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MODULATION OF MEMBRANE CURRENTS BY CYCLIC AMP IN CLEAVAGE-ARRESTED *DROSOPHILA* NEURONS

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Summary

A number of *Drosophila* learning mutants have defective intracellular second-messenger systems. In an effort to develop techniques that will allow direct measurement of the effects of these mutations on whole-cell neuronal membrane currents, the perforated-patch whole-cell (PPWC) technique has been applied to cleavage-arrested cultured embryonic *Drosophila* neurons. This technique permits the measurement of membrane currents without disturbing the intracellular environment. As a result of the maintenance of the intracellular environment, *Drosophila* neuron currents are found to be much more stable than when measured using the conventional whole-cell (CWC) patch-clamp technique. Ca^{2+} channel currents, which typically 'wash out' within a few minutes of the beginning of CWC recording, are stable for the duration of the seal (tens of minutes) when measured using the PPWC technique. Since the learning mutations *dunce* and *rutabaga* disrupt cyclic AMP signalling, the actions of externally applied dibutyryl cyclic AMP (db-cAMP) and theophylline on Ca^{2+} and K^+ channel currents were studied. db-cAMP and theophylline enhanced the Ba^{2+} current, carried by Ca^{2+} channels, but had no effect on the K^+ current in the cleavage-arrested neurons. However, the large variability and reduction in density of Ba^{2+} and K^+ currents raise questions about the suitability of using these cleavage-arrested cells as models for *Drosophila* neurons.

Key words: cyclic AMP, calcium channels, voltage-activated currents, *Drosophila*, learning mutants, patch-clamp, modulation.

Introduction

The sophistication of the fly nervous system coupled with the exceptional knowledge that we have of the *Drosophila* genome has made this animal the focus of an ambitious attempt to dissect learning and memory genetically. A number of the *Drosophila* learning mutants isolated in this attempt have been found to have defects in their cellular second-messenger systems (Tully, 1987). While these changes in second-messenger levels probably alter these mutant flies in many ways, their effects on neuronal membrane currents are of special interest, since second messengers have been shown to modulate the excitability of neurons in many different animals. However, studying the excitability of *Drosophila* neurons is complicated by their small size and inaccessibility. To date, cultured embryonic neurons have proved to be most amenable to biophysical analysis of membrane currents. The conventional whole-cell (CWC) patch-clamp technique (Hamill *et al.* 1981) rapidly alters the intracellular environment of such small neurons. Enzymes that determine the levels of second messengers may be quickly lost, and the concentrations of second messengers are probably set by the composition of the pipette solution. The Ca^{2+} current rapidly declines (termed rundown) in neurons studied using the CWC technique (Byerly and Leung, 1988), presumably as a result of the 'wash-out' of some intracellular component. The perforated-patch whole-cell (PPWC) clamp technique offers a method of studying neurons with minimal disruption of the intracellular environment, so that membrane currents can be studied with second-messenger systems intact. The PPWC technique uses an antibiotic such as nystatin or amphotericin-B (Horn and Marty, 1988; Rae *et al.* 1991) to permeabilize the patch, instead of breaking the patch as is done in the CWC technique. We found it very difficult to obtain reliable permeabilization of the patch when using the small-opening electrodes required to study the normal cultured embryonic *Drosophila* neurons, which are quite small (less than 6 μm in diameter). Wu *et al.* (1990) and Saito and Wu (1991) have introduced the cleavage-arrested embryonic neuron as a larger model for *Drosophila* neurons. Culturing the *Drosophila* embryonic cells in the presence of the cytoskeletal disrupter cytochalasin-B results in the presence of larger (diameters of 10–20 μm) cells with many properties typical of neurons. We have found that the PPWC technique can be applied with a satisfactory success rate to these 'giant' cleavage-arrested 'neurons'.

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In this paper, we report studies investigating whether PPWC studies of cleavage-arrested neurons are a feasible avenue for studying the effects of learning mutations on the membrane currents of *Drosophila*. Since study of mutant membranes requires comparisons between populations of cells, we have employed population studies to determine whether modulation is large enough to be identified over the variability that occurs between cells of different cultures. In particular, we focused on the effects of cyclic AMP on K^+ and Ca^{2+} channel currents, since cyclic AMP metabolism is known to be altered in the *Drosophila* learning mutants *dunce* and *rutabaga*, and K^+ and Ca^{2+} currents are known to be modulated by intracellular cyclic AMP levels in many better-studied cells (Siegelbaum and Tsien, 1983; Strong, 1984; Armstrong and Eckert, 1987). *dunce* affects the structural gene for cyclic-AMP-dependent phosphodiesterase, producing higher than normal levels of cyclic AMP *in vivo*; and *rutabaga* affects a membrane-bound, Ca^{2+}/calmodulin-sensitive adenyl cyclase, a defect which tends to lower levels of cyclic AMP (Tully, 1987). Although only wild-type cells are studied, this is the first study of the action of cyclic AMP on voltage-clamped neuronal membrane currents in *Drosophila*. The rapid wash-out of Ca^{2+} channel currents seen under CWC patch-clamp (Byerly and Leung, 1988) is eliminated using the PPWC technique. Extracellular application of dibutyryl cyclic AMP (and theophylline) enhanced the Ba^{2+} currents, but did not affect the K^+ currents, of these cells. The isolation of the Ba^{2+} current was incomplete owing to the activation of a background outward current, which was reduced by cyclic AMP.

However, our study raises questions about the suitability of the cleavage-arrested cells as models for *Drosophila* neurons. The densities of the voltage-activated currents in the cleavage-arrested cells, especially the density of the Ca^{2+} channel current, were reduced compared with those measured for standard cultured embryonic neurons. Even though the average membrane area was increased about six times in the cleavage-arrested cells, the variability in currents between cells was not reduced. Consequently, studies of the effects of cyclic AMP with individual cells are considerably more convincing than comparisons between populations of treated and untreated cells. Thus, these giant cells will not reduce the labor of identifying alterations in mutant membranes. A preliminary report of this work has been published previously (Alshuaib and Byerly, 1992).

Materials and methods

Preparation of cultures

Eggs were collected over a 1.5 h period from *Drosophila melanogaster* (Oregon R) flies maintained in pint milk bottles at 26 °C. Each culture was prepared from the cells of 1–3 gastrulating embryos. 5 h after the beginning of egg collection, the embryos were placed in a 50 % ethanol/50 % chlorox solution for 2 min to sterilize and dechorionate them. The embryos were then repeatedly washed with a modified Schneider's *Drosophila* medium (DM, Salvaterra *et al.* 1987). Two or three embryos were transferred to a drop of DM on a 35 mm tissue culture dish (Falcon 3001). Each embryo was impaled by a hand-held micropipette (tip diameter about 100 μm); the cells were collected by suction and blown onto the surface of the dish. The cells were further dispersed by repeated passage through the tip of a smaller pipette (tip diameter about 50 μm). The cells adhered to the surface of the dish within minutes of dispersal. The culture dish containing the embryonic cells (in a single drop of DM) was kept in a humid container at room temperature (23 °C). Cells were studied electrophysiologically 43–73 h later (43–49 h later for all cyclic AMP studies) at room temperature. The culture dish was used as the recording chamber with a Sylgard form insert in the dish to confine the extracellular solution to a small volume (0.3 ml). Cells were viewed using Hoffman differential interference contrast optics.

