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THE CALCIUM CURRENT AND THE ACTIVATION OF A SLOW POTASSIUM CONDUCTANCE IN VOLTAGE-CLAMPED MOUSE NEUROBLASTOMA CELLS

BY W. H. MOOLENAAR* AND I. SPECTOR

From the Department of Physiology, State University of Leiden, Leiden, the Netherlands and the Department of Neurobiology, Weizmann Institute of Science, Rehovot, Israel

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SUMMARY

1. The Ca^{2+} inward current (I_{Ca}) and a slow outward current in differentiated cells of mouse neuroblastoma clone N1E-115 have been studied under voltage-clamp conditions.

2. I_{Ca} shows voltage- and time-dependent inactivation when evoked by step-wise depolarizations in Na^+-free solution containing high [Ca^{2+}] (20 mM) and tetraethylammonium (TEA, 25 mM). Ba^{2+} and Sr^{2+} can substitute for Ca^{2+}.

3. Holding potentials below -70 mV maximally activate I_{Ca}. Half inactivation occurs at -56 mV and I_{Ca} is completely inactivated beyond holding levels of -30 mV. Maximum peak currents are of the order of 10^{-4} A/cm^2 and the reversal potential ranges from $+40$ to $+60$ mV. The I_{Ca} inactivation time course follows first-order kinetics with a voltage-dependent time constant ranging from 25 to 100 msec.

4. The striking resemblance between I_{Ca} and the Ca^{2+} current in the unfertilized mouse oocyte (Okamoto, Takahashi & Yamashita, 1977) is discussed.

5. A slow outward current with a rise time of several seconds is recorded on voltage steps beyond -20 mV in high [Ca^{2+}] solutions. It is carried primarily by K^+ on account of the value of the reversal potential and its dependence on $[K^+]_0$. This K^+ current is TEA-insensitive and is blocked by Ca^{2+} antagonists.

6. The slow K^+ current ($I_{K(Ca)}$) is suggested to be mediated by Ca^{2+} influx, but the voltage-dependence of the underlying conductance ($G_{K(Ca)}$) differs significantly from the I_{Ca} voltage-dependence.

7. The results are consistent with the hypothesis that $I_{K(Ca)}$ depends both on I_{Ca} and on membrane potential. An alternative hypothesis is briefly discussed.

INTRODUCTION

In the preceding article (Moolenaar & Spector, 1979) properties of the Ca^{2+} action potential and those of a long-lasting spike after-hyperpolarization (a.h.p.) in mouse neuroblastoma cells were examined under constant-current conditions. The Ca^{2+} spike was found to resemble that in other excitable cells and the a.h.p. was

* Address for reprint requests: Laboratory of Physiology, Wassenaarseweg 62, Leiden, Netherlands.

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inferred to be mediated by a Ca^{2+}-dependent and TEA-resistant K^+ current. In the present paper the Ca^{2+} inward current (I_{Ca}) and the Ca^{2+}-dependent K^+ current are investigated with the voltage-clamp technique. The results on I_{Ca} confirm and extend those reported in an earlier voltage-clamp study on neuroblastoma membrane currents (Moolenaar & Spector, 1978), in which I_{Ca} was suggested to be subject to voltage- and time-dependent inactivation.

Here, the inactivation kinetics and pharmacology of I_{Ca} are studied in greater detail. Interestingly, a striking quantitative similarity turns out to exist between the properties of I_{Ca} and those described for the Ca^{2+} inward current in the unfertilized mouse oocyte (Okamoto *et al.* 1977). Furthermore, the Ca^{2+} dependent K^+ current $I_{K(Ca)}$ is identified during voltage-clamp. $I_{K(Ca)}$ exhibits very slow activation kinetics and its $I-V$ relation shows a maximum. However, its voltage-dependence and kinetic behaviour is not easily compatible with that of I_{Ca}. The results are consistent with the hypothesis that the underlying conductance $G_{K(Ca)}$ depends both on I_{Ca} and membrane potential, but an alternative hypothesis that a second kind of Ca^{2+} channels may actually be involved in $G_{K(Ca)}$ activation cannot be ruled out and is briefly discussed.

METHODS

Cells of mouse neuroblastoma clone N1E-115 were cultured and induced to differentiate into mature nerve cells as described previously (Kimhi, Palfrey, Spector, Barak & Littauer, 1976; Moolenaar & Spector, 1978). Details of the constant-current and voltage-clamp techniques were also described in a previous paper (Moolenaar & Spector, 1978). Micro-electrodes of 10–20 MΩ were used in the present experiments. These relatively high resistance values were necessary to prevent a rapid deterioration of the slow currents during the course of the experiment, at the cost, however, of limiting the high-frequency response of the clamping amplifier. As a consequence, membrane currents elicited by stepwise depolarizations had a stabilization time as long as 2–5 msec. This was not considered to be a disadvantage since mainly relatively slowly developing currents were measured in the present study. The composition of the bathing solutions was the same as that described in the preceding paper (Moolenaar & Spector, 1979). Experiments were carried out at temperatures of 20–24 °C.

RESULTS

Separation of the calcium inward current

In normal solution (containing 1·8 mM-Ca^{2+}) the slow inward Ca^{2+} current (I_{Ca}) is usually too small to be measurable under voltage-clamp, so that the fast transient Na^+ current (I_{Na}) is the only inward current component that appears on depolarizing voltage steps (Moolenaar & Spector, 1978). In 20 mM-Ca^{2+}, 90 mM-Na^+ solution, however, the magnitude of I_{Ca} is considerably enhanced and, as a result, the total inward current consists of two additive components each with their own characteristic time course and voltage-dependence (Fig. 1). The slow I_{Ca} component is activated at -55 mV whereas the fast I_{Na} phase becomes prominent at membrane potentials above -25 mV. At -15 mV also delayed outward currents start to develop. Current records such as shown in Fig. 1 provide supporting evidence for the existence of separate Na^{2+} and Ca^+ channels in the neuroblastoma membrane.

