J. Physiol. (1984), **352**, *pp.* 637–652
With 7 text-figures
Printed in Great Britain

INTRACELLULAR CALCIUM IONS AND CALCIUM CURRENTS IN PERFUSED NEURONES OF THE SNAIL, *LYMNAEA STAGNALIS*

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(*Received 12 October 1983*)

SUMMARY

1. Neuronal somata of *Lymnaea stagnalis* were internally perfused and voltage clamped using the suction pipette method. The cells were exposed to internal solutions buffered to various concentrations of Ca^{2+} while the cytoplasmic Ca^{2+} activity ($[Ca^{2+}]_i$) was monitored with a Ca^{2+}-sensitive micro-electrode.

2. $[Ca^{2+}]_i$ was usually about 10^{-7} M when the cell was perfused with a solution buffered to any level of Ca^{2+} from 9×10^{-7} to below 10^{-8} M. With internal solutions buffered to 10^{-6} M-Ca^{2+} or greater, $[Ca^{2+}]_i$ increased rapidly and overshot the perfusate Ca^{2+} activity by up to two orders of magnitude. It was thus virtually impossible to hold $[Ca^{2+}]_i$ steady at any levels other than about 10^{-7} M or 10^{-4} M using internal perfusion of simple ionic internal solutions.

3. The excess Ca^{2+} which caused the overshoot of $[Ca^{2+}]_i$ entered the cell from the external solution through Cd^{2+}-sensitive channels. Cd^{2+} in the external solution prevented or reversed the overshoot of $[Ca^{2+}]_i$ and brought $[Ca^{2+}]_i$ to near the perfusate level.

4. ATP added to the internal solution also prevented $[Ca^{2+}]_i$ from overshooting the perfusate level during perfusion with high-Ca^{2+} buffers.

5. By monitoring $[Ca^{2+}]_i$ with a Ca^{2+}-sensitive micro-electrode, we were able to estimate the relationship between $[Ca^{2+}]_i$ and the Ca^{2+} current (I_{Ca}) measured under voltage clamp. I_{Ca} was completely blocked as $[Ca^{2+}]_i$ was raised to 10^{-6} M.

6. We believe that the discrepancy between our data and other estimates of the I_{Ca} *vs.* $[Ca^{2+}]_i$ relationship using internal perfusion of molluscan nerve cells results from the incorrect assumption that $[Ca^{2+}]_i$ is controlled adequately during internal perfusion.

INTRODUCTION

Increasing the cytoplasmic free Ca^{2+} activity ($[Ca^{2+}]_i$) blocks inward Ca^{2+} currents (Kostyuk & Krishtal, 1977; Plant, Standen & Ward, 1983) and depresses the amplitude of Ca^{2+}-dependent action potentials (Hagiwara & Nakajima, 1966). Accumulation of Ca^{2+} in the submembrane space is responsible for the inactivation

of the Ca^{2+} current (I_{Ca}) which is observed in some types of cells (Brehm & Eckert, 1978; Tillotson, 1979; Ashcroft & Stanfield, 1981).

The technique of internal perfusion of nerve cell bodies (Kostyuk, Krishtal & Pidoplichko, 1975; Lee, Akaike & Brown, 1978; Byerly & Hagiwara, 1982) has enabled the effect of internal Ca^{2+} to be studied in greater detail by allowing Ca^{2+}-buffered solutions to be introduced directly into the cytoplasm and by allowing interfering K^+ currents to be virtually eliminated by replacement of intracellular K^+ with impermeant cations (see, however, Byerly & Hagiwara, 1982; Byerly, Meech & Moody, 1984). Nonetheless, experiments using internal perfusion have yielded widely varying estimates of the quantitative relationship between $[Ca^{2+}]_i$ and I_{Ca}, even in similar preparations. The three estimates of this relationship in *Helix* neurones alone range over more than three orders of magnitude of $[Ca^{2+}]_i$. Kostyuk & Krishtal (1977) reported that a complete block of I_{Ca} occurred at a $[Ca^{2+}]_i$ of $5{\cdot}8 \times 10^{-8}$ M. Akaike, Lee & Brown (1978) found, on the other hand, that raising $[Ca^{2+}]_i$ more than two orders of magnitude higher, to 10^{-5} M, blocked I_{Ca} by less than 50%. Using intact *Helix* neurones impaled with Ca^{2+}-sensitive micro-electrodes and injected with Ca^{2+} buffers, Plant *et al.* (1983) found a relationship intermediate between the above two, with a 50% block of I_{Ca} at a $[Ca^{2+}]_i$ of 10^{-6} M. This last result agrees with the original estimates of Hagiwara & Nakajima (1966) based on Ca^{2+} spike amplitude in barnacle muscle fibres.

We were suspicious that the cause of much of this variability was the assumption that internal perfusion results in adequate control over a highly buffered and tightly regulated ion such as Ca^{2+}. We therefore studied the blocking effect of intracellular Ca^{2+} on I_{Ca} in neurones exposed to internal perfusion of Ca^{2+}-buffered solutions and simultaneously impaled with Ca^{2+}-sensitive micro-electrodes. We have found that the response of the cell to attempts to vary $[Ca^{2+}]_i$ using internal perfusion is highly variable and that the actual cytoplasmic Ca^{2+} activity is not simply predictable from the Ca^{2+} activity of the perfusate. By using direct measurement of $[Ca^{2+}]_i$ during internal perfusion, we have quantified the blocking effect of cytoplasmic Ca^{2+} on the Ca^{2+} current and have found a relationship that is similar to that reported in the early barnacle muscle experiments and in later injection experiments in intact neurones, but different from both results obtained using the internal perfusion technique in neurones.

Some of these results have been presented in preliminary form (Byerly & Moody, 1983).

METHODS

Isolated neurones from the parietal and visceral ganglia of *Lymnaea stagnalis* were studied using the suction pipette method of internal perfusion (Lee *et al.* 1978; Byerly & Hagiwara, 1982). Methods of cell isolation, internal perfusion and voltage clamping were the same as those described by Byerly & Hagiwara (1982). To insure efficient and consistent exchange of internal solutions, the ratio of suction pipette opening to cell diameter was kept as close as possible to 1:3. In earlier experiments (Byerly & Moody, 1982) we found that this ratio yielded a nearly complete exchange of K^+ in 2–3 min and a 90% change of intracellular pH in about 10 min. The cells we used thus required suction pipettes with lumen diameters ranging from 32–40 μm. The electrical resistance of these pipettes was measured prior to aspiration of the cell and ranged from 250 to 400 KΩ. Pipette resistance was also monitored during each experiment by noting the series resistance compensation

required to achieve minimum settling time of the capacitative transients. This value did not increase significantly during perfusion of high-Ca^{2+} internal solutions, indicating that clogging of the pipette lumen did not occur under these conditions. Experiments were performed at room temperature (22–25 °C).

