in their ion selectivity. In both cases the ratio of the peak amplitudes of Ca^{2+}, Ba^{2+}, and Sr^{2+} currents were 1:1.3:1.1 (mean values of 5 cells).

Discussion

One of the advantages of the suction pipette method for internal perfusion (or dialysis) and voltage clamping is the control of the intracellular ionic composition. As has been shown in a variety of cell types (Kostyuk et al. 1978; Undrovinas et al. 1979; Akaike et al. 1978; Hagiwara and Jaffe 1979), this method makes it possible to eliminate outward currents by dialyzing or perfusing the cells with K^+-free solutions. In the present experiments the efficiency of the internal perfusion was documented by the disappearance of the outward current during the perfusion (see Fig. 7) as well as by the close agreement between the graphically estimated and the calculated values for the reversal potential of the sodium current (Fig. 4; see Methods). As mentioned above, an outward current was absent in the experiments described in the Results section and could thus be ruled out as a factor disturbing Na^+ and Ca^{2+} current measurements.

Resting and action potentials in cultured neuroblastoma cell lines are subject to modulation by culture conditions, including factors leading to differentiation (for a review see Spector 1981). In the neuroblastoma × glioma hybrid cells used in the present study functional voltage-sensitive sodium and calcium channels were present in the proliferating stage prior to morphological differentiation by dibutyryl cyclic AMP, and it was only the density of the inward currents passing though these channels, particularly that of the I_{Ca}, that was found to be changed during differentiation.

There have been several reports about a nonspecific outward current (I_{NS}) that complicated the study of I_{Ca} (see Kostyuk 1980). In neuroblastoma × glioma hybrid cells I_{NS} probably carried by anions present in the intracellular perfusion solution (see Kostyuk 1980) was found after exhaustive perfusion only on application of test pulses of exceptionally large amplitudes (going up to +20 — +50 mV) that are likely to damage the cell membrane. This current was also observed in perfused cells on blocking the I_{Ca} completely with 2 mmol/l Ni^{2+}.

The voltage dependence of the inactivation time course of the rapid sodium inward current, which was determined in cells of the line 108CC5, was the same in both differentiated and undifferentiated cells and was nearly identical with that reported by Moolenaar and Spector (1978) in cells of mouse neuroblastoma line N1E-115. This inward current was presently identified as Na^+ current by the selective Na^+ channel blocker tetrodotoxin and by its absolute dependence on the presence of Na^+ in the extracellular solution. The good agreement of the experimentally determined reversal potential of this current with the Nernst potential indicates a high selectivity of the rapid channel for sodium ions.

Voltage dependence (Fig. 9) and steady state activation and inactivation kinetics (Hering et al. 1985) of I_{Ca} in the two neuroblastoma × glioma hybrid cell lines studied were not altered by cultivation with dibutyryl cyclic AMP. This current was identified as I_{Ca} by its sensitivity to the Ca^{2+} channel blocker Ni^{2+} and by its increasing amplitude with increasing extracellular Ca^{2+} concentration (Fig. 10). Its voltage dependence was very similar to that of the Ca^{2+} inward current in differentiated N1E-115 neuroblastoma cells (Moolenaar and Spector 1978, 1979), with Ca^{2+} as charge carrier being replaceable, as in the N1E-115, by Ba^{2+} and Sr^{2+}.

Neither were the inactivation kinetics and steady state inactivation of the I_{Na} changed by the dibutyryl cyclic AMP treatment (Figs. 5, 6). In other words, our results provide no indication for qualitative changes in the properties of the Na^+ and Ca^{2+} inward currents in 108CC5 and 108CC15 cells as a result of treatment with dibutyryl cyclic AMP that led to marked changes in cell morphology.