To study I_{Ca} separately from the total current external Na^+ was replaced by Tris and TEA (25 mM) was added to block the delayed outward K^+ current. Fig. 2*A*

shows records of I_{Ca} in 20 mM-Ca^{2+} solution. Depolarizing voltage pulses lasting 150 msec were elicted at 2 sec intervals from a steady holding potential (V_h) of -80 mV. Increasing the pulse interval (up to 20 sec) was found not to be of any influence on the I_{Ca} patterns. The peak values of I_{Ca} are plotted against membrane potential in Fig. 2*B*.

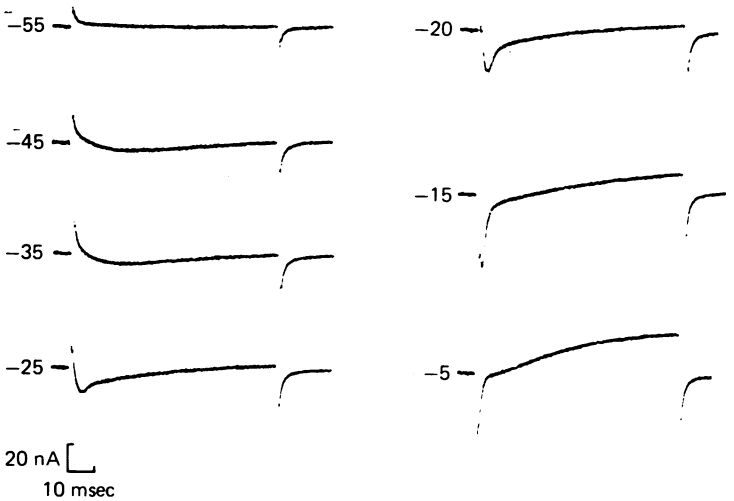

Fig. 1. Typical current records obtained from neuroblastoma cell bathed in 90 mM-Na^+, 20 mM-Ca^{2+} solution, showing superposition of slow Ca^{2+} inward current and fast Na^+ inward current. Delayed outward currents develop beyond -15 mV. Command voltages (mV) are indicated next to the current traces. Holding potential -80 mV.

The maximum peak value of I_{Ca} was reached at -20 mV with a time to peak of about 10 msec and corresponds to a current density of 0·13 mA/cm^2 after leakage correction. Current density values in four other cells ranged between 0·10 and 0·15 mA/cm^2.

A further increase of $[Ca]_o$ to 40 mM in Na^+-free solution did not result in a concommittant increase in I_{Ca} peak values but, in contrast, in a reduction and after prolonged exposure to 40 mM-Ca^{2+} even in a complete abolishment of I_{Ca}. Since in other types of cells Ca^{2+} inward currents are known to be reduced or blocked when the free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) exceeds a level of roughly 10^{-8} M (barnacle muscle: Hagiwara & Nakajima, 1966; molluscan neurones: Kostyuk & Krishtal, 1977*b*; Akaike, Lee & Brown, 1978) it may well be that the observed reduction of I_{Ca} in 40 mM-Ca^{2+} solutions is due to an increased $[Ca^{2+}]_i$. Although no data are available on $[Ca^{2+}]_i$ and its metabolism in neuroblastoma cells, such an increased $[Ca^{2+}]_i$ in high $[Ca^{2+}]_o$, Na^+-free solution is likely to result from enhanced passive Ca^{2+}-influx as well as from a reduced capability of the cell to extrude excess Ca^{2+} out of the cytoplasm due to the absence of external Na^+ (cf. Baker, 1972; Blaustein, 1974).

Following replacement of Ca^{2+} by Ba^{2+} or Sr^{2+} (20 mM) inward currents with virtually the same time course and voltage-dependence as I_{Ca} were observed. Examples of current recordings are illustrated in Fig. 3. Maximum peak current values were again on the order of 10^{-4} A/cm^2. Inward currents were absent when Mg^{2+} (20 mM) was substituted for Ca^{2+}. These results directly confirm the constant-current observations in that the Ca^{2+} channel is readily permeable to Ba^{2+} and Sr^{2+} but not to Mg^{2+} (Moolenaar & Spector, 1979).

I_{Ca} blockers

Inward currents in 20 mM-Ca^{2+}, Na^+-free solutions were blocked by 2 mM-La^{3+}, 10 mM-Co^{2+} or 10 mM-Mn^{2+}. Inward currents in the presence of 20 mM-Ca^{2+} and 20 mM-Mg^{2+} were significantly reduced but not completely blocked. This suggests that the order of effectiveness for blocking is

