Two Different Ionic Mechanisms
Generating the Spike
“Positive” Afterpotential
in Molluscan Neurons

DANTE J. CHIARANDINI and ENRIQUE STEFANI

From the Instituto de Anatomía General y Embriología, Facultad de Ciencias Médicas, Universidad de Buenos Aires, Buenos Aires, Argentina. Dr. Chiarandini’s present address is Laboratory of Neurophysiology, Department of Neurology, College of Physicians & Surgeons, Columbia University, New York. Dr. Stefani’s present address is Department of Biophysics, University College, London, England

ABSTRACT The ionic bases of the “positive” afterpotential (ap) have been examined in the so-called DIH neurons of the central nervous system of Cryptomphallus aspersa. In these cells $E_K$ has been determined and its value compared with the equilibrium potential of the ap ($E_{ap}$). It has been found that in half of the studied cells the $E_K$ value is very close to $E_{ap}$ whereas in another half, the difference ($E_K - E_{ap}$) is large and amounts to circa $-10$ mv. The effects of changes in the concentration gradients of $K^+$, $Cl^-$, and $Na^+$ were assayed in both groups of cells. When the $[K]_i/[K]_o$ ratio is reduced in both groups of neurons, the ap amplitude and the $E_{ap}$ diminished. In cells displaying a large ($E_K - E_{ap}$), $Cl^{-}$-free Ringer’s solution diminished the ap amplitude and $E_{ap}$ but produced no effect in the neurons with a reduced ($E_K - E_{ap}$). A similar effect was observed if $[Cl]_i$ was increased by intracellular injection of NaCl. Changes in both $[Na]_i$ and $[Na]_o$ were ineffective. It is concluded that $K^+$ is the only ion involved in the origin of the ap in the groups of cells with a low value for ($E_K - E_{ap}$). On the contrary, the ap of the neurons presenting large ($E_K - E_{ap}$) is produced by a simultaneous increase in the fluxes of both $K^+$ and $Cl^-$. 

It is known from the work of Gasser and Grundfest (1) and Erlanger and Gasser (2) that the compound action potential recorded extracellularly from myelinated nerves presents in its late descending phase two deflections of opposite polarity named negative and positive afterpotentials. Intracellular recording has shown that the positive afterpotential, which will be referred to
here as the afterpotential, constitutes an increase in the negativity inside the cell \((3, 4)\).

Further work in various nerve cells \((5-7)\) has elucidated the ionic basis of this transient hyperpolarization, showing that it results from an outflux of potassium ions produced by a persistence of the increased membrane permeability to potassium during the falling phase of the action potential. Therefore, the amplitude and polarity of the afterpotential in these cells depends on the difference between the resting membrane potential and the equilibrium potential for potassium \(E_K\).

The present paper deals with the ionic bases of the afterpotential in a group of molluscan central neurons conventionally named DInhi neurons \((8)\). The noncholinergic inhibitory postsynaptic potentials (IPSP’s) recorded in the DInhi cells are solely due to a selective increase in permeability of the subsynaptic membrane to \(K^+\) \((9)\). Therefore, in these cells the equilibrium potential of the IPSP’s \(E_{\text{IPSP}}\) is identical to \(E_K\). It has been found in the present study that two different ionic mechanisms may generate the afterpotential in two different groups of DInhi neurons. One of them consists, as classically described, in an outflux of potassium, whereas the other depends on both potassium and chloride fluxes.

**METHODS**

The periesophageal ganglia of the Argentinian land snail *Cryptomphallus aspersa* were isolated with the associated nerves attached. The nervous system was pinned to the wax bottom of a suitable Lucite chamber of 5 ml volume, and the connective sheaths covering the visceroadominal mass were removed to expose the naked monopolar neuronal somata. The preparation was bathed with a snail Ringer’s solution \((10)\) \((110 \text{ mM NaCl}, 4.9 \text{ mM KCl}, 3.5 \text{ mM MgSO}_4, 18.3 \text{ mM NaHCO}_3, \) and \(6.4 \text{ mM Ca gluconate})\), previously equilibrated with a mixture of 95% \(O_2\) and 5% \(CO_2\). An Ag-AgCl ground electrode was connected to the saline solution via an Agar-Ringer bridge. Ag-AgCl electrodes were used to stimulate the nerves.