Table 1 shows the compositions of the main external, internal, and calibrating solutions used (see Marban, Rink, Tsien & Tsien, 1980). 100 mM-HEPES buffer was used in the internal solutions to control pH because the stability constants of the Ca^{2+} buffers are strongly pH dependent (Harufuji & Ogawa, 1980), and effective control over cytoplasmic pH requires high buffer strengths

TABLE 1. Composition of solutions

Extracellular (mM)

65 Tris	4 Ca^{2+}	4 Mg^{2+}	74 Cl^-	60 glucose	pH 7·4

Intracellular (mM)

			pH	Ca^{2+} buffer	Ca^{2+}	pCa
74 K^+	28 aspartate	100 HEPES	7·3	10 EGTA	0	8
or			7·3	10 HEDTA	5	5·7
74 Cs^+		Calibration	7·29	10 EGTA	5	7
			7·70	10 HEDTA	5	6
			7·39	10 NTA	5	4

(Byerly & Moody, 1982). We found that it was necessary to increase the osmolarity of the external solution to match that of the strongly-buffered internal solutions, even though the internal perfusion apparatus provides an effectively infinite intracellular volume. The swelling of cells aspirated onto the suction pipette when osmolarities were not matched may indicate a restricted diffusion of water through the cytoplasm. Since during the isolation procedure the cells are bathed in normal Ringer solution, we aspirated the cells and ruptured the patch membrane using an internal solution of normal osmolarity with 5 mM-HEPES buffer, and then immediately switched to 100 mM-HEPES internal solution and hyperosmotic external solution. $[Ca^{2+}]_i$ and I_{Ca} were measured in Na^+-free (Tris) external solution and K^+-free (Cs^+) internal solutions. All solutions were filtered (Millipore; 1·2 μm) before use.

Ca^{2+}-sensitive micro-electrodes were prepared using the liquid exchanger for Ca^{2+} (Oehme, Kessler & Simon, 1976) which was kindly provided by Professor W. Simon. Micropipettes were pulled from 2 mm Pyrex glass and the tips broken slightly to 1 μm diameter. Pipettes were heated in a closed container to 250 °C. A drop of chlorotrimethyl silane was added to the container and the pipettes baked for an additional 15–30 min. Siliconized pipettes were backfilled with the pCa 6 calibrating solution (see Table 1) and then a 50–100 μm column of exchanger was drawn into the tip with suction. Electrodes were equilibrated with their tips in pCa 6 solution for at least 1 h before use. Electrodes were calibrated in pCa 4, 6 and 7 solutions (Table 1). A benefit of the K^+-free internal solutions used to eliminate K^+ currents was that the sensitivity of the Ca^{2+} electrodes at low levels of $[Ca^{2+}]$ was greatly increased when Cs^+ replaced K^+. Our electrodes commonly showed responses of 20–25 mV between pCa 6 and 7. Any internal solutions to be used which were not part of the calibration sequence were checked during calibration so that a direct comparison could be made between the Ca^{2+} activity of the perfusate and the actual value of $[Ca^{2+}]_i$ with the same electrode.

The quality of the impalement of the Ca^{2+}-sensitive micro-electrode was critical to the measurement of a meaningful value for the concentration of Ca^{2+} inside the cell. If the membrane did not seal tightly around the electrode, a small influx of Ca^{2+} around the electrode might keep the concentration of Ca^{2+} at the tip of the electrode substantially higher than $[Ca^{2+}]_i$ in the bulk of the cytoplasm. This problem is more severe for the Ca^{2+}-sensitive electrode than other ion-sensitive micro-electrodes because the Ca^{2+} activity being measured is very low and the electrochemical gradient driving Ca^{2+} into the cell is very high. We found evidence suggesting that this problem existed even for impalements that seemed 'good' by electrical criteria, i.e. the Ca^{2+}-sensitive electrode caused only a small increase in holding current when it was inserted into the cell, and it accurately recorded voltage changes imposed across the membrane. With some impalements the recorded $[Ca^{2+}]$ could be made to slowly rise to levels as high as 10^{-5} M by either (1) lowering the concentration of EGTA in the cell from 10 to 0·1 mM (no Ca^{2+} added), with 4 mM-Ca^{2+} outside, or

(2) raising extracellular Ca^{2+} from 4 to 40 mM, with 10 mM-EGTA inside the cell. We do not believe that the bulk cytoplasmic Ca^{2+} activity in fact rose to 10^{-5} M under these conditions, because the holding current did not increase and I_{Ca} was not blocked, effects which are normally seen with a global increase in $[Ca^{2+}]_i$ (see below). For many impalements reducing the intracellular EGTA to 0·1 mM or raising extracellular $[Ca^{2+}]$ to 40 mM had only small, credible effects on the recorded $[Ca^{2+}]_i$. To minimize the possibility of error due to the influx of Ca^{2+} around the Ca^{2+}-sensitive electrode we therefore required that each Ca^{2+} electrode impalement satisfy four requirements: (1) the measured $[Ca^{2+}]_i$ was less than 5×10^{-7} M when the cell had 10 mM-EGTA/0 Ca^{2+} inside and 4 mM-Ca^{2+} outside; (2) the Ca^{2+} electrode accurately measure voltage changes imposed across the membrane; (3) the holding current recover to within less than 1 nA of its initial value upon impalement; and (4) the recorded $[Ca^{2+}]_i$ was insensitive to increasing extracellular $[Ca^{2+}]$ to 40 mM. We also avoided the use of internal solutions which did not have well-buffered Ca^{2+} activities.

We had difficulty obtaining satisfactory calibrations of the Ca^{2+} electrodes after disimpalement. Sensitivity of the electrodes was reduced and response time greatly increased. Electrodes seemed to perform well inside the cell, judging by both the d.c. signals obtained and the speed and magnitude of the response to changes in perfusate $[Ca^{2+}]$. This problem was much worse using internally perfused cells than when similar electrodes were used in intact cells of various types. We suspect that the negative hydrostatic pressure inside the cells results in a backing up of the exchanger into the electrode tip upon disimpalement.

In a few experiments, pH-sensitive micro-electrodes were used. These were constructed exactly as the Ca^{2+} electrodes, but using the H^+ liquid exchanger (Amman, Lanter, Steiner, Schulthess, Shijo & Simon, 1981), also generously provided by Professor W. Simon. The exchanger was equilibrated in 100% CO_2 for 24 h before use.

Peak I_{Ca} amplitude was measured at +20 mV. Currents were leak corrected by subtracting the current recorded during a voltage-clamp step from −40 to −100 mV.

All experiments were performed at the University of Southern California.