For cleavage-arrested cell cultures, 2 μg ml^{-1} of cytochalasin-B (Sigma, St Louis, MO, USA) was added to the DM. The cytochalasin was dissolved in ethanol (0.6 mg ml^{-1}) before adding it to the DM.

Patch-clamp techniques

The CWC patch-clamp technique (Hamill *et al.* 1981) was used to study the membrane currents of both normal and giant (cleavage-arrested) neurons. Electrodes were pulled from 100 μl VWR micropipettes (VWR, Cerritos, CA, USA), coated with Sylgard resin near the tip, and polished to a bubble number (Corey and Stevens, 1983) of 3.0–4.0 for normal neurons and 5.0–5.8 for giant neurons. When filled with potassium aspartate solution (see below), these electrodes had resistances of 6–12 MΩ and 3–6 MΩ, respectively. The application of the CWC patch-clamp to cultured embryonic *Drosophila* neurons has been described in detail by Byerly and Leung (1988). However, in contrast to the results of that previous report, in the present studies we did not find it necessary to use streptomycin on the electrode tip to obtain good seals when aspartate was the major anion in the electrode.

The PPWC patch-clamp technique (Horn and Marty, 1988; Rae *et al.* 1991) was used to study the membrane currents, and especially the Ca^{2+} channel current, of giant neurons. In PPWC experiments, the antibiotic amphotericin-B (Sigma) was used to permeabilize the patch membrane. Amphotericin-B forms selective channels in the patch membrane that allow monovalent ions to pass but exclude divalent ions and non-electrolytes the size of glucose and larger (Cass *et al.* 1970; Holz and Finkelstein, 1970). Each day, a fresh stock solution was made by adding 5 mg of amphotericin-B to 100 μl of dimethyl sulfoxide (DMSO) in a small glass test tube. Just before use, this 50 mg ml^{-1} stock solution was sonicated for 30 s with a Laboratory Supplies sonicator (Hicksville, NY). 2 μl of this stock solution was added to 0.5 ml of a previously filtered (0.45 μm filter) internal solution (see below), making a final antibiotic concentration of 200 μg ml^{-1}. This solution was then sonicated for 30 s and drawn into a 1 ml syringe for back-filling the patch electrodes. This amphotericin-containing internal solution was used to fill electrodes for only 1 h following sonication. The stock solution, which was kept at

1 °C, was resonicated and used to make new internal solutions throughout the day. The tip of the electrode was filled (500–600 μm) with amphotericin-free solution by dipping and, if necessary, suction. After the electrode had been back-filled with the amphotericin-containing solution, the bubble separating the two solutions was dislodged by tapping. Neither positive nor negative pressure was applied to the electrode, while the electrode tip was manipulated to the cell surface. Seals were usually obtained 1.5–2.0 min after dislodging the bubble, which indicates that the amphotericin had not yet reached the tip of the electrode (where it would interfere with seal formation), but permeabilization often began very soon after seal formation (always within 3 min, if any permeabilization occurred). For reasons we do not understand, in 55 % of the cases (180/328) in which a good seal was obtained with amphotericin-B in the pipette, no or very little permeabilization occurred. Adequate permeabilization only occurred in 45 % of the cases with good seals.

Experiments were performed with an Axopatch 1B patch-clamp amplifier (Axon Instruments, Foster City, CA, USA). Data acquisition and analysis were performed using the pCLAMP software (version 5.05, Axon Instruments) on a 286 personal computer. Current recordings were filtered (four-pole Bessel) at 5 kHz (capacitative currents) or 1 kHz (ionic currents) and digitized at 20 or 200 μs intervals, respectively. Passive currents, determined from negative pulses of one-quarter the amplitude of the test pulse ($-P/4$), were subtracted from all of the ionic currents. Series resistance compensation was not used. The largest errors caused by series resistance probably occurred in the measurement of K^+ currents in giant neurons using the PPWC technique, where the true somal membrane potential could be as much as 30 mV less positive than intended.

Solutions

The external solutions used in these experiments, referred to as 'salines,' were adjusted to pH 7.4 with 10 mmol l^{-1} Hepes as buffer and had osmolarities of about 285 mosmol l^{-1} (measured using a 3WII osmometer, Advanced Instruments, Needham Heights, MA). K^+ currents were measured with *Drosophila* saline, which contained (in mmol l^{-1}) 130 Na^+, 6 K^+, 151 Cl^-, 5 Ca^{2+}, 5 Mg^{2+} and 10 glucose. Ca^{2+} channel currents were measured with Ba^{2+} Tris saline, which contained 136 Tris, 151 Cl^-, 5 Ba^{2+}, 5 Mg^{2+} and 10 glucose. Mg^{2+} Tris saline was the same as Ba^{2+} Tris saline, except the external Ba^{2+} had been replaced by Mg^{2+}, making a total of 10 mmol l^{-1} Mg^{2+}. To block Ca^{2+} channels, 0.1 mmol l^{-1} $CdCl_2$ was added to the Ba^{2+} Tris saline. When using the PPWC technique, 1 mmol l^{-1} db-cAMP (Na^+ salt, Sigma) and 2 mmol l^{-1} theophylline (Sigma) were added to the external solution. External solutions were changed during experiments by pipetting 3 ml of the new solution into the 0.3 ml bath; excess solution was removed by a continuous, vacuum-powered exhaust.

The internal solutions were adjusted to pH 7.3 with 10 mmol l^{-1} Hepes as buffer, contained 1 mmol l^{-1} Mg^{2+}, and had low levels (about 10 nmol l^{-1}) of Ca^{2+} (1 mmol l^{-1} EGTA and 0.1 mmol l^{-1} added $CaCl_2$). The osmolarity of the internal solutions was about 10 % lower than that of the external solutions, to improve seal formation (Hamill *et al.* 1981). K^+ currents were measured with potassium aspartate solution, which contained the following in addition to the above (mmol l^{-1}): 156 K^+, 139 aspartate and 6 Cl^-. Ca^{2+} channel currents were measured with caesium aspartate solution, which contained Cs^+ in place of the K^+ in the potassium aspartate solution. The same basic internal solutions were used for PPWC experiments as for CWC experiments. In CWC experiments, the action of cyclic AMP was tested by adding 2 mmol l^{-1} ATP (Na^+ salt, Sigma), 100 μmol l^{-1} db-cAMP and 2 mmol l^{-1} theophylline to the internal solution.

Statistical analyses and data presentation

Since the experiments presented in this paper were performed in preparation for future studies of the membrane currents of cultured neurons from *Drosophila* mutants, we first used comparisons of independent populations of neurons to investigate the effect of cyclic AMP on membrane currents. Comparison of the membrane currents of a mutant with those of the wild-type usually requires a comparison of the currents of independent populations of cells (mutant *versus* wild-type), since in most cases the effects of the mutation cannot be rapidly turned on or off in one cell. We will only be able to detect the effects of mutations that cause changes in membrane current which are large compared with the inherent variability of currents that occurs between independent populations of cultured neurons. Therefore, we are especially interested in determining the inherent variability of the wild-type cultures in this study and frequently present distributions of population data (see Figs 2, 3, 8, 12).