The cells were impaled with double-barreled micropipettes of 1.5 \(\mu\) average over-all tip diameter and 30–50 megohm resistance, pulled with a de Fonbrune microforge (Aloe Scientific, St. Louis, Mo.). One barrel filled with 3 \(M\) KCl was used for recording and was connected to standard dc recording equipment, while the second barrel, filled with 0.6 \(M\) NaCl, was employed to set the resting potential of the cells to desired levels by applying inward or outward currents across the cell membrane. The coupling resistance between the two barrels of the microelectrode was less than 100 kilohm and introduced a slight error in the recording of the membrane potential when current was injected through one barrel. This error was always corrected.

Noncholinergic IPSP’s were evoked generally by stimulating the visceral or anal nerves \((8)\). The \(E_{\text{IPSP}}\) was measured as has been reported previously \((9)\). Antidromic spikes \((11)\) were obtained by stimulating the visceral, anal, or left pallial nerves and recorded on one trace of a dual beam oscilloscope. The other beam was set as a reference of zero potential level. The afterpotential amplitude was measured as the differ-
ence between the initial membrane potential (resting membrane potential) and the maximal hyperpolarization observed.

Chloride-free Ringer's solution was made by replacing all the NaCl by an equivalent amount of Na$_2$SO$_4$ and adding sucrose to keep the osmolarity constant. When the preparation was immersed in Cl-free Ringer's solution, a change in the junction potentials was observed. In some cases this change was corrected by subtracting from the recorded membrane potential values the drift observed at the beginning of the experiment with the microelectrode immersed in the saline solution. In many experiments, the device of Hutter and Noble (12) was used to correct this drift in the junction potential. Potassium-free Ringer's solution was made by omitting KCl. A high potassium concentration solution, (10 mM), was made by adding KCl to the normal saline solution.

A Ringer's solution with the 25% of the normal content of Na (low Na Ringer's solution) was made by replacing 96 mM/liter of NaCl by an isosmotic amount of sucrose.

The modified saline solutions were applied by injecting 50 ml into the chamber within 30-40 sec. After every change in the ionic composition of the external medium, the preparation was washed with snail Ringer's solution until both the original membrane potential and the firing frequency were restored.

The intracellular ionic concentration was modified by injecting ions ionophoretically into the neurons. To reduce internal potassium concentration, Na$^+$ was injected into the neuron by passing a weak current between the Na$_2$SO$_4$ barrel and the earth electrode. It has been shown (5) that with this procedure the current which passes across the neuronal membrane is carried mainly by K$^+$. This results in a considerable depletion of the intracellular K.

To increase internal chloride concentration ([Cl]$_i$), NaCl was injected by interbarrel current flow (intracellular “interbarrel” injection) (13, 14) making the KCI-filled barrel negative and the Na$_2$SO$_4$ one positive. To increase sodium inner concentration ([Na]$_i$), the intracellular “interbarrel” injection was done between the barrels of a double microelectrode filled with NaCl in one side and Na$_2$SO$_4$ in the other. This Na$_2$SO$_4$ injection avoided an increase of [Cl]$_i$ when Na was injected.

When the bathing solutions were changed, the spikes were recorded 5-20 sec after the total replacement of the solution. In the case of intracellular injections, the records were performed 15-30 sec after interrupting the injecting current.

RESULTS

Equilibrium Potential of the Spike Afterpotential ($E_{ap}$) of DInhi Neurons

The amplitude of the spike afterpotential (ap) depends on the membrane potential level, as illustrated in Fig. 1, for the antidromic spike of a DInhi neuron. In this case, the cell presented a resting membrane potential of -43 mv (b), and Fig. 1 shows the characteristics of the ap when the membrane potential was preset at different membrane potential levels, between -36 and -56 mv. The depolarization of the cell to -36 mv (Fig. 1, a) caused an increase in the ap amplitude whereas hyperpolarization (Fig. 1,
c and d) produced a diminution. Since in most of the studied cells the soma spike became blocked when the neuron was hyperpolarized beyond \(-60\) or \(-65\) mV, it was not possible to measure \(E_{ap}\) directly by simple hyperpolarization of the cell. For this reason \(E_{ap}\) was obtained applying the procedure used by Coombs, Eccles, and Fatt (5) in motoneurons. Plotting the ap amplitude against the initial membrane potential, a straight line was drawn as shown in Fig. 2 (filled circles). \(E_{ap}\), the membrane potential level at which the ap amplitude becomes null, was obtained by extrapolation. In the case of the neuron of Fig. 2, \(E_{ap}\) was \(-68\) mV. This extrapolation is valid since in the Dlnhi neurons the membrane resistance is "ohmic" between \(-50\) and \(-100\) mV, as shown in the direct current-voltage plot (15) of Fig. 2, inset.