RESULTS

Resting $[Ca^{2+}]_i$ and Ca^{2+} transients in perfused neurones

Using Ca^{2+}-sensitive micro-electrodes, we found $[Ca^{2+}]_i$ values of between 5×10^{-8} and 4×10^{-7} M in neurones exposed to our normal internal solutions (10 mM-EGTA/0 Ca^{2+}), which has a Ca^{2+} activity of less than 10^{-8} M (Fig. 1*A* and *B*). $[Ca^{2+}]_i$ was never as low as the Ca^{2+} activity of the perfusate, measured with the same Ca^{2+} electrode (see also Fig. 5). Since our measured values of intracellular Ca^{2+} are close to those reported in intact molluscan neurones (Christoffersen & Simonsen, 1977; Alvarez-Leefmans, Rink & Tsien, 1981; Plant *et al.* 1983), we assume that 10 mM-EGTA is an insufficiently strong buffer to reduce $[Ca^{2+}]_i$ much below the value set by intrinsic cytoplasmic buffers.

Attempts to raise $[Ca^{2+}]_i$ by exposure of the cell to internal solutions buffered to higher values of $[Ca^{2+}]$ gave variable and surprising results. If the internal solution was buffered to a value of $[Ca^{2+}]$ only slightly above the normal range of $[Ca^{2+}]_i$ values measured with 10 mM-EGTA/0 Ca^{2+} internal solution, there was very little effect on $[Ca^{2+}]_i$. For example, when one cell was internally perfused with a 10 mM-EGTA/9 mM-Ca^{2+} solution ($[Ca^{2+}] = 9 \times 10^{-7}$ M), the steady-state $[Ca^{2+}]_i$ measured was 8×10^{-8} M; so the cell could hold $[Ca^{2+}]_i$ below that of the internal perfusate. If the internal solution was buffered to values of $[Ca^{2+}]$ greater than or equal to 10^{-6} M, the response of most cells was dramatically different, as illustrated in Fig. 1*A*. In this cell, after 15 min of perfusion with low-Ca^{2+} buffer, $[Ca^{2+}]_i$ had stabilized below 10^{-7} M. The internal solution was then changed to one buffered to 2×10^{-6} M (pCa 5·7; 10 mM-HEDTA/5 mM-Ca^{2+}, pH 7·3). After a short delay $[Ca^{2+}]_i$ began to increase, rapidly

overshooting the Ca^{2+} activity of the perfusate and after 15 min reached a value greater than 10^{-5} M. Prolonged exposure of the cell to this internal solution caused $[Ca^{2+}]_i$ to decrease only slightly from its peak value. When the low-Ca^{2+} buffer was reintroduced, $[Ca^{2+}]_i$ rapidly returned to less than 10^{-7} M. Virtually all cells studied showed this overshooting response to internal solutions buffered to $[Ca^{2+}]$ values above 10^{-6} M. This was seen with a variety of Ca^{2+} buffers. Internal solutions buffered with HEDTA seemed to produce the overshooting response at lower values of $[Ca^{2+}]$

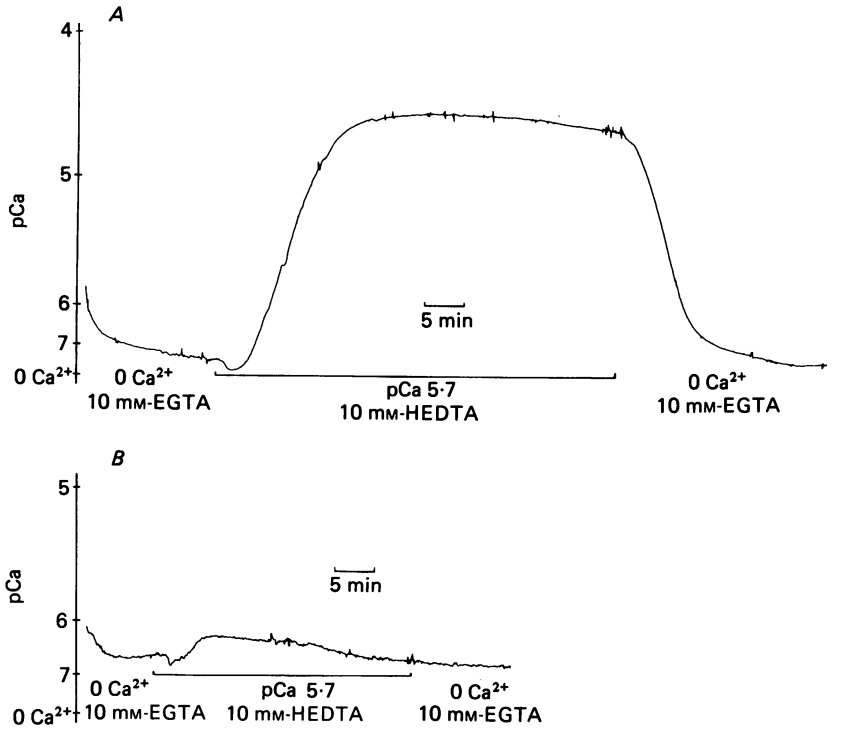

Fig. 1. Chart recordings of experiments showing the effects on $[Ca^{2+}]_i$ of perfusion with a pCa 5·7 internal solution. In both experiments, the cells were impaled with the Ca^{2+}-sensitive micro-electrode during exposure to a 0 Ca/10 mM-EGTA internal solution. After the Ca^{2+} electrode signal had stabilized, the internal solution was changed to one buffered to pCa 5·7 (see Table 1). The cell in *A* showed an overshooting response, with $[Ca^{2+}]_i$ exceeding 10^{-5} M and remaining at this level with only a slight decline throughout the exposure to high Ca^{2+}. The record in *B* illustrates the less common response, in which $[Ca^{2+}]_i$ is only slightly and transiently affected by the high-Ca^{2+} internal solution. In both experiments, the internal solution contained K^+. In this and all experiments, the holding potential was −50 mV.

than did solutions buffered with EGTA. Internal solutions with 10 mM-HEDTA/3 mM-Ca^{2+} ($[Ca^{2+}] < 9 \times 10^{-7}$ M) regularly produced the overshooting response (see Fig. 2*A*), while 10 mM-EGTA solutions with slightly higher values of $[Ca^{2+}]$ allowed the cell to keep $[Ca^{2+}]_i$ near 10^{-7} M. Occasionally, and especially in cells which had been exposed to internal perfusion (with the low-Ca^{2+} buffer) for less than 10 min (see below), exposure to pCa 5·7 internal solution resulted in a slow and sometimes