$$La^{3+} > Co^{2+} \simeq Mn^{2+} \gg Mg^{2+}.$$

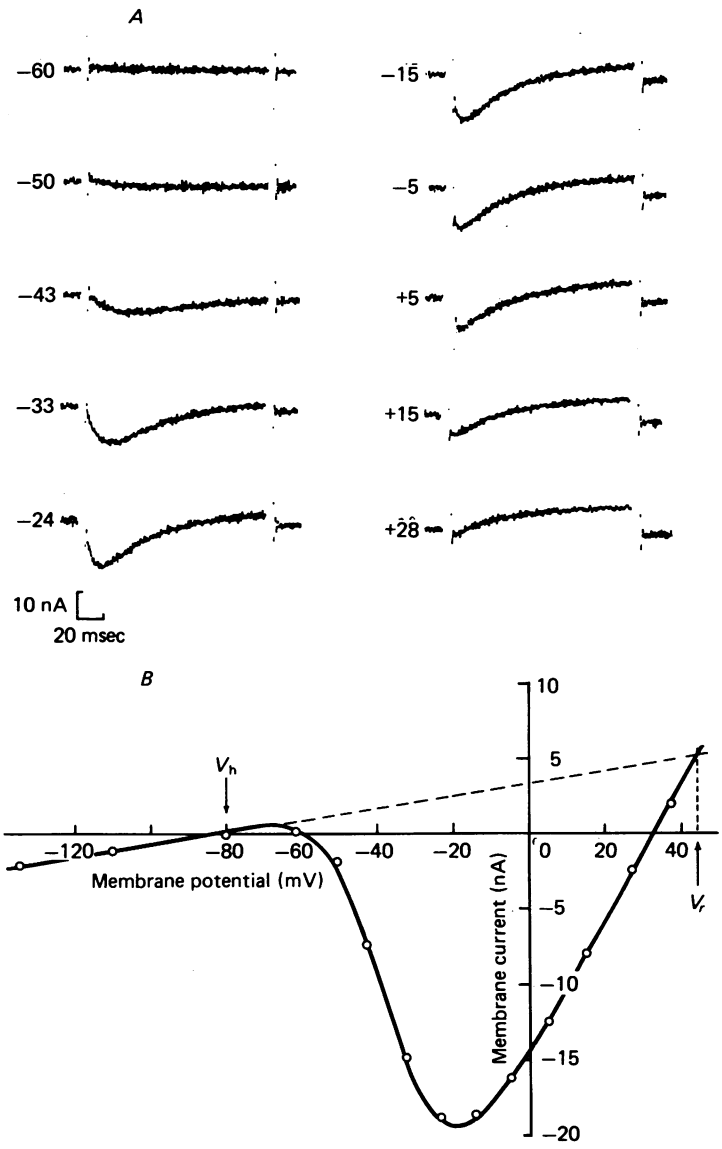

Fig. 2. *A*, Ca^{2+} inward currents (I_{Ca}) in Na^+-free solution containing 20 mM-Ca^{2+} and 25 mM-TEA. Command voltages are indicated next to the current traces. Holding potential −80 mV. *B*, peak values of I_{Ca} as a function of membrane potential for the same cell as in *A*. Dashed line represents linear extrapolation of leakage current as measured by hyperpolarizing voltage steps. V_h, holding potential; V_r, reversal potential of I_{Ca}. Estimated membrane surface area $1{\cdot}7 \times 10^{-4}$ cm^2.

A similar sequence has been reported for the Ca^{2+} channel in other excitable membranes where the multivalent ions are assumed to competitively occupy the Ca^{2+} binding sites (Hagiwara & Takahashi, 1967; Hagiwara, 1973). The organic Ca^{2+} antagonists Verapamil and D-600 (20 μg/ml.; Tritthart, Fleckenstein, Herbst & Grün, 1969) did not reduce I_{Ca} significantly.

Fig. 3. Inward Ba^{2+} and Sr^{2+} currents in Na^+- and Ca^{2+}-free solution containing 25 mM-TEA. Holding potential -80 mV.

Reversal potential of I_{Ca}

The current–voltage curve crosses the extrapolated leakage current curve (dashed line in Fig. 2*B*) at V_r, the 'reversal potential' (actually the 'null potential' since no inversion of I_{Ca} was recorded). V_r ranged between $+40$ and $+60$ mV in most cells. Assuming that $[Ca^{2+}]_i$ in neuroblastoma cells is not far different from the average value found in other animal cells (10^{-7} M or less; reviewed by Baker, 1972) the application of the Nernst equation for a Ca^{2+}-selective channel would result in a value of the equilibrium potential (E_{Ca}) of at least $+120$ mV in 20 mM-Ca^{2+} solution at 20 °C. Thus the empirical value of V_r is far too negative to agree with a reasonable estimate of E_{Ca}.

One source that may explain the relatively low value of V_r arises from the procedure of leakage current subtraction. Thus far the leakage current–voltage (I_1–V) characteristic has been taken as linear for positive potentials by simply extrapolating the I_1–V curve as determined by large hyperpolarizing steps. In many cells, however, blocking of all voltage-dependent currents in 20 mM-Ca^{2+}, Na^+-free solution containing TEA and Co^{2+} revealed a rectifying I_1–V characteristic (Fig. 4); that is, more current is flowing for strong depolarizing test potentials than expected from a simple symmetry around the holding potential. Similar observations have been reported for the leakage current in squid giant axon (Adelman & Taylor, 1961) and for *Myxicola* axons (Goldman & Binstock, 1969). Obviously, this leakage current rectification may give rise to a considerable under-estimation of the value of V_r.

Another source that may contribute to an underestimation of V_r is non-ideal ionic selectivity of the neuroblastoma Ca^{2+} channel. Although relatively little is known about Ca^{2+} channel selectivity, a recent study on the voltage-dependent Ca^{2+} channel in mammalian heart muscle has revealed a small relative permeability to monovalent cations, which has been shown to be sufficient to account for a discrepancy between V_r and E_{Ca} of roughly the same magnitude as in neuroblastoma cells (see Reuter & Scholz, 1977).

Finally, the presence of a counteracting TEA-resistant outward current might contribute to a net reduction of the I_{Ca} values and thereby to a lowering of V_r. Although such a current will be shown to be present ($I_{K(Ca)}$, see below), its extremely slow rise time seems to preclude any significant reduction of the I_{Ca} peak values.

Kinetics of I_{Ca}

By analogy with the Hodgkin–Huxley (1952) formulation, the kinetics of I_{Ca} was assumed to be governed by two independent parameters representing an activation and an inactivation process respectively. The properties of the latter process were

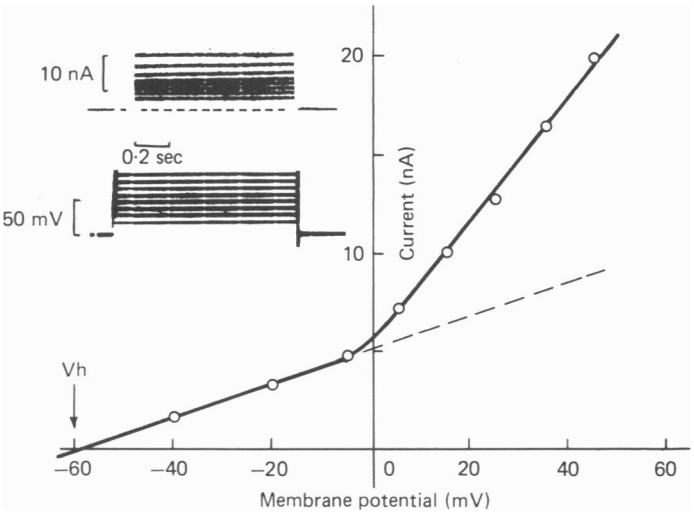

Fig. 4. Leakage current–voltage relation for cell bathed in 20 mM-Ca^{2+}, Na^+-free solution containing 10 mM-Co^{2+} and 25 mM-TEA. Current traces are displayed in inset; high frequency noise was eliminated by means of a low-pass filter (24 db/octave, cut-off frequency 300 Hz) resulting in prolonged capacitive current transients which are omitted from the current records.

investigated by stepping from various holding potentials to a fixed level of -20 mV and normalizing the largest I_{Ca} peak value to one. This procedure yields the steady-state inactivation function h_∞ (V). The results of such an experiment in 20 mM-Ca^{2+}, Na^+-free solution are illustrated in Fig. 5.