As has been mentioned, the IPSP's recorded in Dlnhi neurons are due exclusively to an increase of the subsynaptic membrane permeability to K\(^+\) (9) and thence the \(E_{IPSP}\) is identical to \(E_K\). If, as in many other excitable tissues (5-7), the ap in these neurons would be due to the persistence of an increased K\(^+\) conductance during the late phase of the action potential, an \(E_{ap}\) value very close to the \(E_{IPSP}\) would be expected. In order to test this hypothesis both the \(E_{IPSP}\) (which will be referred to here as \(E_K\)) and the \(E_{ap}\) were successively measured in a series of Dlnhi neurons, and the difference between both values was computed. In these experiments it became evident that whereas in some Dlnhi neurons the difference between \(E_K\) and \(E_{ap}\), \((E_K - E_{ap})\), was very small, in some other Dlnhi cells it was \(-10\) mV or more. Fig. 3 shows the frequency distribution of \((E_K - E_{ap})\) computed in a series of 47 neurons. From this histogram it appears that the neuronal population is composed of two groups of cells. This heterogeneity was confirmed by plotting the experimental data on a probability graph (16) as shown in Fig. 4. An S-shaped curve was obtained (filled circles) instead of a straight line, confirming that two populations were present. If the experimental data shown in Fig. 3 are treated as belonging to two different groups of neurons, one comprising the neurons in which \((E_K - E_{ap})\) was between 6.15 and \(-3.85\) mV and the other, the cells with an \((E_K - E_{ap})\) larger than \(-3.85\) mV, two
straight lines are obtained. One of them (Fig. 4, open circles) corresponds to the data of 23 neurons in which the $(E_K - E_{ap})$ has a mean value of $-0.26 \pm 0.44$ mV ($P > 0.1$). These cells will be referred to as neurons with reduced $(E_K - E_{ap})$. The second line (Fig. 4, triangles) represents the data of a group formed by 24 neurons with a mean $(E_K - E_{ap})$ of $-11.1 \pm 0.63$ mV ($P < 0.001$). These cells will be denoted as neurons with large $(E_K - E_{ap})$.

The resting potential of both groups of cells was very similar: $-43.2 \pm 2.06$ mV for the cells having a reduced $(E_K - E_{ap})$ and $-42.2 \pm 2.02$ mV for the other group. $E_K$ in both groups was also very close: $-69.8 \pm 0.93$ mV for the first group and $-71.1 \pm 0.69$ mV for the cells with large $(E_K - E_{ap})$.

\footnote{Mean $\pm$ standard error.}
$E_{\text{ap}}$). On the contrary, $E_{\text{ap}}$ was $-70.0 \pm 1.09$ mV in the cells with reduced $(E_K - E_{\text{ap}})$ and $-60.1 \pm 0.76$ mV in the other group. The difference between both values is statistically significant ($P < 0.001$).

A statistically significant difference ($P < 0.001$) has been also observed between the amplitudes of the ap in both types of neurons. The mean amplitude of the ap in 13 neurons presenting a reduced $(E_K - E_{\text{ap}})$ was $11.5 \pm 0.8$ mV while the ap amplitude in 13 cells with a large $(E_K - E_{\text{ap}})$ was $5.8 \pm 0.4$ mV. The ap amplitude was always recorded at the same membrane potential.

**Changes in the Potassium Concentration Gradient**

To investigate the role of K\(^+\) in the generation of the afterpotential, the potassium concentration gradient was modified by changing both its outer ([K\(_o\)]) and inner concentrations ([K\(_i\)]) and the modifications of the ap amplitude and $E_{\text{ap}}$ were analyzed in these conditions.

Fig. 5, a shows an antidromic spike recorded in a DInhi neuron, which when immersed in normal Ringer's solution, presented a resting membrane potential.
potential of $-48$ mv. Fig. 5, $a'$ shows the ap at higher amplification. When \([K]_o\) was raised from 4.9 mm to 10 mm, the neuron was depolarized by 7 mv and both the rate of fall of the spike ($b$) and the ap amplitude ($b'$) were diminished, in spite of the cell depolarization. This reduction of the ap became more evident when the depolarization produced by the high $[K]_o$ was corrected by again driving the membrane potential to $-48$ mv. The spike and its ap in this condition are shown in Fig. 5, $c$ and $c'$: the ap has been reduced to 35% of its original amplitude.