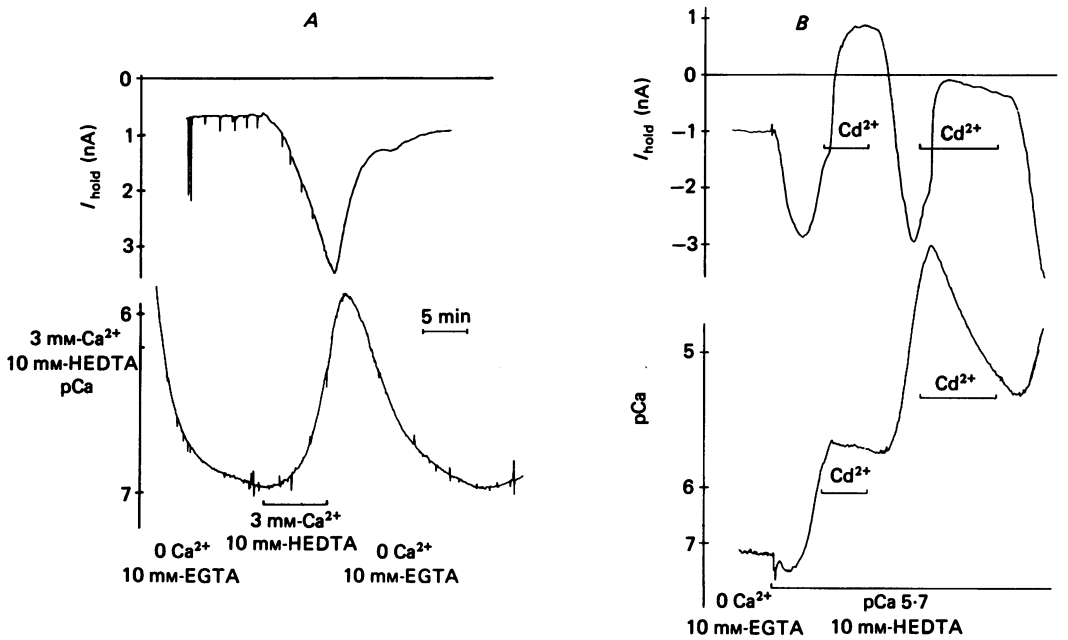

Fig. 2. Chart recordings of experiments showing the increase in inward holding current (I_{hold}) caused by perfusion with high-Ca^{2+} internal solutions, and the effect of extracellular Cd^{2+} on that current and the Ca^{2+} transient. *A*, holding current (upper trace) and $[Ca^{2+}]_i$ (lower trace) records from a cell during exposure to pCa 6·2 internal solution. During exposure, $[Ca^{2+}]_i$ increases rapidly, overshooting the buffer value, and the holding current increases from 0·75 to 3·5 nA. Both effects are rapidly reversible. The extracellular solution was hyperosmotic *Lymnaea* Ringer solution containing Na^+, and the internal solution contained Cs^+ aspartate. *B*, holding current and $[Ca^{2+}]_i$ records from a cell exposed to pCa 5·7 internal solutions. During exposure to this solution, $[Ca^{2+}]_i$ increased rapidly to pCa 5·75 and the holding current increased from 1·0 to 2·9 nA and then spontaneously decreased to 1·5 nA and began to stabilize. At this time, 1 mM-Cd^{2+} was added to the extracellular solution, causing $[Ca^{2+}]_i$ to stop increasing and the holding current to decrease rapidly and reverse direction. When Cd^{2+} was washed out, the holding current returned rapidly to its previous large inward value, and $[Ca^{2+}]_i$ began to increase again, reaching nearly 10^{-4} M. Cd^{2+} was reapplied, causing the holding current to decrease to near zero and $[Ca^{2+}]_i$ to decrease to near the buffer value. The internal solution contained K^+ aspartate.

transient increase in $[Ca^{2+}]_i$ which did not attain the measured Ca^{2+} level of the perfusate (Fig. 1*B*).

Thus, even in well perfused cells (see Methods for criteria) $[Ca^{2+}]_i$ does not bear a fixed relation to the Ca^{2+} activity of the perfusate. Depending on the Ca^{2+} activity of the perfusate, $[Ca^{2+}]_i$ is either around 10^{-7} M (perfusate $[Ca^{2+}] < 10^{-6}$ M) or greater than 10^{-5} M (perfusate $[Ca^{2+}] > 10^{-6}$ M). When the perfusate $[Ca^{2+}]$ is near 10^{-6} M, the actual steady-state $[Ca^{2+}]_i$ is either near 10^{-7} M or above 10^{-5} M, but not in between.

The source of excess Ca^{2+}

The excess Ca^{2+} which causes the overshoot of $[Ca^{2+}]_i$ described above could either be released from intracellular stores or enter the cell from the extracellular medium.

Our evidence indicates that it enters the cell from the outside through Cd^{2+}-sensitive channels activated by high internal Ca^{2+}.

Fig. 2*A* shows the large inward current present at high levels of $[Ca^{2+}]_i$, triggered in this cell by an internal solution buffered to pCa 6. A several-fold increase in inward holding current was always seen when $[Ca^{2+}]_i$ was increased above 10^{-6} M. This current returned to its normal level when $[Ca^{2+}]_i$ was decreased. In some cells, the

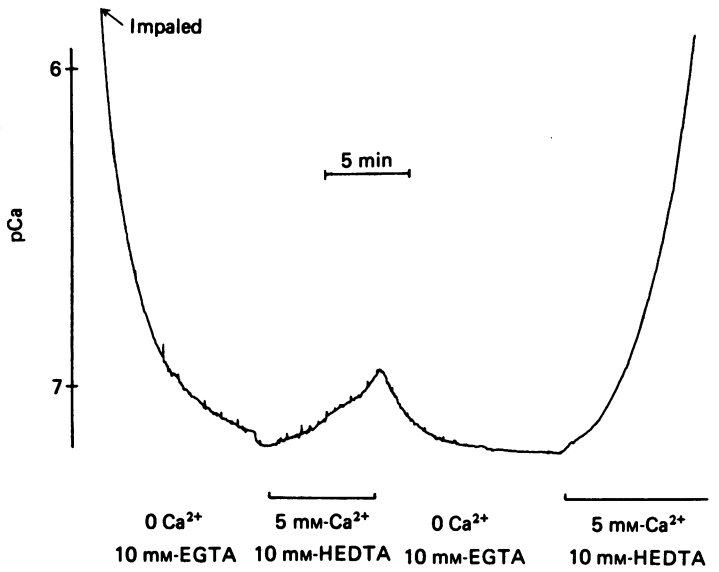

Fig. 3. Chart recordings showing the effects of perfusion with a pCa 5·7 internal solution at different times after breaking the membrane patch. Exposure to this solution 12 min after the beginning of the experiment caused only a small increase in $[Ca^{2+}]_i$, whereas 11 min later a large overshooting response was seen in the same cell with the same internal solution. The internal solutions contained Cs^+ aspartate.

inward holding current decreased spontaneously as $[Ca^{2+}]_i$ climbed to higher levels (see Fig. 2*B*). We do not know the source of this apparent inactivation, although it might be the activation of an outward current by higher levels of $[Ca^{2+}]_i$. Fig. 2*B* shows that 1 mM-Cd^{2+} in the external solution shifted the holding current in the outward direction as though the inward current was blocked. At the same time, Cd^{2+} caused $[Ca^{2+}]_i$ to stop increasing, or, if it was above the value of the perfusate, to decrease rapidly toward this value. Both effects of Cd^{2+} were readily reversible.