I_{Ca} reached its maximum peak values when elicited from holding potentials more negative than -70 mV, whereas inactivation was complete at potentials more positive than -30 mV. The solid line in Fig. 5 is drawn according to eqn. (1):

$$h_\infty = 1 \Big/ \left(1 + \exp \frac{(V - V_h)}{K_h}\right), \tag{1}$$

where the half-inactivation potential V_h is -56 mV. The slope parameter K_h is 4·8 mV for an e-fold change in h_∞. Since the activation of I_{Ca} starts at -55 mV (cf. Fig. 2*B*) the h_∞ (V) curve will be partially overlapped by the steady-state activation curve. This implies that in the voltage range between -55 and -30 mV some voltage-dependent fraction of the Ca^{2+} channels is in a steadily non-inactivated (i.e. open) state.

Qualitatively similar h_∞ (V) functions have been described for Ca^{2+} inward currents in a variety of preparations, including cardiac muscle (Reuter, 1973; Trautwein, McDonald & Tripathi, 1975), molluscan neurones (Standen, 1974, 1975; Akaike *et al.* 1978), crustacean muscle (Mounier & Vassort, 1975; Henček & Zachar, 1977) and tunicate eggs (Okamoto, Takahashi & Yoshii, 1976).

The time course of I_{Ca}, corrected for leakage, at different test potentials is shown semi-logarithmically in Fig. 6*A*. The decaying phase of I_{Ca} fits a straight line, indicating that the inactivation kinetics follows a first-order process with a single time constant (τ_h) which depends on voltage. Fig. 6*B* shows a plot of τ_h against membrane potential as determined from the slope of the lines in Fig. 6*A*. It is also clear from

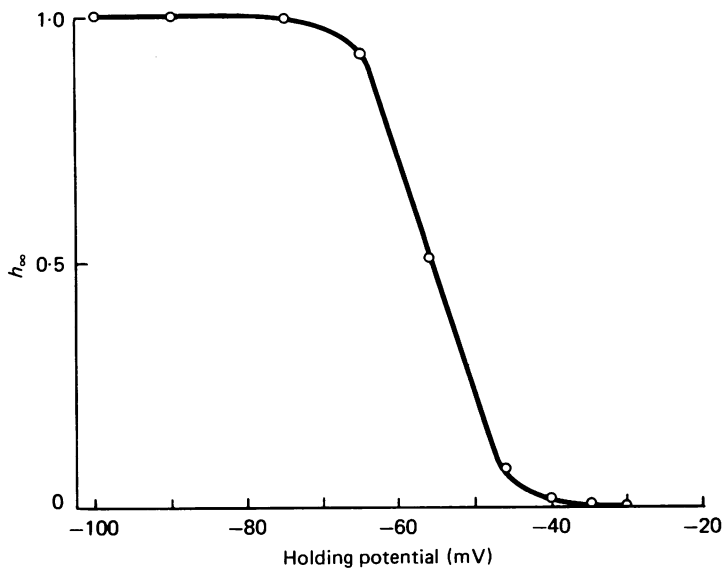

Fig. 5. Steady-state inactivation curve h_∞ (V) of the Ca^{2+} current; abscissa: holding potential; ordinate: peak Ca^{2+} current (normalized to 1) during test pulse from the holding potential to −20 mV.

Fig. 6*A* that at longer times and particularly at larger depolarizations the inactivation time course deviates from a straight line in positive (i.e. outward) direction. The most plausible explanation for this deviation is that at larger depolarizations a TEA-resistant outward current is initiated with a relatively slow rise time. In the next section the existence of such a current is confirmed and its kinetic and pharmacological properties are investigated.

Activation of a slow outward current

In high [Ca^{2+}] solutions containing 25 mM-TEA about half of the cells tested produced slowly rising outward currents upon prolonged depolarizations beyond −20 mV (Fig. 7). The rise time of these currents was on the order of a few seconds which is considerably slower than that of the delayed K^+ current $I_{K(V)}$ (Moolenaar & Spector, 1978). The rise time tended to decrease with increasing depolarizations, though in a much less prominent way than in case of $I_{K(V)}$. Although variations in the activation time course were relatively small among different cells, the intensity of the slow current varied greatly from cell to cell. For example, in the cell used for I_{Ca} analysis (Fig. 6*A*) the slow current was not well developed, whereas in other cells with almost identical I_{Ca} patterns the relative intensity of the slow current appeared to be much more increased (see Fig. 7*A*). In a few cells the slow current intensity was large enough to appear clearly superimposed on the delayed K^+ current $I_{K(V)}$ thereby

allowing a direct confirmation of the differences between the time courses of both outward currents (Fig. 7*C*). In most other cells, however, the slow current was at least one order of magnitude smaller in intensity than $I_{K(V)}$ so that its observation was only possible after blockage of $I_{K(V)}$ by external TEA (Fig. 7*A*, *B*, *E*).