The increase of $[K]_o$ to 10 mm depolarized DInhi neurons by circa 9 mv and generally produced a reduction in the $E_{ap}$ of circa 8 mv. An experiment of this type has been plotted in Fig. 2 (open circles) in which the extrapolation of the straight line drawn from the experimental values shows that the $E_{ap}$ has been reduced from $-68$ to $-60$ mv. Table I shows that the effects
of raising $[K]_o$ to 10 mM on each one of the neuronal types described is similar: in the group of cells in which the $(E_K - E_{ap})$ was 0.25 mv the change in the $E_{ap}$ amounted to about 9 mv, and in the group of cells that presented a $(E_K - E_{ap})$ of -9.8 mv, it was about 8 mv.

The alterations of the $E_{ap}$ amplitude produced by the removal of K from the Ringer's solution bathing the preparation are shown in Fig. 6. It may be seen that the control $E_{ap}$ (Fig. 6, a) was increased by the removal of $K^+$ from the saline solution (b). The K-free solution hyperpolarized the neuron in 3

### Table I

**CHANGE OF $E_{ap}$ BY EFFECT OF HIGH $[K]_o$**

<table>
<thead>
<tr>
<th>Neurons with reduced $(E_K - E_{ap}) = 0.25 \pm 0.08$ mv*</th>
<th>Neurons with large $(E_K - E_{ap}) = -9.8 \pm 0.71$ mv</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control $E_{ap}$</td>
<td>$[K]_o$</td>
</tr>
<tr>
<td>-----------------</td>
<td>--------</td>
</tr>
<tr>
<td>$m_0$</td>
<td>$m_0$</td>
</tr>
<tr>
<td>-66.5</td>
<td>-59.5</td>
</tr>
<tr>
<td>-64.0</td>
<td>-58.0</td>
</tr>
<tr>
<td>-67.5</td>
<td>-58.0</td>
</tr>
<tr>
<td>-74.0</td>
<td>-64.0</td>
</tr>
<tr>
<td>-78.0</td>
<td>-66.0</td>
</tr>
<tr>
<td>-80.0</td>
<td>-73.0</td>
</tr>
<tr>
<td>-81.0</td>
<td>-74.0</td>
</tr>
</tbody>
</table>

-8.6 ± 0.9 mv‡

-7.6 ± 0.9 mv‡

* Mean ± standard error.
‡ Difference statistically significant ($P < 0.001$).
mv. When the resting potential was corrected to the control value (−40 mv) by injecting outward current, the increase in the ap amplitude (Fig. 6, c) amounted to about 40% of the initial amplitude.

Inner potassium concentration was reduced by injecting Na⁺ ionophoretically into the cell by passing a current of 40 namp during 120 sec (5). Fig. 7 illustrates the effect of this procedure in a DInhi neuron with a resting potential of −46 mv. The ap of this cell before the injection is shown in a. 15 sec after the end of the injection, the cell was depolarized in 7 mv and the ap amplitude was much reduced (b). The ap amplitude recovered exponentially with a half time of 38 sec.

In most of the cells studied, the conduction velocity became diminished when [K]₀ or [K]ᵢ was modified. This finding clearly appears by comparing the difference in delay of the antidromic spike discharge between the control records (Fig. 5, a, Fig. 6, a, and Fig. 7, a) and the records in the same cells after changing [K]₀ or [K]ᵢ, (Fig. 5, b, Fig. 6, b, and Fig. 7, b).
Changes in the Chloride Concentration Gradient

The large difference between the $E_K$ and the $E_{ap}$ found in one group of the examined neurons may be explained by assuming that $K^+$ is not the only ion involved in the genesis of the ap. Since in this group of cells $E_{ap}$ was consistently closer to the resting potential than the $E_K$, any other ion associated with the ap should be distributed in such a way that its equilibrium potential must be closer to the zero level than $E_K$. It was highly probable that only two ions could fulfill this condition, $Na^+$ and $Cl^-$. There is no information about the distribution of $Na^+$ in DInhi neurons but on the basis that an outward, active $Na^+$ transport was demonstrated (17) in the so-called H neurons (18) an $E_{Na}$ more positive with respect to $E_K$ would be expected. That chloride ions may also participate was suggested by some existing evidence. DInhi neurons are always depolarized and excited by acetylcholine (8) and it was demonstrated that this depolarizing action of acetylcholine (ACh potential) in the so-called D neurons of molluscs (18) is due to a $Cl^-$ activation (13, 19). Moreover, it appears that in DInhi neurons the ACh potential is $Cl^-$ dependent, these cells having an equilibrium potential for the ACh potential ($E_{ach}$) of circa $-30$ mv.