In cultures of 108CC15 cells (Furuya et al. 1983) and of the neuroblastoma × glioma hybrid cell line 108CC25 (Reiser and Hamprecht 1982) several days of exposure to dibutyryl cyclic AMP were required for the development of the ability to generate Ca^{2+} and Na^+ action potentials. Similarly, in a mouse neuroblastoma × clonal rat liver cell hybrid (NBr10-A) possessing neuronal properties an uptake of $^{45}Ca^{2+}$ on depolarization was observed after 6 days of cultivation with dibutyryl cyclic AMP or prostaglandin E_1 and theophylline, but not in logarithmically dividing cells (Nirenberg et al. 1983). The absence in these experiments of Ca^{2+} and Na^+ channel activity during exponential growth and at the early stage of dibutyryl cyclic AMP treatment could be explained on the ground that at the low measured resting potential of exponentially growing neuronal cells (−10 to

−25 mV, see Spector 1981; −40 mV, see Furuya et al. 1983), these channels are inactivated (cf. Fig. 11) and that it takes days for the resting membrane potential to attain levels (Furuya et al. 1983) at which activation becomes possible. In our voltage clamp experiments, on the other hand, functional Ca^{2+} and Na^+ channels were manifestly present to a certain extent in exponentially growing, morphologically undifferentiated cells. This shows that these cells were at least partially differentiated electrophysiologically. Voltage-sensitive Ca^{2+} channels were also reported to be present in 108CC5 cells growing in Eagle's minimum essential medium supplemented with fetal calf serum and glutanine and, in further general agreement with the present results, a depolarization-induced net uptake of $^{45}Ca^{2+}$ by these cells was increased 4-fold following differentiation by chronic treatment with prostaglandin E_1 and 1-methyl-3-isobutylxanthine (Freedman et al. 1984).

Interventions causing elevations of cellular cyclic AMP levels were found in several laboratories to augment the slow inward Ca^{2+} current in cardiac muscle cells with little or no delay (see Reuter 1983), presumably by enhancing the probability of the individual Ca^{2+} channels to open during depolarization (Reuter 1983). In perfused dorsal root ganglion neurons of young rats (Fedulova et al. 1981) and perfused snail neurons (Doroshenko et al. 1982) the addition of cyclic AMP together with ATP and Mg^{2+} to the perfusion medium was found to antagonize within minutes the run-down of the Ca^{2+} inward current. In contrast, a rapid action of cyclic AMP and derivatives has not been observed in cultured neuroblastoma × glioma hybrid cells. Nirenberg et al. (1983) reported that it took days for dibutyryl cyclic AMP to raise the percentage of NG108-15 (108CC15) cells capable of displaying Ca^{2+} action potentials. Similarly, as found in the present experiments, prolonged exposure to dibutyryl cyclic AMP was required to increase Ca^{2+} and Na^+ current density in this and the 108CC5 hybrid cell line, and a short-term internal perfusion with Mg ATP in combination with cyclic AMP or the catalytic subunit of cyclic AMP-dependent protein kinase was ineffective. A similar lack of influence of these interventions on the Ca^{2+} current in N1E-115 neuroblastoma cells was explained on the ground that in these cells, like in the 108CC15 hybrids (Hering et al. 1985), inactivation of this current is not Ca^{2+}-dependent and the current therefore not subject to regulation by cyclic AMP and protein kinase (P. G. Kostyuk, personal communication).

The acute effects of the cyclic AMP-protein kinase system on Ca^{2+} (see above) and also on Na^+ (Catterall 1984) and K^+ (see Nestler Greengard 1983) conductances of excitable cell membranes have all been attributed to phosphorylation of proteins forming part of, or being closely linked to the respective ion channels. In contrast, the mechanism of the long-term action of cyclic AMP on ion channel activity in cultured nerve cell lines is less clear. As a next step in the electrophysiological approach to this problem we have explored the possibility that changes in the kinetics of activation and inactivation of the I_{Ca} might be responsible

for the presently observed increase in the density of this current following prolonged treatment with dibutyryl cyclic AMP (Hering et al. 1985). However, on the basis of the results obtained this possibility has to be dismissed. The cause of the increase in density of the Ca^{2+} current and probably also of that of the Na^{+} current (see Figs. 5 and 6) must be sought elsewhere.

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