Clearly, Ca^{2+} ions enter the cell under these conditions more rapidly than the perfusion and intrinsic regulating mechanisms of the cell can remove them. The cell's intrinsic Ca^{2+} buffering capability appears to decrease substantially during prolonged exposure to internal perfusion. Exposure to high-Ca^{2+} internal solutions often yielded a larger and more rapid increase in $[Ca^{2+}]_i$ at longer times after aspiration of the cell onto the suction pipette and rupture of the patch membrane. This effect is often quite dramatic, as illustrated in Fig. 3. Exposure for 6 min to a pCa 5·7 buffered internal solution 12 min after rupture of the patch membrane caused only a small increase in $[Ca^{2+}]_i$ in this cell. Eleven minutes later, the same internal solution caused a rapid,

large increase in $[Ca^{2+}]_i$ which after 6 min had reached 10^{-6} M and was still increasing at its maximum rate.

The ability of the cell to regulate $[Ca^{2+}]_i$ during perfusion with high-Ca^{2+} internal solutions can be markedly increased by addition of ATP to the internal solution. Fig. 4 shows two examples of this effect. In Fig. 4*A*, 5 mM-ATP was added after dialysis with pCa 5·7 Ca^{2+} buffer had caused $[Ca^{2+}]_i$ to reach pCa 6·0. The climb of

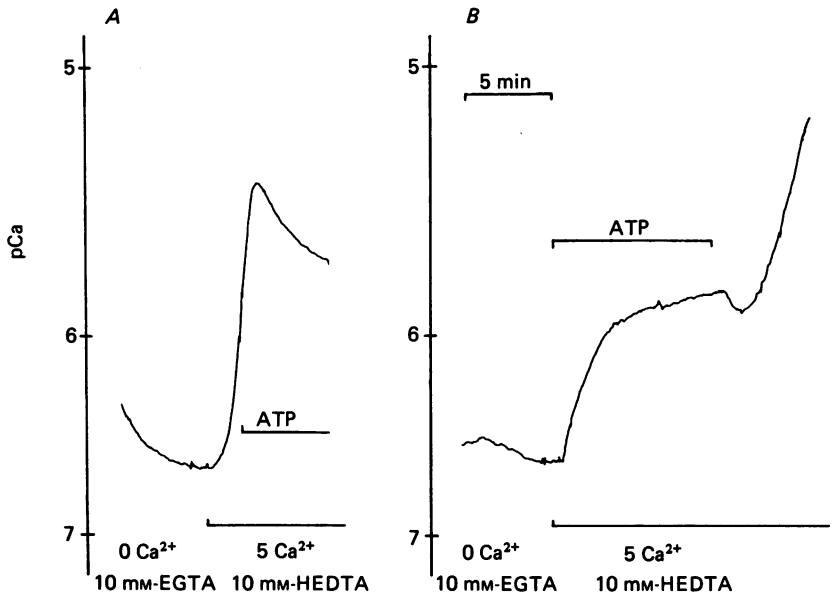

Fig. 4. Effect of intracellular ATP (5 mM) on $[Ca^{2+}]_i$ transients produced by perfusion with pCa 5·7 buffer. *A*, after $[Ca^{2+}]_i$ had increased above the perfusate value, ATP was introduced into the perfusate, causing a rapid decrease of $[Ca^{2+}]_i$ toward pCa 6. *B*, perfusion with pCa 5·7 internal solution was carried out in the presence of intracellular ATP. $[Ca^{2+}]_i$ increased smoothly up to pCa 6 with no overshoot. Removal of ATP caused a rapid increase in $[Ca^{2+}]_i$ to values higher than the perfusate. The transient decrease in $[Ca^{2+}]_i$ upon removal of ATP is caused by the small amount of Ca^{2+} present in the ATP solution. The internal solutions contained Cs^+ aspartate.

$[Ca^{2+}]_i$ almost immediately began to slow; $[Ca^{2+}]_i$ reached a maximum value of pCa 5·4 and then began falling toward pCa 5·7. In Fig. 4*B*, perfusion with the same internal solution in the presence of ATP caused a slow increase in $[Ca^{2+}]_i$ toward the perfusate value. Removal of ATP in the presence of high-Ca^{2+} buffer caused an immediate rapid increase in $[Ca^{2+}]_i$ to values above that of the perfusate. (The ATP contains a small amount of Ca^{2+}, which explains the transient decrease in $[Ca^{2+}]_i$ upon ATP removal.) ATP does not act by blocking the Ca^{2+}-dependent inward holding current, since it reduces this current much less than does Cd^{2+} while affecting the $[Ca^{2+}]_i$ transient similarly.

We thought that an effect similar to that of ATP might be achieved by returning Na^+ to the extracellular solution, thus allowing Na^+–Ca^{2+} exchange to operate (Blaustein, 1974). However, this merely slowed the increase in $[Ca^{2+}]_i$ without preventing its overshoot (see Fig. 2*A*).

Thus, in internally perfused neurones, intracellular Ca^{2+} regulating systems are substantially disrupted, probably due to the removal of intracellular metabolites such as ATP. The effect of various internal solutions on $[Ca^{2+}]_i$ may therefore depend critically on metabolites and energy sources added to the internal solutions, the time during which the cell has been exposed to internal perfusion, and the metabolic state of the cell, as well as the efficiency of diffusional contact between the suction pipette and the cytoplasm. These effects will be difficult to predict in a given preparation and only by direct monitoring can $[Ca^{2+}]_i$ be known.

Effect of internal Ca^{2+} on the Ca^{2+} current

The variable effects on $[Ca^{2+}]_i$ of perfusion with Ca^{2+}-buffered internal solutions probably explains the wide range of estimates of the relationship between $[Ca^{2+}]_i$ and the Ca^{2+} current. We reinvestigated this question by directly monitoring cytoplasmic Ca^{2+} activity during internal perfusion while simultaneously measuring I_{Ca} under voltage clamp. These experiments were hampered by two technical difficulties. First, I_{Ca} disappears, or 'washes out' during prolonged internal perfusion (Byerly & Hagiwara, 1982; Fenwick, Marty & Neher, 1982; Doroshenko, Kostyuk & Martynyuk, 1982). Under our conditions of perfusion, 90 % reduction of I_{Ca} occurred in 25–40 min after rupture of the membrane patch (see Figs. 5 and 6*A*). The time required to increase and decrease $[Ca^{2+}]_i$ is a significant fraction of this survival time. This means that any block caused by internal Ca^{2+} is seen against a background of continuously decreasing I_{Ca}. Secondly, as we have shown above, little control is exerted over $[Ca^{2+}]_i$ using internal perfusion. It is virtually impossible to hold $[Ca^{2+}]_i$ constant anywhere between pCa 6·5 and 4·5. In addition, the rapidity with which $[Ca^{2+}]_i$ changes indicates that large spatial gradients probably exist within the cytoplasm. The addition of ATP to the internal solution might allow us to obtain steady values between pCa 6·5 and 4·5, but this creates additional problems. The time course of the change in $[Ca^{2+}]_i$ in the presence of ATP is often even slower than shown in Fig. 4*B*, so that the normal decrease of I_{Ca} obscures the effect of high Ca^{2+}. Furthermore, the presence of ATP might provide an indirect pathway for Ca^{2+} action on the Ca^{2+} channel via Ca^{2+}-dependent biochemical reactions requiring ATP.