To obtain information on the possible role of $Cl^-$ in the genesis of the ap, the effect of changes in the $Cl^-$ concentration gradient on both the ap amplitude and $E_{ap}$ was studied. These changes were obtained in two different ways: (a) by replacing $Cl^-$ by $SO_4^-$ in the bathing solution and (b) by increasing [$Cl$]$_i$ by intracellular injections of NaCl.

The effect of a $Cl^-$ free Ringer's solution on the ap amplitude was studied in 19 DInhi cells. This Ringer's solution produced a slight depolarization of 3-5 mv but the membrane potential was adjusted to its initial value before recording the ap amplitude.

In eight neurons with a mean $(E_K - E_{ap})$ of $-0.93 \pm 0.57$ mv the removal of $Cl^-$ from the external medium did not modify the ap amplitude. The neuron shown in Fig. 8 is an example of this behavior: the control ap in a was not altered by the removal of $Cl^-$ (a'). On the contrary, in another group of 11 neurons presenting a statistically significant value of $(E_K - E_{ap})$ of $-11.1 \pm 1.0$ mv, the $Cl^-$ free Ringer's solution reduced the ap amplitude. Fig. 8 shows the recording of the ap in a neuron of this type before (b) and after (b') removing $Cl^-$ from the external solution.

Table II summarizes the effect of a $Cl^-$ free Ringer's solution on the $E_{ap}$ of neurons of both groups. It may be appreciated that in the group of six neurons in which the $(E_K - E_{ap})$ was circa $-1$ mv, the removal of $Cl^-$ from the saline solution did not significatively modify the $E_{ap}$ value. These findings are also illustrated in the plot of Fig. 9 a which correspond to a DInhi neuron

\[^{2}\text{Chiarandini, D. J. Data to be published.}\]
having a control $E_{ap}$ of $-73.5$ mv (filled circles) which was reduced to $-72.0$ mv in Cl-free Ringer's solution (open circles).

On the contrary, in the second group of eight cells presented in Table II showing an $(E_K - E_{ap})$ of about $-10$ mv, the removal of Cl⁻ reduced the $E_{ap}$ in $6.0$ mv, this difference being statistically significant. In Fig. 9 b this change is illustrated in a DInhi cell whose control $E_{ap}$ of $-57$ mv (filled circles) was reduced to $-47$ mv by the removal of Cl⁻ from the external medium (open circles).

The Cl⁻ concentration gradient was also modified in both types of neurons

| TABLE II |

EFFECT OF A CI-FREE RINGER’S SOLUTION ON THE $E_{ap}$ |

| Neurons with reduced $(E_K - E_{ap}) = -0.93 \pm 0.59$ mv | Neurons with large $(E_K - E_{ap}) = -10.2 \pm 0.42$ mv |

<table>
<thead>
<tr>
<th>Control $E_{ap}$</th>
<th>$[\text{Cl}]<em>o = 0$ mm $E</em>{ap}$</th>
<th>Change in $E_{ap}$</th>
<th>Control $E_{ap}$</th>
<th>$[\text{Cl}]<em>o = 0$ mm $E</em>{ap}$</th>
<th>Change in $E_{ap}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$-60.5$</td>
<td>$-60.0$</td>
<td>$-0.5$</td>
<td>$-63.0$</td>
<td>$-58.0$</td>
<td>$-5.0$</td>
</tr>
<tr>
<td>$-66.0$</td>
<td>$-69.0$</td>
<td>$3.0$</td>
<td>$-60.0$</td>
<td>$-54.0$</td>
<td>$-6.0$</td>
</tr>
<tr>
<td>$-74.0$</td>
<td>$-72.5$</td>
<td>$1.5$</td>
<td>$-57.0$</td>
<td>$-47.0$</td>
<td>$-10.0$</td>
</tr>
<tr>
<td>$-64.0$</td>
<td>$-64.0$</td>
<td>$0$</td>
<td>$-59.0$</td>
<td>$-54.0$</td>
<td>$-5.0$</td>
</tr>
<tr>
<td>$-74.0$</td>
<td>$-74.0$</td>
<td>$0$</td>
<td>$-57.0$</td>
<td>$-53.0$</td>
<td>$-4.0$</td>
</tr>
<tr>
<td>$-66.5$</td>
<td>$-66.0$</td>
<td>$-0.5$</td>
<td>$-62.0$</td>
<td>$-54.0$</td>
<td>$-8.0$</td>
</tr>
<tr>
<td>$-59.0$</td>
<td>$-54.0$</td>
<td>$-5.0$</td>
<td>$-59.0$</td>
<td>$-54.0$</td>
<td>$-5.0$</td>
</tr>
</tbody>
</table>