Throughout the Results, population data are presented as the mean ± S.D., even when the distributions are clearly not normal. Standard errors of the mean are never given. The means of two populations are compared using a two-tailed Student's *t*-test for independent samples. A difference is considered statistically significant if the probability that both samples came from the same distribution is less than 5 % ($P<0.05$). Graphics were generated with SYSTAT (Systat, Inc.) and Freelance (Lotus) software packages.

All membrane potentials given are nominal values that ignore the liquid junction potential that existed between the pipette and bath solutions at the beginning of the experiment. Using a 3 mol l^{-1} KCl reference electrode, the junction potential between an aspartate internal solution and the external solution was −10.5 mV for *Drosophila* saline and −14.5 mV for Ba^{2+} Tris saline (Byerly and Leung, 1988). Thus, the somal membrane potentials were actually 10–15 mV more negative than those given. In addition, there are errors due to uncompensated series resistance of about 5 mV per 100 pA in the PPWC experiments and of smaller values in the CWC experiments.

Results

Giant versus *normal neurons*

Having determined that giant (cytochalasin-B-treated)

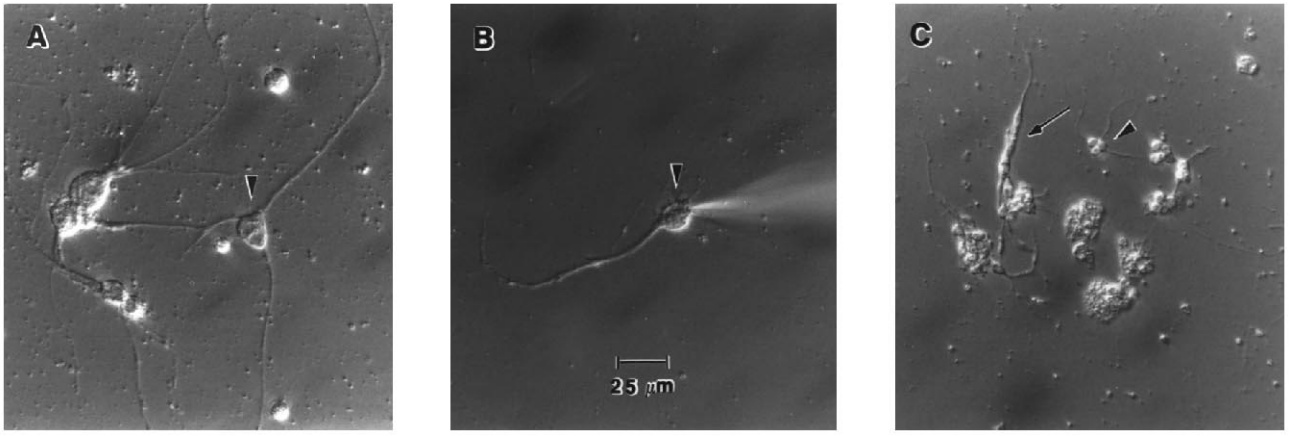

Fig. 1. Photographs of giant and normal cultured embryonic *Drosophila* neurons. All photographs were taken using Hoffman modulation contrast optics at the same magnification (×400). (A,B) Arrowheads indicate typical examples of the giant neurons that were studied. Cells were cultured in a cytochalasin-B-containing medium. (C) Normal neurons from a culture not containing cytochalasin-B. The arrowhead indicates the type of neuron chosen for patch-clamp study. An arrow points to a myotube; such myotubes are not seen in the cytochalasin-B cultures. Scale bar in B (25 μm) applies to all three photographs.

Drosophila neurons offer considerable advantage for the application of the PPWC voltage-clamp technique, we made a quantitative comparison of the capacitative and K^+ currents of giant neurons with those of the normal embryonic cells that have previously been characterized (Byerly and Leung, 1988). Wu *et al.* (1990) and Saito and Wu (1991) have demonstrated that these giant cells are able to differentiate neuron-specific properties including neurites, neuronal antigens and voltage-activated Na^+, Ca^{2+} and K^+ currents. Our purpose was to compare the quality of the voltage-clamp and the K^+ current properties between giant and normal neurons, using the CWC patch-clamp technique. Fig. 1A,B (arrowheads) shows two giant neurons typical of those studied. The average diameters of the cells studied ranged from 10 to 20 μm, and each cell had 1–4 neurites. The normal neurons studied in this comparison had diameters of 4–6 μm and 1–3 neurites (Fig. 1C, arrowhead). In general, neurons with isolated cell bodies (not in contact with other cells) were chosen for study, since they were easier to describe and to approach with the patch electrode. The choice of cells to study may be one of the major sources of inconsistencies between the membrane currents observed by different experimenters in studies of embryonic *Drosophila* neurons.

The area of membrane under effective voltage control was about six times larger for the giant neurons studied than for the normal neurons. The capacitance of neurons was determined from the transient current recorded when the membrane potential was stepped from the holding potential (−60 mV) to −110 mV. Typical examples of these capacitative currents are shown in Fig. 2 for normal and giant cells. For both types of cell, the capacitative current consists of a large-amplitude rapidly decaying component and a small-amplitude slowly decaying component; the first component corresponds to the charging of the soma and proximal parts of neurites and the second component corresponds to the charging of the distal parts of neurites. We calculated both a fast capacitance (C_F) and a total capacitance (C_T) for each cell by integrating the capacitative current for the first millisecond (arrows in Fig. 2) and for the first 8 ms, respectively. Histograms of the fast capacitance measured for the 29 normal neurons and 30 giant neurons studied in this comparison are presented in Fig. 2. The fast capacitance was 1.7±0.6 pF (mean ± S.D.) for the normal neurons and 10.7±4.9 pF for the giant neurons. The ratio C_F/C_T is a measure of the quality of the voltage-clamp. As the amount of poorly clamped membrane (distal neurites) increases, C_F/C_T decreases in magnitude. The quality of the clamp was slightly better for the normal neurons than for the giant neurons; C_F/C_T was 0.78±0.20 for the normal and 0.64±0.14 for the giant neurons (a statistically significant difference, $P<0.01$). The whole-cell resistance of the giant cells (2.5±3.7 GΩ) was about one-fifth that of the normal cells (12.3±11.2 GΩ). This suggests that the specific resistances of the giant and normal neuronal membranes are very similar, given that the relative capacitances indicate that the membrane area of the giant cell is six times that of the normal cell.