Ionic dependence

The slowly declining tail currents following a depolarizing step beyond −20 mV in 20 mM-Ca^{2+}, 25 mM-TEA solution reversed their direction in a holding potential range between −70 to −85 mV (Fig. 7*D*), that is the range of the K^+ equilibrium

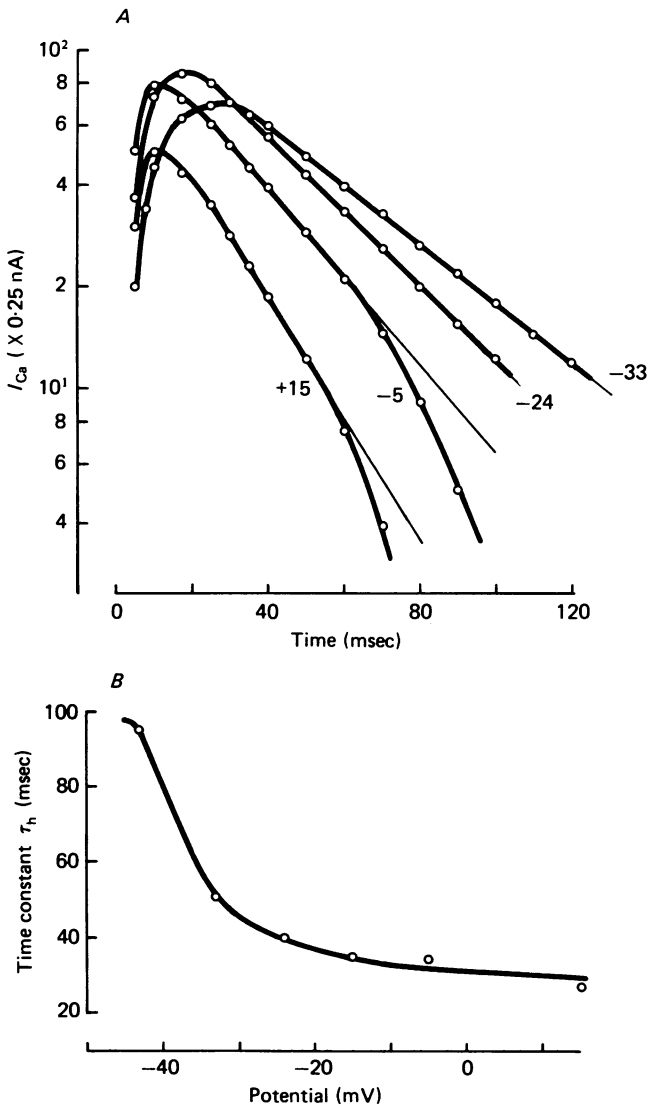

Fig. 6. *A*, semilogarithmic plot of the time course of the Ca^{2+} current at different test potentials. *B*, inactivation time constant τ_h plotted against membrane potential; τ_h was obtained from the slope of the straight lines in *A*.

potential (Moolenaar & Spector, 1978). Increasing external $[K^+]$ from 5·5 to 55 mM and setting the holding potential at -70 mV shifted the value of the reversal potential to -25 mV (two cells) that is a 45–60 mV increase per ten-fold change in $[K^+]_o$. This strongly suggests that K^+ is the main charge carrier for the slow current.

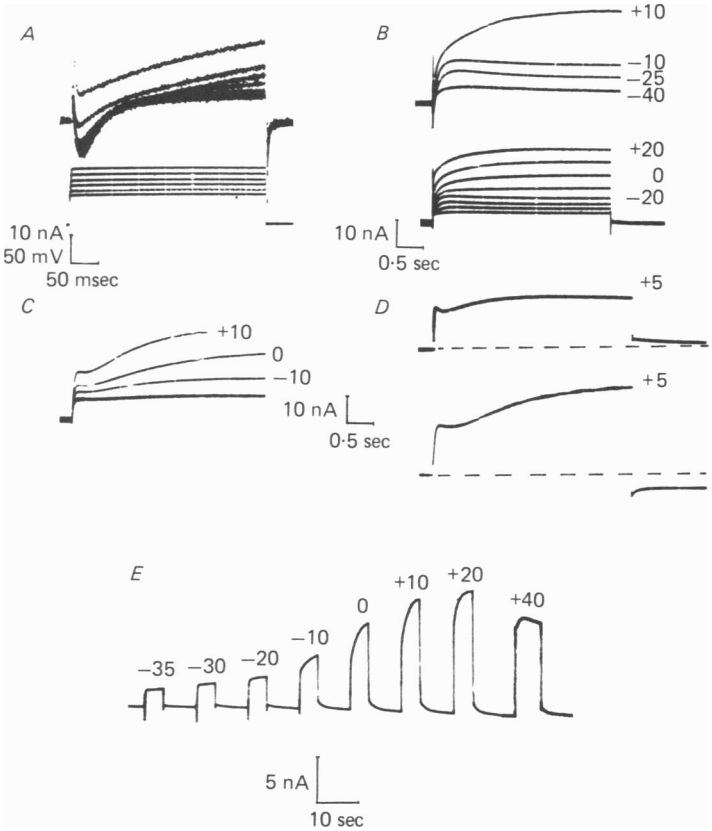

Fig. 7. Slow outward current $I_{K(Ca)}$ during prolonged depolarizations in 20 mM-Ca^{2+}, Na^+-free solution from a holding potential (V_h) of -70 mV (except in *D*). Current filter cut-off frequency 3 kHz (*A*) or 300 Hz (*B–E*). Delayed K^+ current $I_{K(V)}$ was blocked by 25 mM-TEA (*A*, *B*, *E*). In *C* and *D*, $I_{K(V)}$ was only partially blocked (2·5 mM-TEA) allowing visual separation of $I_{K(V)}$ and $I_{K(Ca)}$. Slow tail current reversal is shown in *D* (upper trace: $V_h = -55$ mV; lower trace: $V_h = -95$ mV). Decrease of $I_{K(Ca)}$ with increasing steps beyond $+20$ mV is shown in *E*.

As mentioned above, the slow current was particularly pronounced in the presence of increased levels of extracellular Ca^{2+}. In Ca^{2+}-free solutions (containing 1·8 mM-Co^{2+} as a substituting ion) or in high $[Ca^{2+}]$ solutions containing 2 mM-La^{3+}, sufficient to block I_{Ca}, outward currents were always reduced to the level of the leakage current (see inset of Fig. 4). Furthermore, depolarizations from holding potentials more positive than -20 mV at which I_{Ca} is completely inactivated (cf. Fig. 5), were not capable to initiate the slow current.