$-0.08 \pm 0.62$ mv*

$-6.1 \pm 0.80$ mv†

* Difference not statistically significant ($P > 0.1$).
† Difference statistically significant ($P < 0.001$).
by changing \([\text{Cl}]_i\) by intracellular "interbarrel" injections of NaCI. This salt was injected using currents of 50 namp during 90 sec. Table III and Fig. 10 a and 10 b illustrate the results obtained with this procedure. Table III corresponds to a group of four neurons with an \(E_{ap}\) very close to \(E_K\) in which the increase of \([\text{Cl}]_i\) did not modify the value of the \(E_{ap}\). Fig. 10 a illustrates this fact in a DInhi cell in which the control \(E_{ap}\) (filled circles) was only modified by 1 mv after the injection (open circles). Another group of six neurons with a difference between \(E_K\) and \(E_{ap}\) of about \(-10\) mv (see Table III) shows, on the contrary, that the increase of \([\text{Cl}]_i\) reduces the \(E_{ap}\) by 7 mv. Fig. 10 b corresponds to a plot obtained from a cell of this group in which the control \(E_{ap}\) (filled circles) was reduced by 6 mv after increasing \([\text{Cl}]_i\) (open circles).

The removal of \(\text{Cl}^-\) from the external solution affects in different ways the membrane resistance of both groups of cells. In a group of five cells having afterpotentials insensitive to changes in the ratio \([\text{Cl}]_o/[\text{Cl}]_i\), different modifications in the membrane resistance were observed: in two cells it was reduced by 15% while it remained unchanged in the other three cells. A similar situation was observed in seven cells having afterpotentials sensitive to

---

**Figure 9.** Effect of a Cl-free medium on the \(E_{ap}\). The \(E_{ap}\) values were obtained as in Fig. 2. 9 a: effect on a neuron with a reduced \((E_K - E_{ap}) = -0.5\) mv; resting potential, \(-53\) mv. Filled circles were obtained when the preparation was bathed with a normal Ringer's solution and the \(E_{ap}\) determined was \(-73.5\) mv. Open circles were obtained after the removal of \(\text{Cl}^-\) from the saline solution, measuring an \(E_{ap}\) of \(-72.5\) mv. Notice that the \(E_{ap}\) was practically not affected by the removal of \(\text{Cl}^-\) from the solution. 9 b: an analogous plot obtained in a neuron presenting a large \((E_K - E_{ap}) = -15.0\) mv; resting potential, \(-46\) mv. Notice that in this case the removal of \(\text{Cl}^-\) (open circles) reduced the control \(E_{ap}\) (filled circles) from \(-55\) to \(-45\) mv.
changes in the ratio $[\text{Cl}]_o/[\text{Cl}]_i$: the resistance fell about 24% in three cells and did not change in the other four neurons.

**Changes in the Sodium Concentration Gradient**

To analyze the possible participation of $\text{Na}^+$ in the origin of the ap, $[\text{Na}]_i$ was increased by an intracellular "interbarrel" injection of $\text{Na}_2\text{SO}_4$ (50 namp, 90 sec). This treatment did not modify either the ap amplitude or the $E_{ap}$ in a group of three neurons with large $(E_K - E_{ap})$. The Na injection hyperpolarized the cells by about 8 mv.

**TABLE III**

**EFFECT OF INCREASING $[\text{Cl}]_i$ ON THE $E_{ap}$**

<table>
<thead>
<tr>
<th>Neurons with reduced $(E_K - E_{ap})$</th>
<th>Neurons with large $(E_K - E_{ap})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$-70.0$</td>
<td>$-70.0$</td>
</tr>
<tr>
<td>$-60.4$</td>
<td>$-59.4$</td>
</tr>
<tr>
<td>$-67.5$</td>
<td>$-68.5$</td>
</tr>
<tr>
<td>$-80.0$</td>
<td>$-78.0$</td>
</tr>
</tbody>
</table>

- $0.50 \pm 0.65$ mv$^*$
- $7.1 \pm 1.32$ mv$^\dagger$

$^*$ Difference not statistically significant ($P > 0.1$).
$^\dagger$ Difference statistically significant ($P < 0.001$).