The voltage-activated K^+ currents of the giant and normal neurons had a similar voltage-dependence and time course, but the K^+ current density was reduced in the giant cells. Fig. 3 shows typical examples of the K^+ currents recorded from a normal and a giant cell at potentials from −40 to +60 mV. It should be pointed out that the current records of Fig. 3 may also include Na^+ and Ca^{2+} currents, but these currents are generally smaller than K^+ currents and are insignificant at +60 mV, the potential at which we measure the K^+ currents. The magnitude of the K^+ current varied greatly from cell to cell. Although the K^+ currents recorded in the giant cells (453±307 pA at +60 mV) were larger than those of normal neurons (153±121 pA at +60 mV), the K^+ current density (obtained by dividing the current by the fast capacitance) was smaller for the giant neurons (Fig. 3). Since Byerly and Leung (1988) concluded that most of the K^+ current of embryonic *Drosophila* neurons was under good voltage control, it is appropriate to calculate current

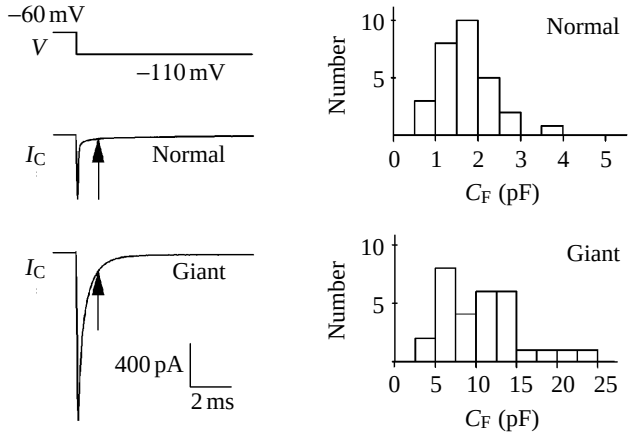

Fig. 2. Comparison of capacitative currents of giant neurons with those of normal neurons. The current traces on the left show the capacitative currents (I_C) flowing in response to a 50 mV hyperpolarizing voltage step (V, upper trace) of a normal neuron (cell no. 1822c02) and a giant neuron (cell no. 1D05c19). The calibration bars apply to both current records. The arrows indicate 1 ms after the hyperpolarizing step; the integral of the current up to this time is used to calculate the fast capacitance (C_F) of each neuron. The histograms on the right show the distributions of fast capacitances measured for 29 normal and 30 giant neurons. Electrode capacitances, determined by the transient currents recorded before the patches were broken, have been subtracted from these measurements. All these studies used the CWC patch-clamp technique.

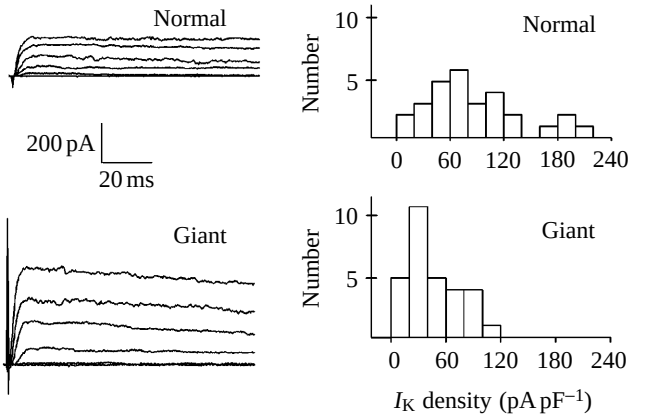

Fig. 3. Comparison of voltage-activated K^+ currents in giant and normal neurons. On the left are currents recorded from a normal neuron (cell no. 1N21c29) and a giant neuron (cell no. 2106c25) during 100 ms pulses to −40 (bottom trace), −20, 0, 20, 40 and 60 mV (top trace). On the right, histograms of the K^+ current (I_K) density at 60 mV are presented for 29 normal and 30 giant neurons. K^+ currents were recorded using potassium aspartate solution in the pipette and *Drosophila* saline in the bath. Passive currents (leakage and capacitative) have been subtracted from these records. Holding potential was −80 mV. All these studies used the CWC patch-clamp technique.

density using the fast capacitance to quantify membrane area. At +60 mV, the K^+ current density was 46±28 pA pF^{-1} for the 30 giant neurons and 85±53 pA pF^{-1} for the 29 normal cells. Errors in membrane potential due to uncompensated series resistance would cause us to underestimate the K^+ currents of the giant cells more than those of the normal cells, but not by enough to explain the observed difference. The voltage-dependence of activation was similar for the K^+ currents of normal and giant neurons; in both types of cell, K^+ current was clearly activated at 0 mV, but only weakly activated at −20 mV. This voltage-dependence of activation is consistent with that reported by Byerly and Leung (1988), recognizing that the membrane potentials have not been corrected for the liquid-junction potentials (about −13 mV) in this report. The time course of activation and inactivation of the K^+ currents appeared to be the same for both giant and normal neurons. The time course of inactivation was quantified by calculating the percentage of the peak current that had inactivated 100 ms from the beginning of a pulse to +60 mV. This value of inactivation was 22±17 % for the normal and 15±14 % for the giant cells. The rare rapidly inactivating K^+ currents reported by Byerly and Leung (1988) were not encountered in the 29 normal cells included in this study. It is interesting that, even though giant cells had six times the membrane area of normal cells, the relative variation in the K^+ current density between cells was as large for the giant cells (S.D./mean = 0.61) as for the normal cells (S.D./mean = 0.62). Thus, giant cells do not provide an advantage in obtaining a statistically significant sample of the membrane currents from a particular *Drosophila* mutant genome.

Perforated-patch versus *conventional whole-cell studies*

The PPWC voltage-clamp of *Drosophila* giant cells was slower than the CWC voltage-clamp, but still allowed comparable control of the membrane potential. The extent of permeabilization of the patch membrane was monitored by the amplitude of the transient current resulting from the negative voltage pulse continuously applied to the electrode; the series resistance, which was primarily the resistance of the permeabilized patch membrane, was estimated by dividing the magnitude of the voltage step by the amplitude of the transient current. Fig. 4 compares the transient capacitative current recorded during a 50 mV hyperpolarizing step of a PPWC clamp with that of a typical CWC clamp. Permeabilization of the patch was considered adequate to allow measurement of whole-cell current when the series resistance of the voltage-clamp dropped below 80 MΩ. Studies of ionic currents were begun when the series resistance reached a constant level. Although in some cases this occurred in less than 3 min after the seal was formed, more typically 5–10 min was required. The series resistance of the patches in 26 PPWC studies was 56±23 MΩ. The ratio of fast to slow capacitance (quality, defined above) was 0.52±0.20 for the 26 PPWC studies compared with 0.64±0.14 for 30 CWC studies. The series resistance of the perforated patch extended the duration of the early capacitative transient beyond 1 ms. Once the resistance of the permeabilized patch reached a steady level, the capacitative currents recorded under the PPWC condition were very stable, showing almost no change for the duration of the