Following substitution of Ba^{2+} or Sr^{2+} (20 mM) for external Ca^{2+}, depolarization failed to activate outward currents in 25 mM-TEA solutions (six cells). These results

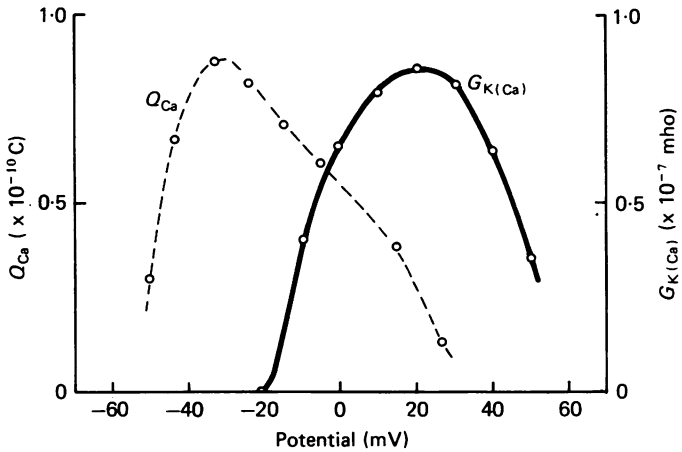

Fig. 8. The 'steady-state' value of the Ca^{2+}-dependent K^+ conductance ($G_{K(Ca)}$) plotted against membrane potential (continuous line). $G_{K(Ca)}$ was determined from eqn. (2). Dashed line represents Q_{Ca}, i.e. the amount of charge carried by Ca^{2+} into the cytoplasm during a clamp pulse; Q_{Ca} was estimated from the time course of I_{Ca} as

$$Q_{Ca} = \int I_{Ca}\,dt.$$

indicate that the activation of the slow outward K^+ current ($I_{K(Ca)}$) is specifically linked to voltage-dependent influx of Ca^{2+} into the cytoplasm.

Voltage-dependence

The steady-state I–V characteristic of the slow current was hard to measure since most cells deteriorated when subjected to prolonged (> 2 sec) depolarizations beyond about +30 mV. For cells that did allow higher test potentials the current intensity decreased at higher depolarizations (Fig. 7*E*). It is seen from Fig. 7*E* that the $I_{K(Ca)}$ time course during prolonged depolarization to +40 mV reaches a peak value after a few seconds and then slowly declines. Technical difficulties prevented us to establish if this decline is due to a true inactivation of $I_{K(Ca)}$ or to a gradual reduction of E_K.

The most direct way to analyse the $I_{K(Ca)}$ voltage-dependence is to plot the Ca^{2+} dependent K^+ conductance $G_{K(Ca)}$ against voltage (Fig. 8) thereby eliminating the contribution of the driving force $(V-E_K)$. $G_{K(Ca)}$ was calculated from eqn. (2):

$$G_{K(Ca)} = I_{K(Ca)}/(V-E_K) \qquad (2)$$

where E_K was taken as −75 mV and $I_{K(Ca)}$ values were taken at 2·5 sec after pulse onset. A typical plot of $G_{K(Ca)}$ against voltage is shown in Fig. 8 (solid line). The $G_{K(Ca)}$ *vs.* voltage curve shows a distinct maximum at about +30 mV and declines towards the zero current axis with increasing depolarizations.

The shape of the $G_{K(Ca)}$–V graph clearly differs from that of the $G_{K(V)\infty}$–V graph (see Fig. 8 in Moolenaar & Spector, 1978). Since $G_{K(Ca)}$ depends on Ca^{2+} influx for its activation, the plot in Fig. 8 is expected to reflect somehow the voltage-dependence of I_{Ca}.

Relationship between $G_{K(Ca)}$ and I_{Ca}

If $G_{K(Ca)}$ is activated by I_{Ca} in some direct way, then $G_{K(Ca)}$ and I_{Ca} will exhibit the same voltage-dependence. This, however, is certainly not the case (compare Figs. 2*B* and 8): the maximum of the $G_{K(Ca)}$–V curve occurs at a much higher potential (+30 mV) than the maximum of the I_{Ca}–V curve (−20 mV). Thus the observed voltage-dependence of $G_{K(Ca)}$ is quantitatively difficult to reconcile with an exclusive role of I_{Ca} in the activation of $G_{K(Ca)}$. Also illustrated in Fig. 8 is the voltage-dependence of the amount of net charge transferred by Ca^{2+} into the cytoplasm (Q_{Ca}). Q_{Ca} was calculated from the I_{Ca} time course at various potentials as

$$Q_{Ca} = \int I_{Ca}\, dt$$

and might represent an indirect measure for the increase in $[Ca^{2+}]_i$ during a clamp pulse. However, the Q_{Ca}–V and $G_{K(Ca)}$–V curves are seen to be very different. Thus, although the results indicate that Ca^{2+} influx is a prerequisite for the activation of $G_{K(Ca)}$, the voltage-dependence of I_{Ca} as well as that of Q_{Ca} is not easily compatible with a model in which I_{Ca} or the resulting rise in $[Ca^{2+}]_i$ solely underlies $G_{K(Ca)}$ activation. Additional parameters have, therefore, to be considered to account for the quantitative discrepancy between the properties of I_{Ca} and those of $G_{K(Ca)}$. On the basis of these results two hypotheses on $G_{K(Ca)}$ activation can be envisaged: (1) $G_{K(Ca)}$ activation depends both on I_{Ca} and on voltage; (2) $G_{K(Ca)}$ is voltage-independent and is activated by Ca^{2+} influx through a second population of Ca^{2+} channels. Both hypotheses will be discussed below.

DISCUSSION

In the present study the neuroblastoma Ca^{2+} current and a Ca^{2+}-dependent K^+ current underlying respectively the Ca^{2+} spike and the prolonged after-hyperpolarization (Moolenaar & Spector, 1979), have been examined with the voltage-clamp technique. A preliminary description of the Ca^{2+} inward current was already given in a previous voltage-clamp study on neuroblastoma membrane currents (Moolenaar & Spector, 1978). The activation of a Ca^{2+}-dependent K^+current, however, went unnoticed in earlier studies on neuroblastoma excitability.