Because of indications that prolonged perfusion eliminated some of the cell's Ca^{2+} buffering systems, we were first concerned that the normal 'wash-out' of the I_{Ca} was caused by a gradual increase in $[Ca^{2+}]_i$ during perfusion with low-Ca^{2+} buffers. Fig. 5 shows that this is not the case. In this experiment $[Ca^{2+}]_i$ and I_{Ca} were recorded in the same cell for 30 min after rupture of the membrane patch. No increase in $[Ca^{2+}]_i$ occurred during this time, while I_{Ca} gradually disappeared.

To estimate the relationship between $[Ca^{2+}]_i$ and I_{Ca}, a transient increase in $[Ca^{2+}]_i$ similar to that shown in Fig. 2*A* was brought about by perfusion with pCa 5·7 (10 mM-HEDTA/5 mM-Ca^{2+}) solution. Intracellular Ca^{2+} activity was continuously recorded with the Ca^{2+}-sensitive micro-electrode and I_{Ca} at +20 mV was measured once each minute. The range of normal wash-out times for I_{Ca} in the absence of increased $[Ca^{2+}]_i$ is shown in Fig. 6*A*. Fig. 6*B* shows the effect of a brief exposure to pCa 5·7 internal solution. Within a few minutes, I_{Ca} began to decrease abruptly, reaching 10 % of its initial value in about 8 min. Upon wash-out of the high-Ca^{2+} internal solution, I_{Ca} increased to 50 % of its original value. In Fig. 6*B* the envelope

of I_{Ca} values in low-Ca^{2+} internal solution, before and after exposure to high Ca^{2+}, falls within the range of normal wash-out kinetics shown in Fig. 6*A*. In such instances we considered the high-Ca^{2+} block to be fully reversible; such brief exposures of the cell to high Ca^{2+} do not appear to accelerate the wash-out process. However, invariably in cells in which I_{Ca} was reduced to zero in high-Ca^{2+} perfusates the block of I_{Ca} was not reversible, even though the measured $[Ca^{2+}]_i$ returned to its original

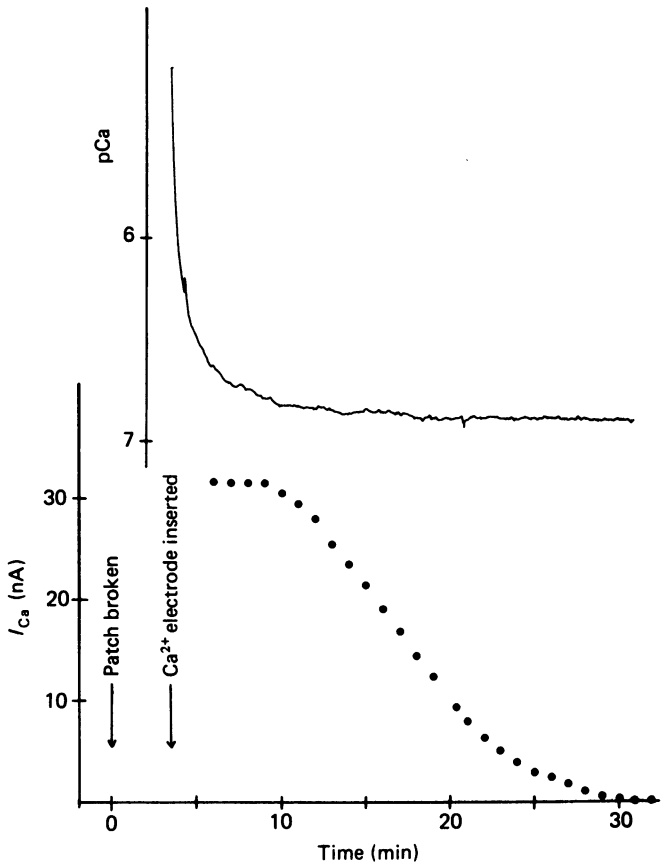

Fig. 5. Experiment showing that the 'wash-out' of I_{Ca} is not caused by a slow increase in $[Ca^{2+}]_i$ during prolonged perfusion. The top panel shows the Ca^{2+} electrode record and the bottom plotted values of I_{Ca} (at +20 mV) from the same cell (see Methods for measurement of I_{Ca}). The same time scale applies to both panels. The internal solution was 10 mM-EGTA/0 Ca^{2+}, Cs^+ aspartate.

value at a time at which we would have expected appreciable Ca^{2+} current to remain in the cell.

A series of experiments like those shown in Fig. 6*B* and *C* were carried out with the Ca^{2+} electrode in place. In most cells, we allowed I_{Ca} to be completely blocked in order to get values for $[Ca^{2+}]_i$ over the full range of the effect. We normally began the high-Ca^{2+} perfusion at 10 min or earlier after patch rupture so that I_{Ca} wash-out would contribute little error to the measurements. In our interpretation of these experiments, we assume that the action of intracellular Ca^{2+} on I_{Ca} is a rapid,

reversible block. However, since the measurements are not made under steady-state conditions, other interpretations are possible (see Discussion).

The results of five such experiments are plotted in Fig. 7. We do not know the source of the scatter of the curves along the horizontal axis, but it probably represents a d.c. error in the Ca^{2+} electrode measurement. The results show that a complete block of I_{Ca} occurs as $[Ca^{2+}]_i$ is raised from near 10^{-7} M to 10^{-6} M. This contrasts with the