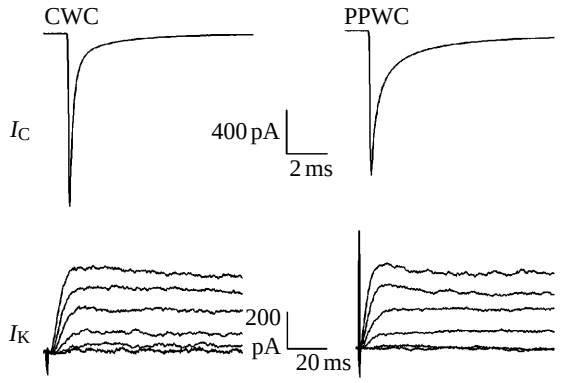

Fig. 4. Comparison of PPWC and CWC clamp currents. On the left are shown the capacitative current (I_C) and K^+ currents (I_K) for a typical giant neuron studied using the CWC technique (cell no. 1D12c21, C_F=10.6 pF, C_F/C_T=0.67, series resistance=31 MΩ). On the right are shown the capacitative current and K^+ currents for a typical giant neuron studied using the PPWC technique (cell no. 2210c09, C_F=12.4 pF, C_F/C_T=0.52, series resistance=39 MΩ). Corresponding records are shown at the same current and time scales for the two techniques (see calibration bars). The capacitative currents were recorded during a 50 mV hyperpolarizing step. The K^+ currents were recorded at −40 (bottom trace), −20, 0, 20, 40 and 60 mV (top trace); holding potential was −80 mV. Passive currents have been subtracted from these K^+ current records and from all current records presented in the following figures.

seal, often 20–30 min. This contrasts markedly to the CWC condition, where both the magnitude and time course of the capacitative transient usually changed in a few minutes. It was also noted that the PPWC technique caused little, if any, change in the appearance of the neurons, while the appearance of neurons studied using the CWC technique often changed dramatically, with the cell appearing to round up and sometimes to slide into the pipette. Both observations support the conclusion that the PPWC technique is less disruptive to the normal intracellular environment than the CWC technique.

The voltage-activated K^+ currents recorded under PPWC conditions appeared to be the same as those recorded with the CWC clamp. Fig. 4 shows typical K^+ currents recorded from giant cells using the two types of voltage-clamp. The time course and voltage-dependence of the K^+ currents recorded in the two types of studies were very similar. The percentage inactivation of the K^+ current that occurred by the end of the 100 ms pulse was 10±10 % for the PPWC studies compared with 15±14 % for the CWC studies. The K^+ current density at +60 mV was 57±28 pA pF^{-1} in 26 PPWC studies and 46±28 pA pF^{-1} in 30 CWC studies. The similarity of the K^+ current under PPWC and CWC conditions suggests two conclusions: (1) that the PPWC technique is fast enough to study, at least qualitatively, the voltage-activated currents in these cells, and (2) that the K^+ currents of these cells are not very sensitive to intracellular second messengers. In CWC clamp studies, intracellular Ca^{2+}, cyclic AMP, ATP, etc. are reduced to very low levels in the regions near the patch electrode, while these messengers and metabolites remain at endogenous levels and the levels are free to change when the PPWC technique is used. The absence of large Ca^{2+}-activated K^+ currents in these cells was also suggested by the results of applying 0.1 mmol l^{-1} Cd^{2+} to the external solution. The outward currents at +60 mV were actually somewhat larger in the presence of Cd^{2+} (increases of 5 % and 45 % in two experiments). Since this level of Cd^{2+} is known to block the Ca^{2+} current (Byerly and Leung, 1988), the outward K^+ current appears to have little dependence on Ca^{2+} influx.

In contrast to the similarity of CWC and PPWC K^+ currents, the inward Ba^{2+} currents measured using the two techniques were markedly different; Ba^{2+} current was much more stable under the PPWC technique. Ba^{2+} currents were measured with a caesium aspartate solution in the patch electrode and an external solution in which Ca^{2+} was replaced with Ba^{2+} and Tris replaced both K^+ and Na^+ (Ba^{2+} Tris saline). These Ba^{2+} currents are thought to flow through voltage-activated Ca^{2+} channels. We chose Ba^{2+}, instead of Ca^{2+}, to carry current through the Ca^{2+} channels because Ba^{2+} currents are larger and Ba^{2+} also blocks K^+ channels. Ba^{2+} currents can only be recorded for a few minutes after the CWC clamp is achieved (Fig. 5A,C). This rapid 'wash-out' of the Ba^{2+} current in giant neurons under CWC conditions is very similar to the wash-out of Ca^{2+} currents reported for CWC studies of normal *Drosophila* neurons (Byerly and Leung, 1988). However, the Ba^{2+} current recorded under PPWC conditions is stable for as long as the seal is maintained, periods often exceeding 20 min (Fig. 5B,C). This stability of the Ba^{2+} current obtained by using the PPWC technique allows us to study modulation of the *Drosophila* Ca^{2+} channels for the first time.

The Ba^{2+} currents recorded in the giant neurons were surprisingly small. Although some cells were found with net inward currents of 40–80 pA (see Figs 6, 7, 9, 10), many giant neurons had no net inward current. Figs 6 and 7 show the currents recorded at various potentials from two selected giant neurons, which had considerable net inward current under the conditions of internal caesium aspartate solution and external Ba^{2+} Tris saline. In all such neurons, the maximum inward current was recorded at approximately 0 mV. The early inward currents seen in Figs 6A and 7A at +40 mV probably result from the slow time course with which the membrane containing the Ca^{2+} channels is depolarized; consequently, the average membrane current measured from 30 to 40 ms is plotted (Figs 6C, 7C) as the best representation of the current for each potential. We did not calculate Ba^{2+} current densities, because there is considerable evidence that many Ca^{2+} channels are not under good voltage control and may primarily reside in the processes of the cells.

Two types of experiments established that the inward current recorded in these experiments is carried by Ba^{2+} through Ca^{2+} channels. When external Ba^{2+} is replaced by Mg^{2+} (an ion that is impermeant to Ca^{2+} channels), the inward current disappears (Fig. 7B,C). The inward current is also eliminated by adding 0.1 mmol l^{-1} Cd^{2+} (a universal Ca^{2+} channel blocker) to the Ba^{2+} Tris saline (Fig. 6B,C). Both replacement of Ba^{2+} with Mg^{2+} and addition of Cd^{2+} shift the current in the outward

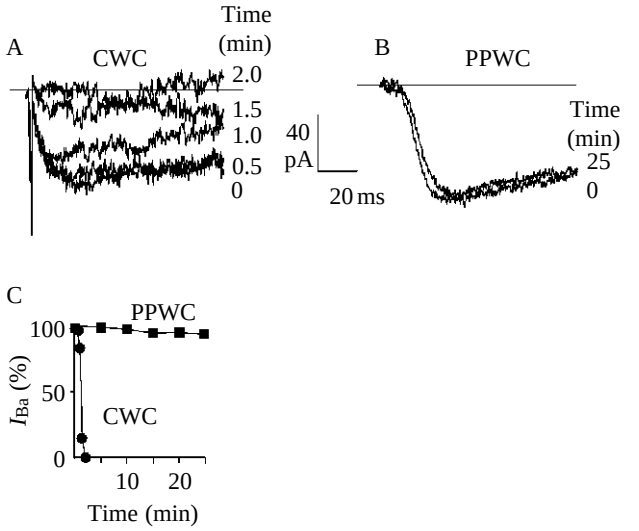

Fig. 5. Comparison of the stability of Ba^{2+} currents recorded by PPWC and CWC techniques. (A) Ba^{2+} currents recorded at 0 mV from a giant neuron using the CWC technique (cell no. 2722c03). The first current (0 min) was recorded immediately after rupture of the patch membrane; four records taken at 30 s intervals following the first record show a rapid decline in the inward current. (B) Ba^{2+} currents recorded at 0 mV from a similar neuron studied using the PPWC technique (cell no. 2422c25). The Ba^{2+} current recorded immediately after patch permeabilization (0 min) was only slightly larger than the current recorded 25 min later. Horizontal lines in A and B indicate the zero current levels. The larger noise seen in the CWC recordings (A) is consistently observed and is partially due to the lower series resistance of the CWC technique. (C) Plots of Ba^{2+} current (I_{Ba}) magnitude (average measured between 30 and 40 ms) at 0 mV against time for the same two cells. Holding potential was −80 mV for all Ba^{2+} current studies.