The Ca^{2+} inward current

The properties of I_{Ca} were found to be most conveniently studied in Na^+-free, 25 mM-TEA solutions containing 20 mM-Ca^{2+}, that is some ten-fold increase of the Ca^{2+} concentration in normal solution (1·8 mM). The voltage-dependence of I_{Ca} in 20 mM-Ca^{2+} solutions was not significantly different from that in 10 mM-Ca^{2+} solutions (Fig. 11*B* in Moolenaar & Spector, 1978) but its magnitude was clearly enhanced. The maximum peak Ca^{2+} current density in 20 mM-Ca^{2+} is of the order of 10^{-4} A/cm^2 (reached at −20 mV), which is about one order of magnitude smaller than the average Na^+ current density of neuroblastoma cells in standard solution (0·8 mA/cm^2, reached at −5 mV, Moolenaar & Spector, 1978). Similar values have been inferred for the Ca^{2+} current density in the presynaptic terminal of the squid

giant synapse (Katz & Miledi, 1969) and in barnacle muscle fibres (Hagiwara, Hayashi & Takahashi, 1969).

Estimation of the value of the reversal potential is subject to some underestimation because the leakage current contribution at potentials near V_r is in most cells larger than expected on account of symmetry around the holding potential. Identification of V_r with E_{Ca} is further obscured since the theoretical value of E_{Ca} is unknown, in particular under conditions of elevated $[Ca^{2+}]_o$ in Na^+-free medium; an E_{Ca} value well above +100 mV does not seem unlikely, however, since such values are commonly found in non-mammalian neurones (e.g. Standen, 1975; Akaike *et al.* 1978). Other sources possibly contributing to the low value of V_r and already discussed in the Results, are non-ideal selectivity of the Ca^{2+} channel and, more unlikely, the opposing outward $I_{K(Ca)}$. In summary, rectifying leakage, elevated $[Ca^{2+}]_i$ and non-ideal Ca^{2+} selectivity are the most likely sources underlying the relatively low value of V_r.

The pharmacological properties of the Ca^{2+} channel are very similar to those described for many other preparations: Ba^{2+} and Sr^{2+} can easily pass, whereas La^{3+}, Mn^{2+} and, to a lesser extent, Mg^{2+} all block I_{Ca}, presumably by competitive occupation of Ca^{2+}-binding sites (Hagiwara, 1973, 1975; Akaike *et al.* 1978).

The results of the present study confirm our earlier suggestion that I_{Ca} is subject to time- and voltage-dependent inactivation: conditioning depolarization reduces the magnitude of I_{Ca} during the test pulse and preceding hyperpolarization enhances it. Furthermore, the steady-state inactivation $h_\infty(V)$ curve for I_{Ca} is of the form used by Hodgkin & Huxley (1952) to describe Na^+ inactivation. The time course of the I_{Ca} inactivation at a certain voltage is characterized by a single time constant τ_h, that decreases with increasing depolarizations, a common characteristic of inward current inactivation processes. The resemblance with the inactivation time course of I_{Ba} and I_{Sr}, both incapable of initiating a counteracting K^+ current, support the view that the I_{Ca} decline is due to true inactivation and not to $I_{K(Ca)}$ activation.

In molluscan neurones the 'early' Ca^{2+} channels that contribute to the rising phase of the action potential have also been reported to be subject to time- and voltage-dependent inactivation (Geduldig & Gruener, 1970; Standen, 1974, 1975; Kostyuk & Krishtal, 1977*a*; Akaike *et al.* 1978). In other preparations the inactivation of the Ca^{2+} channel appears to be less pronounced (squid giant axon: Baker, Meves & Ridgway, 1973; squid giant synapse: Llinás, Steinberg & Walton, 1976; barnacle muscle: Keynes, Rojas, Taylor & Vergara, 1973; Hagiwara, Fukuda & Eaton, 1974). A slow and more or less persistent Ca^{2+} influx has recently been detected in molluscan neurones, showing a kinetic behaviour that clearly contrasts with Hodgkin & Huxley (1952) kinetics (Eckert & Lux, 1976; Heyer & Lux, 1976*a*; Eckert, Tillotson & Ridgway, 1977). This suggests that molluscan neurones may have two sets of Ca^{2+} channels (see however Akaike *et al.* 1978), although direct evidence for this hypothesis is lacking as yet.

Resemblance with the Ca^{2+} channel in the mouse oocyte membrane

Voltage-dependent Ca^{2+} channels have recently been identified in the membrane of various unfertilized oocytes, including the mouse oocyte, under voltage-clamp conditions (Okamoto *et al.* 1976, 1977). Interestingly, in particular the Ca^{2+} current

described in the mouse egg membrane (Okamoto *et al.* 1977) shows a remarkable quantitative similarity with I_{Ca} in mouse neuroblastoma cells. The resemblance includes the current–voltage relation, kinetic behaviour and pharmacological properties.

The I–V curve in 20 mM-Ca^{2+}, Na^+-free solution at 33 °C in the mouse oocyte (Text-fig. 4*B* in Okamoto *et al.* 1977) strikingly resembles the corresponding I–V curve in mouse neuroblastoma cells (Fig. 2*B*): numerical values of critical activation voltage (−55 mV), voltage of maximum I_{Ca} (−20 mV) and reversal potential (40–60 mV) are almost identical in both types of membranes. Rough calculations on the mouse egg current records as shown in Text-fig. 3 of Okamoto *et al.* (1977), taking into account the reported Q_{10} of 4 for the time constants, showed us that the value of the peak time and the inactivation time constant τ_h at a certain voltage are similar as well (cf. Fig. 6*B*). Finally, both types of Ca^{2+} channels are permeable to Ba^{2+} and Sr^{2+} and are blocked by La^{3+} and Mn^{2+}.