In a group of five neurons of the same type, the effect of lowering the concentration of Na in the external medium ([Na]$_o$) on the $E_{ap}$ was analyzed. Since the low Na Ringer's solution had also a low concentration of Cl (18.9 mm, see Methods), the experiments were performed by bathing first the preparation with a Ringer's solution containing 18.9 mm of Cl and a normal Na content and after, with the low Na Ringer's solution. In this way it was possible to differentiate the effects due to the reduction of [Na]$_o$ from those produced by lowering [Cl]$_o$. No changes were observed in the $E_{ap}$ that could be attributed to the reduction of [Na]$_o$ alone. The removal of Cl$^-$ depolarized the cells by 3 mv. The replacement of the low Cl$^-$ solution by the low Na solution hyperpolarized the neurons by circa 5 mv.

**Calculation of Cl/K Permeability Ratio During the ap**

The above results show that in the DInhi neurons presenting a large $(E_K - E_{ap})$ the ap is due to an increase of the membrane permeability to both K$^+$ and Cl$^-$, so that their $E_{ap}$ is given by the following equation:
\[
E_{ap} = \frac{RT}{F} \ln \left( \frac{[K]_o + \alpha [Cl]_o}{[K]_i + \alpha [Cl]_i} \right)
\]

where \( R, T, \) and \( F \) have the usual meaning, \( E_{ap}, [K]_o, \) and \( [Cl]_o \) are known, and \( \alpha \) is the \( Cl/K \) permeability ratio. \( [K]_i \) may be calculated taking \(-70\) mV as the mean value of the \( E_K \) and using the relation \( [K]_i = [K]_o \exp \left( -\frac{E_K}{F/RT} \right) \). This yields a \( [K]_i \) equal to 78.5 mM. Assuming that in DInhi neurons \( E_{ACh} = E_{Cl} \), \( [Cl]_i \) may be obtained from equation \( [Cl]_i = [Cl]_o \exp \left( \frac{E_{Cl}}{F/RT} \right) \).

\( F/RT \). Since \( E_{ACh} \) is \(-30\) mV, \( [Cl]_i \) would be 35.0 mM. By substituting all the known values in equation (1) and solving for \( \alpha \) a value near 0.1 is obtained.

**DISCUSSION**

Coombs, Eccles, and Fatt (5) have examined the ionic basis of the ap in mammalian motoneurons by studying the effects of intracellular injections of \( Cl^- \) and \( Na^+ \) on the ap amplitude. These authors demonstrated that the ap of motoneurons was insensitive to changes in both \( [Cl]_i \) and \( [Na]_o \), concluding that \( K \) was the only ion involved in the genesis of the ap. This mechanism has also been found in other excitable cells. In squid giant axon (6) it has been observed that the membrane potential level reached by the ap was very
close to the $E_K$. Blackman, Ginsborg, and Ray (7) were able to show in sympathetic ganglionic neurons in vitro that their AP was reduced in amplitude by increasing $[K]_o$, but it was not affected by the removal of $Cl^-$ from their environment. Finally, in both the cockroach giant axon (20) and in frog motoneurons (21) it has been observed that the AP amplitude diminished with increasing $[K]_o$. Since in all these preparations $[K]_i$ is higher than the concentration which would be in equilibrium with the resting potential, it may be concluded that $K^+$ leaves all these cells during the AP.