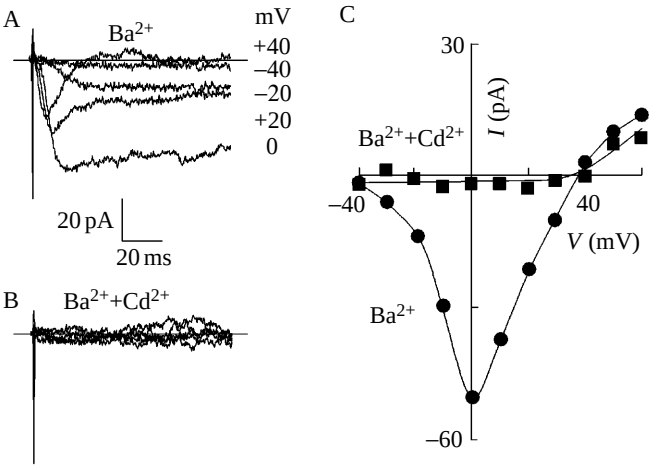

Fig. 6. Current–voltage (*I–V*) relationships for the Ba^{2+} current and the background current, using Cd^{2+} to block the Ba^{2+} current. All currents were recorded from the same cell (cell no. 2911c35) using the PPWC technique and caesium aspartate solution in the pipette. (A) Currents recorded with Ba^{2+} Tris saline (Ba^{2+}) in the bath. Membrane potentials at which the currents were recorded are given at the end of the traces. (B) Currents recorded after 0.1 mmol l^{-1} Cd^{2+} had been added to the Ba^{2+} Tris saline (Ba^{2+}+Cd^{2+}). (C) Average currents (*I*) measured between 30 and 40 ms after the beginning of the pulse are plotted against the potential (*V*) of the pulse; Ba^{2+} (●), Ba^{2+}+Cd^{2+} (■).

direction more at 0 mV than at +60 mV; this is expected for elimination of the Ca^{2+} channel current, because the current through open Ca^{2+} channels at +60 mV is very small compared with the current at 0 mV (Hagiwara and Byerly, 1981; Fenwick *et al.* 1982; Lee and Tsien, 1982).

The prominent outward currents recorded at +60 mV in some cells (Fig. 7) indicate that the Ca^{2+} channel current is contaminated by an outward background current under the conditions of internal caesium aspartate solution and external Ba^{2+} Tris saline. The magnitude of this background current can be determined at +60 mV, where the Ca^{2+} channel current is negligible. The mean value of this background current at +60 mV is about 40 pA and almost never larger than 100 pA (Fig. 8, upper right histogram). The cell shown in Fig. 6 has little background current (14 pA at +60 mV), while that shown in Fig. 7 has a larger background current (53 pA at +60 mV). The voltage-dependence of the background current can be inferred from the currents remaining after elimination of the Ca^{2+} channel currents (Figs 6, 7) and from cells which have very few functional Ca^{2+} channels (indicated by the absence of a negative slope region or net inward current in Ba^{2+} Tris saline). These current–voltage (*I–V*) relationships indicate that the outward background current increases rapidly at positive potentials. Outward current at 0 mV was less than 20 % of that at +60 mV and was seldom larger than 20 pA.

For studies of Ca^{2+} channel function, we have selected giant neurons with prominent net inward currents, usually 40–60 pA at 0 mV. Since we estimate the outward background current at 0 mV to be 5–10 pA (20 % of outward currents measured at +60 mV), the Ca^{2+} channel current in these cells is approximately 10 (range 5–20) times larger than the background current at 0 mV. This conclusion is supported by the observation that the maximum inward current occurs near 0 mV in all these cells.

We are uncertain as to which channels or ions carry the background current. The background current recorded at +60 mV in Ba^{2+} Tris saline is stable over periods greater than 10 min, suggesting that it is not carried by K^+, which should be depleted over this period since there is no K^+ in either the internal or external solutions. This conclusion is also supported by the observation that these background currents are not sensitive to the addition of K^+ channel blockers (10 mmol l^{-1} TEA^+ and 5 mmol l^{-1} 4-aminopyridine) to the Ba^{2+} Tris saline. We suspect that the outward current is a non-specific cation current resulting from a partial loss of selectivity of K^+ channels due to the removal of external Ca^{2+} (Armstrong and Lopez-Barneo, 1987; Armstrong and Miller, 1990). This hypothesis is supported by the observation that replacing external Ba^{2+} by Mg^{2+} increases the outward background current measured at +60 mV by 30–100 % (Fig. 7) and that replacing external Ca^{2+} by Mg^{2+} (without exposure to Ba^{2+}) increases the outward current at +60 mV by 200–3000 % (see Fig. 11).

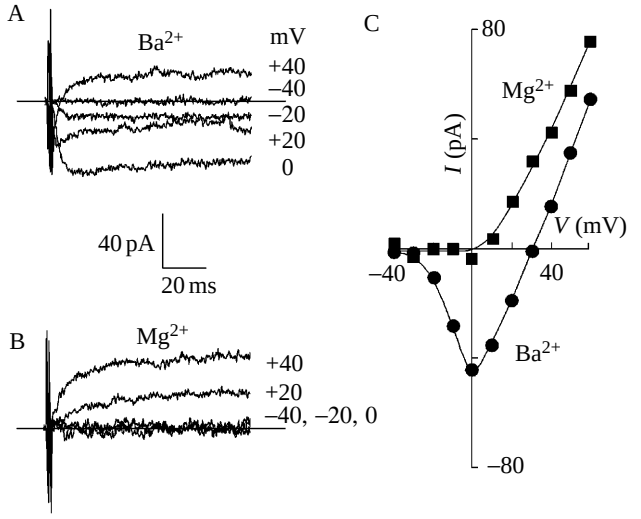

Fig. 7. Current–voltage relationships for the Ba^{2+} current and the background current, using Mg^{2+} substitution to eliminate the Ba^{2+} current. All currents were recorded from the same cell (cell no. 2723c44) using the PPWC technique and caesium aspartate solution in the pipette. (A) Currents recorded with Ba^{2+} Tris saline (Ba^{2+}) in the bath. Membrane potentials at which the currents were recorded are given at the end of the traces. (B) Currents recorded after Ba^{2+} Tris saline had been replaced with Mg^{2+} Tris saline (Mg^{2+}). (C) Average currents measured between 30 and 40 ms after the beginning of the pulse are plotted against the potential of the pulse; Ba^{2+} (●), Mg^{2+} (■).