This suggests a remarkable constancy for the molecular structure of the Ca^{2+} channel during embryonic development from unfertilized egg to mature sympathetic nerve cell. Preliminary experiments (unpublished) have revealed the presence of Ca^{2+} channels in growing, undifferentiated neuroblastoma cells as well, thereby further supporting the contention that the Ca^{2+} channel is not a characteristic of the differentiated state (see also Miyake, 1978). Our data indicate that a voltage-dependent fraction of the Ca^{2+} channels is permanently in a non-inactivated state between −55 and −30 mV, thus contributing to the steady Ca^{2+} influx into the cytoplasm and possibly assisting in the regulation of the free intracellular Ca^{2+} level. Free intracellular Ca^{2+} has been shown to be a primary trigger for mammalian egg activation and subsequent development (Fulton & Wittingham, 1978). Furthermore, intracellular Ca^{2+} has been implicated as a 'second messenger' in the control of cell growth (for references see Rasmussen & Goodman, 1977). Viewed in this context it is tempting to speculate that the Ca^{2+} channels play a role not only during 'early' developmental processes such as fertilization and egg cleavage but also during growth and maturation of cells originating from the neural crest, such as neuroblastoma cells.

Although evidence for participation of Ca^{2+} channels in the regulation of $[Ca^{2+}]_i$-dependent growth processes is lacking at present, the hypothesis is attractive and may, in principle, be tested by means of tissue culture techniques.

The activation of $I_{K(Ca)}$

The slowly rising outward current, observed in many cells under conditions of an enhanced voltage-dependent Ca^{2+} influx, is carried predominantly by K^+ on account of the value of the reversal potential and its dependence on $[K^+]_o$. Its relatively low intensity and easy deterioration during prolonged test pulses made it difficult, however, to study its properties in all detail. $I_{K(Ca)}$ contrasts sharply with the earlier described delayed outward K^+ component $I_{K(V)}$ (Moolenaar & Spector, 1978) in its kinetics, its insensitivity to external TEA and, particularly, in its dependence on external Ca^{2+}. Any manipulation that blocks or steadily inactivates I_{Ca} abolishes $I_{K(Ca)}$. Furthermore, $I_{K(Ca)}$ is absent when Ca^{2+} is replaced by Ba^{2+} or Sr^{2+}. Obviously, $I_{K(Ca)}$ depends on Ca^{2+} influx or on the resulting increase in $[Ca^{2+}]_i$. However, the present results seem to contradict a *direct* relationship between $I_{K(Ca)}$ and I_{Ca}:

neither the I_{Ca}–V nor the Q_{Ca}–V plots fit quantitatively the $I_{K(Ca)}$–V curve. Only above a certain critical voltage level (−20 mV) Ca^{2+} influx is capable of activating $I_{K(Ca)}$.

These results can be accounted for by a model in which the underlying conductance $G_{K(Ca)}$ depends both on I_{Ca} and membrane potential. Alternatively, one could hypothesize that $G_{K(Ca)}$ is activated solely by Ca^{2+} influx through a separate population of Ca^{2+} channels which exhibit a voltage dependence similar to that of $I_{K(Ca)}$ rather than that of I_{Ca}. Although any direct evidence for the existence of two distinct types of Ca^{2+} channels in neuroblastoma cells is lacking, one should realize that the inherent inseparability of this second Ca^{2+} current and $I_{K(Ca)}$, both by voltage dependence and pharmacology, will obscure its presence in conventional voltage-clamp experiments. In molluscan neurones, for instance, the slow and persistent Ca^{2+} current (Eckert & Lux, 1976) which activates a component of K^+ current (Heyer & Lux, 1976*b*), has first been detected by indirect K^+ efflux measurements using extracellular K^+-sensitive electrodes (Lux & Eckert, 1974). The application of similar techniques in conjunction with voltage-clamp results may be necessary to distinguish unequivocally between both hypotheses on $I_{K(Ca)}$ activation in neuroblastoma cells.

Comparison with other cells

The occurrence of Ca^{2+}-dependent K^+ conductances or permeabilities appears to be widespread among various types of cells including red blood cells (Whittam, 1968; Lew, 1974), leech neurones (Jansen & Nicholls, 1973), molluscan neurones (Meech, 1972, 1974*a*; Meech & Standen, 1975; Heyer & Lux, 1976*b*; Thompson, 1977), vertebrate motoneurones (Barrett & Barrett, 1976; Krnjević *et al.* 1978), amphibian sympathetic neurones (Busis & Weight, 1976), skate electroreceptors (Clusin & Bennett, 1977), cardiac Purkinje fibres (Isenberg, 1977) and the protozoan *Paramecium* (Brehm, Dunlap & Eckert, 1978). The slow outward K^+ current component in frog skeletal muscle fibres (Adrian, Chandler & Hodgkin, 1970) may be Ca^{2+} mediated as well (Meech, 1976).

As for the molecular basis of Ca^{2+}-activated K^+ conductances recent evidence obtained from molluscan neurones (Hofmeier & Lux, 1978; Lux & Hofmeier, 1978) suggests that at least two different types of Ca^{2+}-dependent K^+ conductances have to be distinguished: the first type is activated by $[Ca^{2+}]_i$ and shows a relatively slow time course (Meech, 1972, 1974*b*; Krnjević *et al.* 1978; Gorman & Thomas, 1978; Hofmeier & Lux, 1978); the second type is triggered by Ca^{2+}-influx (Heyer & Lux, 1976*b*; Lux & Hofmeier, 1978; see, however, Eckert & Tillotson, 1978) shows a faster time course and is depressed by $[Ca^{2+}]_i$ (Heyer & Lux, 1976*b*; Eckert & Lux, 1977). On this basis a Ca^{2+}-dependent K^+ conductance of the first type underlies the post-activity hyperpolarization in certain molluscan and vertebrate neurones (Meech, 1974*b*; Krnjević *et al.* 1978; Gorman & Thomas, 1978). By analogy, $G_{K(Ca)}$ underlying the a.h.p. in neuroblastoma cells is then likely to be activated by an increase in $[Ca^{2+}]_i$ rather than by Ca^{2+} influx itself. Additional voltage-dependence of $G_{K(Ca)}$, as proposed here for the activation of $G_{K(Ca)}$ to fit with in I_{Ca}, has not been put forward in the afore-mentioned studies on other cell types. Clearly, more experiments will be necessary to gain further insight into the physical mechanism of $G_{K(Ca)}$ activation in neuroblastoma cells.

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