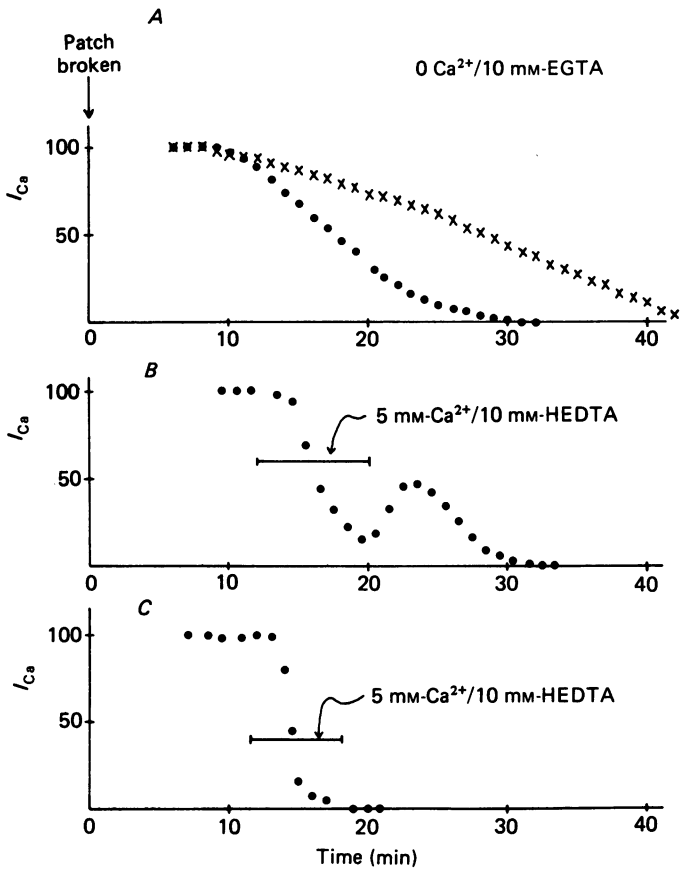

Fig. 6. Effect of perfusion with pCa 5·7 internal solution on I_{Ca}. Normalized I_{Ca} is plotted *vs.* time after the membrane patch under the suction pipette was broken. *A*, I_{Ca} plotted from two cells exposed only to 10 mM-EGTA/0 Ca^{2+} internal solution, illustrating the normal range of I_{Ca} wash-out times. *B*, a cell in which pCa 5·7 buffer was perfused long enough to block I_{Ca} by 85 %. Block reverses to a point expected from normal I_{Ca} wash-out times. *C*, a cell in which pCa 5·7 buffer was perfused long enough to block I_{Ca} completely. No reversal was seen. The internal solutions contained Cs^+ aspartate.

relationship reported, also in *Lymnaea*, by Kostyuk & Krishtal (1977), which showed a complete block of I_{Ca} when $[Ca^{2+}]_i$ reached $5{\cdot}8 \times 10^{-8}$ M. Although we cannot say by how much I_{Ca} would increase in our experiments if $[Ca^{2+}]_i$ were decreased from our initial values (when perfused with 10 mM-EGTA/0 Ca^{2+}), we believe that it is unlikely to be the order of magnitude required to make our results consistent with Kostyuk & Krishtal (1977), considering that our maximum current amplitudes were

similar to theirs. Furthermore, as discussed below, we suspect that the actual $[Ca^{2+}]_i$ values in their experiments differed substantially from the nominal pCa values of their perfusates. The relationship shown in Fig. 7 also differs from that reported by Akaike *et al.* (1978), which showed less than 20% block of I_{Ca} at a $[Ca^{2+}]_i$ of 10^{-6} M.

Low intracellular pH has been reported to block Ca^{2+} currents (Umbach, 1982), and we were concerned that, in spite of the high pH buffer strength used in the internal solutions, the change in $[Ca^{2+}]_i$ might be sufficient to produce a significant

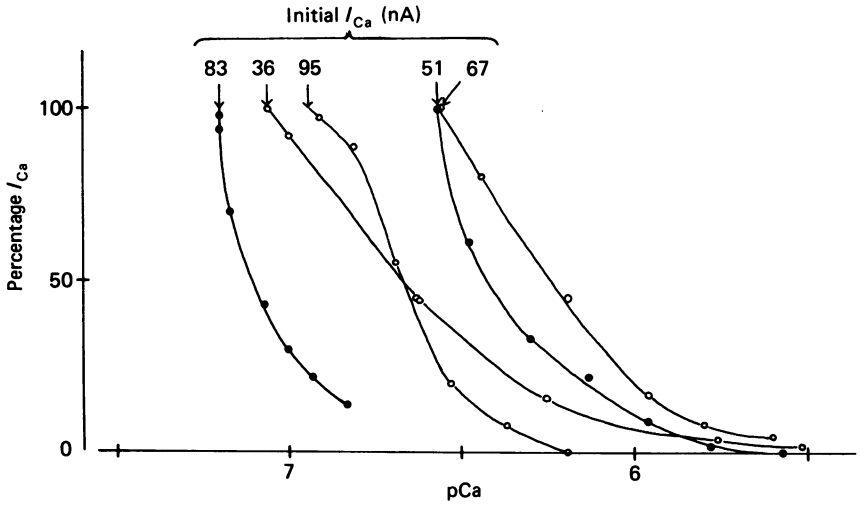

Fig. 7. Plots of normalized I_{Ca} *vs.* $[Ca^{2+}]_i$ from five cells in which I_{Ca} was blocked by perfusion with pCa 5·7 buffer and $[Ca^{2+}]_i$ was recorded simultaneously. Open circles indicate experiments in which the Ca^{2+} electrode was placed near the suction pipette, closed circles those in which the Ca^{2+} electrode was placed at or beyond the mid-point of the cell measured from the suction pipette ('equator'). The internal solutions contained Cs^+ aspartate.

change in internal pH and thus contaminate the I_{Ca} block. However, experiments with pH-sensitive micro-electrodes showed that less than 0·1 unit change in intracellular pH occurred during perfusion with pCa 5·7 internal solutions.

Two changes of relationships are evident in Fig. 7, depending on the location of the Ca^{2+} electrode relative to the perfusion pipette. For penetrations away from the suction pipette (filled circles) a significant block of I_{Ca} was seen before any rise in $[Ca^{2+}]_i$ was recorded. This indicates that there is a substantial gradient of $[Ca^{2+}]$ across the cell, so that Ca^{2+} channels near the suction pipette are blocked before those at the other side of the cell. Penetrations with the Ca^{2+} electrode near the suction pipette (open circles) revealed an increase in $[Ca^{2+}]_i$ as soon as I_{Ca} began to be blocked. Ca^{2+} electrode penetrations away from the suction pipette should give a fairly good estimate of the $[Ca^{2+}]_i$ required for complete block of I_{Ca}, since when I_{Ca} is blocked in all parts of the cell, the lowest $[Ca^{2+}]_i$ recorded must still be sufficient to completely block I_{Ca}. We are therefore confident that our estimate of the concentration of cytoplasmic Ca^{2+} required to completely block I_{Ca} is reasonably accurate. Since penetrations near the suction pipette never showed an increase in $[Ca^{2+}]_i$ without a

reduction of I_{Ca}, it seems safe to conclude that intracellular Ca^{2+} partially blocks I_{Ca} at concentrations as low as 10^{-7} M. The existence of substantial spatial gradients of $[Ca^{2+}]_i$ across the cell during perfusion makes it very difficult to determine the shape of the $[Ca^{2+}]_i$ *vs.* I_{Ca} relation with any accuracy. A better determination could be made if the wash-out of I_{Ca} could be slowed, while retaining rapid perfusion of ions, so that more prolonged and hence spatially uniform increases in $[Ca^{2+}]_i$ could be used. In fact, the wash-out of I_{Ca} is greatly slowed by reducing the temperature below 10 °C, but measurements with the Ca^{2+}-sensitive micro-electrode showed that our ability to change $[Ca^{2+}]_i$ by perfusion had been slowed by roughly an equal factor.