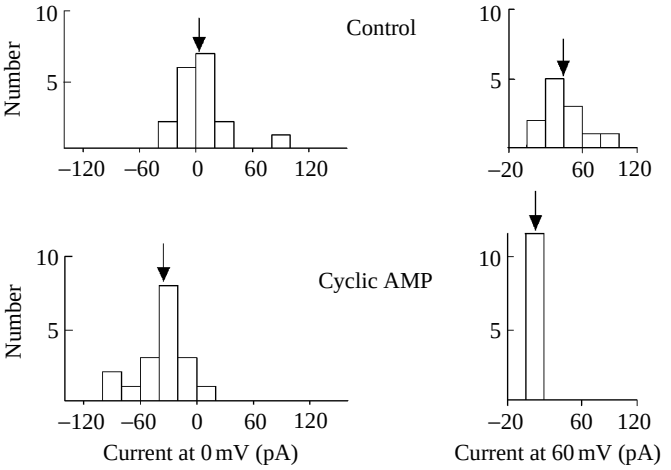

Fig. 8. Effect of db-cAMP and theophylline on Ba^{2+} and background currents shown by comparison of two populations of cells. The net currents at 0 mV (left-hand histograms) and 60 mV (right-hand histograms) were recorded using the PPWC technique with caesium aspartate solution in the pipette. Eighteen cells were studied in standard Ba^{2+} Tris saline (control), and 18 cells were studied in Ba^{2+} Tris saline to which 1 mmol l^{-1} db-cAMP and 2 mmol l^{-1} theophylline had been added (Cyclic AMP); however, currents at 60 mV were measured in only 11 cells of each population. Arrows indicate the means of each histogram, +3 pA (0 mV, control), −36 pA (0 mV, cyclic AMP), +39 pA (60 mV, control) and +11 pA (60 mV, cyclic AMP).

Cyclic AMP enhances Ba^{2+} current

Giant neurons bathed in an external solution that should increase intracellular cyclic AMP levels have, on average, larger Ba^{2+} currents than do cells bathed in the control solution. The net current at 0 mV was measured from 18 neurons under control conditions (internal caesium aspartate solution and external Ba^{2+} Tris saline) and from 18 different neurons in which 2 mmol l^{-1} theophylline and 1 mmol l^{-1} db-cAMP were added to the bath. Theophylline and db-cAMP were added to the bath at least 15 min before the membrane currents were measured. This experiment mimics the situation that might be expected in an attempt to determine whether the *dunce* mutation affects neuronal Ba^{2+} currents. All cells for which a good perforated patch-clamp was obtained were accepted, irrespective of the magnitude and direction of the net current measured at 0 mV. The net current at 0 mV in cells bathed in the control solution was very small, +2.7±26 pA (N=18), while the current measured in cells bathed in the solution containing db-cAMP and theophylline was inward and much larger, −36±28 pA, (N=18; Fig. 8, left-hand histograms). This difference is statistically significant (P<0.001). The outward currents at +60 mV were measured in 11 cells from each of these two populations. The control current at +60 mV was 39±25 pA and that in db-cAMP- and theophylline-treated cells was 11±4 pA (Fig. 8, right-hand histograms); this reduction is also significant (P<0.05). The larger inward shift of current at 0 mV (39 pA) than at +60 mV (28 pA) indicates that db-cAMP and theophylline are enhancing the Ba^{2+} current and not just reducing the outward background current, because the background current is much larger at +60 mV than at 0 mV. Although this comparison of currents from two populations of cells strongly suggests that cyclic AMP enhances Ca^{2+} channel activity, the large variation in Ba^{2+} current magnitude between cells motivated us to attempt to measure the effect of cyclic AMP in single cells.

Addition of db-cAMP and theophylline to the extracellular solution almost immediately increased the Ba^{2+} currents of individual neurons. After determination of the current–voltage relationship for a cell, the current at 0 mV was continuously monitored at 30 s intervals while db-cAMP and theophylline were added to the bath (solution change was complete in less than 1 min). Fig. 9 shows plots of current at 0 mV *versus* time for two such experiments. In each of the six experiments of this type, the current at 0 mV became more inward; the mean Ba^{2+} current was −58.3±37 pA before and −74.8±36 pA after addition of db-cAMP and theophylline. (Only cells which exhibited net inward currents under control conditions were used in these experiments.) Note that the mean increase in inward current is 2–4 times larger than the mean background current at 0 mV. Thus, db-cAMP and theophylline must act on the Ca^{2+} channel current and not only on the background current. This enhancement of the Ba^{2+} current was fast, reaching a steady state within 2 min after the solution change (as can be seen in Fig. 9), but not readily reversible when db-cAMP and theophylline were removed from the bath (results not illustrated). Neither the time

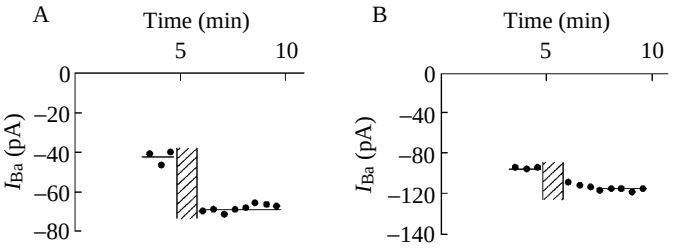

Fig. 9. Effect of db-cAMP and theophylline on Ba^{2+} current demonstrated in single cells. The Ba^{2+} current measured at 0 mV is plotted against time, before and after addition of db-cAMP and theophylline to the Ba^{2+} Tris saline in the bath. Two examples of this experiment are given, cell no. 2730c50 (A), for which the Ba^{2+} current increases from −40 pA to −70 pA, and cell no. 2715c24 (B), for which the current increases from −95 pA to −117 pA. The hatched bars indicate the periods during which the bath solution was changed. Currents were measured every 30 s, except during the solution change. Horizontal lines indicate the mean values for the measurements through which they are drawn.

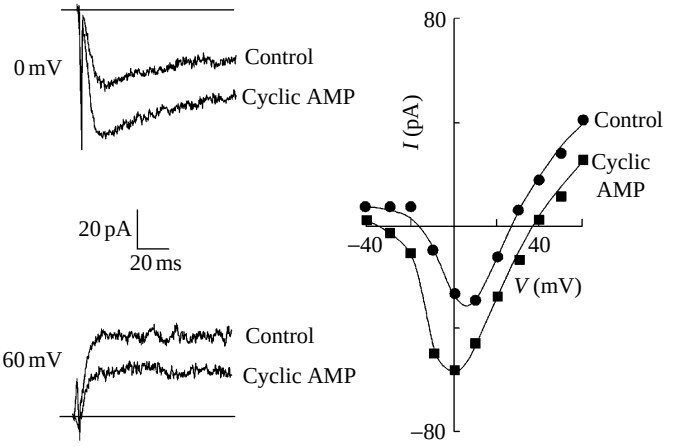

Fig. 10. Current–voltage relationships of Ba^{2+} and background currents before (Control) and after addition of db-cAMP and theophylline. On the left, comparisons of the currents measured before and after addition of db-cAMP and theophylline (Cyclic AMP) are presented for 0 mV and 60 mV. On the right, current before the addition (●) and after the addition (■) are plotted against membrane potential; current is the average of the values measured between 30 and 40 ms after the beginning of the pulse. Results are for cell no. 2730c50, the same cell that was used in Fig. 9A.

course nor the voltage-dependence of the Ba^{2+} current appeared to be greatly changed by the addition of db-cAMP and theophylline (Fig. 10). The slight shift of the *I–V* relationship to the left for the enhanced Ba^{2+} current is expected because of the appreciable series resistance.