In the present series of experiments, the possible difference existing between the $E_K$ and the $E_{ap}$ has been measured in a large number of DIInhi neurons. It has been rather surprising to find that there were two different populations of DIInhi cells, one presenting a reduced $(E_K - E_{ap})$ and the other with a large $(E_K - E_{ap})$. Statistical analysis confirmed the existence of these groups. This difference in the value of the $(E_K - E_{ap})$ appears as a clear difference in the amplitude of the AP between both groups of cells. Moreover, when changes in the $Na^+$, $K^+$, and $Cl^-$ concentration gradients were assayed on both groups of neurons, it was found that they behaved differently. The AP of neurons presenting a reduced $(E_K - E_{ap})$ was only sensitive to modifications of the $K^+$ concentration gradient, while the AP of the cells showing large $(E_K - E_{ap})$ underwent changes when either the $K^+$ or the $Cl^-$ concentration gradient was modified. The AP of the last group of cells was not affected by changes in the $Na^+$ concentration gradient. The replacement of chloride in the Ringer's solution by sulfate may produce a drastic reduction of the ionic $Ca^{++}$ concentration (22) which may in turn affect the membrane permeability. However it is known from a previous work (9) that a similar Cl-free Ringer's solution does not affect the IPSP's or $E_{IPSP}$ in DIInhi neurons, demonstrating that at least the permeability to $K^+$ remains unaffected. Furthermore, in the experiments reported here, the membrane resistance was almost unaffected by the Cl-free Ringer's solution, indicating that the ionic permeability was not markedly affected. Therefore, it seems possible to discard the possibility that the modifications of the AP amplitude recorded in certain neurons, when a Cl-free Ringer's solution was used, are due to a secondary effect in the membrane permeability, instead of an effect due to the change in the chemical gradient of $Cl^-$. It may be concluded that the DIInhi neurons with reduced $(E_K - E_{ap})$ present a $K^+$-dependent AP whereas the AP of the DIInhi neurons showing large $(E_K - E_{ap})$ depend on both $K^+$ and $Cl^-$. However, since the present studies were only concerned with the mechanism of the AP, a change in $Cl^-$ permeability during the descending phase of the spike cannot be ruled out on the basis of the data here presented. Grundfest (23), analyzing the possible ionic mechanisms of electrogenesis, predicted the possibility that $Cl^-$ may participate in the AP. The present results confirm this hypothesis.
The change of the $[K]_o/[K]_i$ ratio affects the a.p. of both kinds of cells, but when $[K]_o$ was raised from 4.9 to 10.0 mM, the observed reduction of the $E_{ap}$ was smaller than that theoretically predicted by the Nernst equation. For instance, in the case of DInhi neurons presenting a K-dependent ap the expected reduction in the $E_{ap}$ would be $RT/F \ln (10.0/4.9) = 18$ mv, instead of the actual reduction of 8.6 mv. Some causes may explain this discrepancy: (a) the $[K]_i$ may accommodate to the new K concentration of the bathing solution and the actual value of $[K]_i$ may be not constant as it was assumed for the calculations, and (b) the increase of $[K]_o$ may have enhanced the neuronal permeability to Na$^+$ to a significant level. A similar discrepancy has been reported in sympathetic ganglion cells (7).

DInhi neurons have been considered a homogeneous group of cells (24) since they present a very constant pattern of response to orthodromic stimulation and a typical pharmacological specificity (8). However, the results here presented strongly suggest a dual mechanism of origin of their ap. To confirm this duality it was necessary to discard some possible artifacts which could have produced it. Since in some experiments the same electrode was used for recording from both types of neurons, differences arising from leakages of chloride ions from the recording electrode may be discarded. Furthermore, the increase of $[Cl]_o$ affects only one group of cells. Neuron damage, another possibility of artifact, is considered unlikely since DInhi neurons presenting K-dependent ap and DInhi neurons presenting K-Cl-dependent ap had similar membrane potentials and similar $E_K$ values, which remain constant during the experiments.

It is of interest now to analyze the ionic fluxes that give rise to the K-Cl-dependent ap. The effect provoked by changes in the $[Cl]_o/[Cl]_i$ ratio only evidences a participation of Cl$^-$ in the genesis of the ap but gives no information about the possible direction of the Cl$^-$ fluxes. Since in the neurons presenting this type of ap, $E_{ap}$ is always less negative than $E_K$, it may be assumed that the Cl$^-$ flux participating in the ap has an outward direction and antagonizes the K$^+$ outflux.

Comparing the values of the resting potential with the $E_K$ in both kinds of DInhi neurons it was found that the former is generally some 25 mv lower than the $E_K$. The fact that the membrane potential does not follow the Nernst equation for K$^+$ would indicate that other ions besides K participate in the genesis of the resting potential. This view agrees with the observed sensitivity of the membrane potential to changes in both Na$^+$ and Cl$^-$ concentration gradients: with increasing $[Na]_o$ or decreasing $[Na]_i$, the neurons became hyperpolarized while with decreasing $[Cl]_o$ or increasing $[Cl]_i$ they became depolarized. All these effects strongly suggest that K$^+$, Cl$^-$, and Na$^+$ all would participate in the genesis of the resting potential.
The authors want to express their gratitude to Dr. H. M. Gerschenfeld for his unfailing interest and helpful criticism. They are also indebted to Mr. A. von Ellenrieder from the Instituto del Cálculo, Facultad de Ciencias Exactas, Universidad de Buenos Aires, for his help in the statistical analysis and to Dr. M. Cereijido for reading the manuscript.

This work was supported by grants of the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina, and from the U.S. Air Force Office of Scientific Research (Grant AF-AFOSR-656-65).

Dr. Chiarandini is a fellow of the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.

Dr. Stefani is a fellow of the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.

Received for publication 13 June 1966.

REFERENCES