DISCUSSION

Our results show that the cytoplasmic Ca^{2+} activity responds in a complex manner to internal perfusion with simple Ca^{2+}-buffered solutions. The value of $[Ca^{2+}]_i$ is not simply predictable from the Ca^{2+} activity of the perfusate and even for individual neurones, the response to high-Ca^{2+} internal solutions varies with successive exposures. The most common response that we observed in *Lymnaea* neurones was an increase in $[Ca^{2+}]_i$ to values one to two orders of magnitude greater than the Ca^{2+} activity of the internal solution. The membrane channels through which extracellular Ca^{2+} enters the cell in this situation may be similar to the non-specific cation channel reported in cardiac muscle (Colquhoun, Neher, Reuter & Stevens, 1981) and neuroblastoma cells (Yellen, 1982), which is activated by internal Ca^{2+} at levels near 10^{-6} M. Although this channel does not pass Ca^{2+} in neuroblastoma cells, a Ca^{2+}-activated inward current has been reported in *Helix* neurones (Hofmeier & Lux, 1981) which is not carried by Na^+ and may represent a channel similar to the one which we see in *Lymnaea*. The entry of Ca^{2+} becomes regenerative in *Lymnaea* neurones during perfusion with high-Ca^{2+} internal solutions, resulting in a form of Ca^{2+}-induced Ca^{2+} release into the cytoplasm, which, however, can overwhelm the cell's intrinsic Ca^{2+} regulating systems only after a period of internal perfusion (with low-Ca^{2+} solutions) has somehow inactivated them. Addition of ATP to the cytoplasm can at least partially restore these Ca^{2+} buffering systems and prevent $[Ca^{2+}]_i$ from going out of control during perfusion. Since supplying extracellular Na^+ does not have this effect, the Ca^{2+} regulating system involved seems to be more similar to the Ca^{2+}-ATPase reported in squid axon (Beaugé, Dipolo, Osses, Barnola & Campos, 1981) than to a Na^+–Ca^{2+} exchange (Blaustein, 1974). Addition of ATP to the perfusate thus allows $[Ca^{2+}]_i$ to be controlled more closely, but at the expense of lengthening the time needed to raise $[Ca^{2+}]_i$. This time can be critical when studying Ca^{2+} currents, which disappear from the internally dialysed cell rapidly.

Although our experiments show that I_{Ca} is eliminated when $[Ca^{2+}]_i$ is raised to 10^{-6} M, they do not determine the mechanism by which intracellular Ca^{2+} acts. We have discussed the data assuming that Ca^{2+} acts via a rapid, reversible binding directly to the channel. However, Ca^{2+} may act indirectly, possibly inhibiting intracellular reactions that increase the number of functional Ca^{2+} channels or accelerating intracellular reactions that decrease their number. If I_{Ca} wash-out reflects changes in the rates of such intracellular reactions (Byerly & Hagiwara, 1982; Doroshenko *et al.* 1982), the wash-out rate might be sensitive to intracellular Ca^{2+}.

It has been reported that when internal solutions buffered to 8×10^{-6} M-Ca^{2+} are used, the wash-out of Ca^{2+} currents occurs more rapidly than when internal solutions are buffered to lower levels (Fenwick *et al.* 1982). We do not believe that the data presented by these authors (see their fig. 17 *A* and *B*) distinguishes a faster wash-out from a gradual block of I_{Ca} as $[Ca^{2+}]_i$ increases from its value before patch rupture to whatever value results from the 8×10^{-6} M buffer. Our results indicate that moderate increases in $[Ca^{2+}]_i$, sufficient to reduce I_{Ca} by no more than 60%, do not necessarily speed the wash-out process. When $[Ca^{2+}]_i$ is increased further, however, the block of I_{Ca} becomes irreversible, suggesting that Ca^{2+} may act at these levels to accelerate the process which normally results in I_{Ca} wash-out. Since we do not know why Ca^{2+} currents disappear in perfused cells, we cannot say what the relation is between the reversible block of I_{Ca} by intracellular Ca^{2+} and the effect of Ca^{2+} in speeding I_{Ca} wash-out. Until we have this information, we cannot be certain that the mechanism of the Ca^{2+} effect on I_{Ca} over the entire range of the curves in Fig. 7 is identical to that observed in intact cells.

Our estimate of the relationship between $[Ca^{2+}]_i$ and I_{Ca} disagrees with other results obtained using internal perfusion of molluscan neurones (Kostyuk & Krishtal, 1977; Doroshenko & Tsyndrenko, 1978; Akaike *et al.* 1978). We believe that the discrepancy of these results with each other and with our data results from the incorrect assumption that $[Ca^{2+}]_i$ can be made to follow the Ca^{2+} activity of the pipette solution during perfusion. For example, data indicating a complete block of I_{Ca} at a $[Ca^{2+}]_i$ of $5{\cdot}8 \times 10^{-8}$ M (Kostyuk & Krishtal, 1977) was obtained by perfusion with 10 mM-EGTA buffers containing differing amounts of free Ca^{2+}. In our hands such buffers never reduced the measured cytoplasmic Ca^{2+} activity to the perfusate value and seldom brought it below 10^{-7} M. Our results agree best with those of Plant *et al.* (1983) who injected Ca^{2+} into intact *Helix* neurones and measured $[Ca^{2+}]_i$ with Ca^{2+}-sensitive micro-electrodes. Our data are also consistent with the earlier estimates made by Hagiwara & Nakajima (1966) using injected barnacle muscle fibres. The ability of this system to adequately control $[Ca^{2+}]_i$ probably results from the very high buffer strengths used (100 mM-EGTA), the absence of perfusion to deplete other intracellular molecules, and possibly from the substantial Ca^{2+} buffering capabilities of muscle.

It is possible that better control over $[Ca^{2+}]_i$ could be achieved in our experiments by increasing the amount of Ca^{2+} buffer in the perfusate. This, however, would be done at the expense of decreasing pH buffering strength. The combination of these two is likely to result in the appearance of substantial pH_i changes whenever attempts are made to increase $[Ca^{2+}]_i$. We feel that at present the limitations of the internal perfusion technique for studying the effects of intracellular Ca^{2+} activity are severe, and that only in the presence of an independent measure of $[Ca^{2+}]_i$ can results of such experiments be properly interpreted. This situation may change as more is understood about the intracellular molecular environment required for the normal functioning of Ca^{2+} currents and Ca^{2+} buffering systems in these cells.

These experiments were supported by U.S.P.H.S. grants NS 15341 to L. Byerly, HD 17486 to W. J. Moody, and NS 09012 to S. Hagiwara. W.J.M. was also supported by a Muscular Dystrophy Association postdoctoral fellowship.

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