The reduction in the net outward current recorded at +60 mV caused by db-cAMP and theophylline application (Figs 8, 10) suggests that this treatment may also affect the background current, since there is very little inward current through Ca^{2+} channels at +60 mV. When the Ba^{2+} current was blocked by Cd^{2+} (as in Fig. 6B), the addition of db-cAMP and theophylline caused no change in the current at any potential (N=5); the current at +60 mV was 10.4±6.0 pA before and 10.0±6.0 pA after addition of db-cAMP and theophylline. This control experiment supports the interpretation that the increase in inward current at 0 mV caused by db-cAMP and theophylline treatment is primarily due to an increase in the Ba^{2+} current; however, it does not rule out the possibility that an outward background current that was suppressed when Cd^{2+} was present in the external saline might be reduced by db-cAMP and theophylline.

The large outward currents activated at positive voltages in individual neurons in Mg^{2+} Tris saline are reduced by db-cAMP and theophylline application. When external Ba^{2+} is replaced with Mg^{2+} (Mg^{2+} Tris saline), not only is the inward Ba^{2+} current at 0 mV lost, but the outward current at more positive potentials becomes larger (Fig. 7). In neurons which have never been exposed to Ba^{2+}, replacing external Ca^{2+} with Mg^{2+} produces even larger outward currents. The outward current in Mg^{2+} Tris saline may be a good model for the outward background current in Ba^{2+} Tris saline, because the outward current in Mg^{2+} Tris saline is much larger than the background current in Ba^{2+} Tris saline and both may result from K^+ channels losing selectivity owing to the absence of external Ca^{2+} (Armstrong and Lopez-Barneo, 1987; Armstrong and Miller, 1990). Fig. 11 shows an example of the currents recorded in Mg^{2+} Tris saline before and after the application of db-cAMP and theophylline. db-cAMP and theophylline application reduced the outward currents at +60 mV by 44±7 % (N=4). The *I–V* relationships of Fig. 11B show that the current that is sensitive to db-cAMP and theophylline is much larger at +60 mV than at 0 mV. These results suggest that db-cAMP and theophylline inhibit the outward background current observed in Ba^{2+} Tris saline, as well as enhancing the Ba^{2+} current. However, the increase of inward current at 0 mV is primarily due to enhancement of Ca^{2+} channel activity.

The K^+ current of giant neurons is not sensitive to cyclic AMP

Since the voltage-activated K^+ currents are stable under CWC voltage-clamp, we first tested the effects of cyclic AMP application by comparing the K^+ currents of 11 neurons studied using pipettes containing the standard potassium aspartate internal solution with the K^+ currents of 11 neurons studied using pipettes containing this standard solution supplemented with 2 mmol l^{-1} ATP, 100 μmol l^{-1} db-cAMP and 2 mmol l^{-1} theophylline. The external solution (*Drosophila* saline) contained Ca^{2+} so that the large outward current (discussed above, Fig. 11) was absent. Of the outward current first measured after patch rupture (achieving the whole-cell configuration) 95 % disappears during the first few seconds of dialysis when the pipette contains caesium aspartate solution with external *Drosophila* saline, thus demonstrating that this outward current is carried almost exclusively by K^+ (see Byerly and Leung, 1988). The Ca^{2+} channel current at +60 mV is very small. The time course and voltage-dependence of the K^+ currents in the two populations of cells were quite similar. The

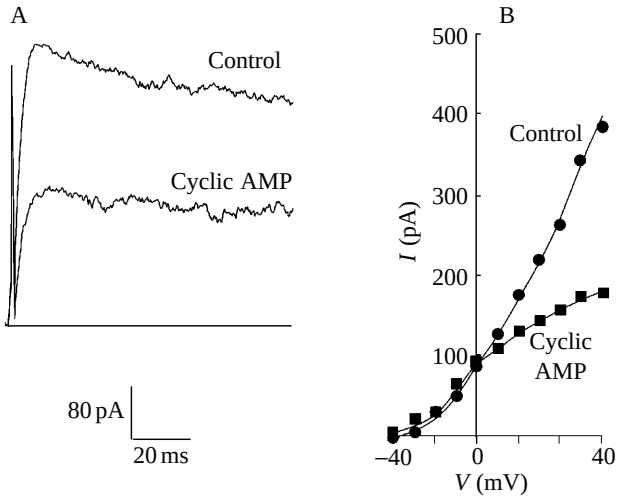

Fig. 11. Large outward currents, which may be related to the background current in Ba^{2+} Tris saline, are reduced by db-cAMP and theophylline application (Cyclic AMP). The neuron was bathed in Mg^{2+} Tris saline (without previous exposure to Ba^{2+} Tris saline); caesium aspartate solution was in the pipette. (A) The current recorded at 60 mV was reduced by about half by addition of db-cAMP and theophylline (Cyclic AMP) to the bath. (B) Currents before the addition (●) and after the addition (■) are plotted against membrane potential; current is the average of values between 30 and 40 ms. Results are for cell no. 2828c14.

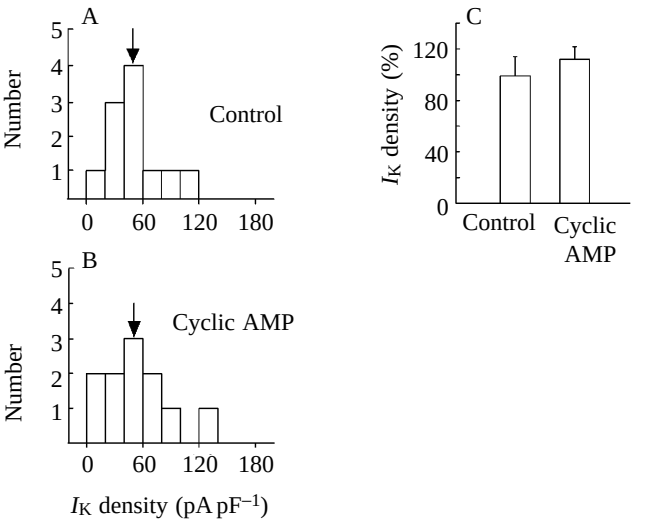

Fig. 12. K^+ currents studied using the CWC technique are not sensitive to the addition of ATP, db-cAMP and theophylline to the patch pipette. K^+ current densities were determined for each cell at 60 mV. The patch electrode solution was potassium aspartate solution and the bath solution was *Drosophila* saline. All studies used the CWC technique on giant neurons. (A,B) Histograms of the K^+ current densities measured within 1 min of breaking the patch membrane for 11 control neurons (A) and for 11 neurons studied with 2 mmol l^{-1} ATP, 0.1 mmol l^{-1} db-cAMP and 2 mmol l^{-1} theophylline added to the potassium aspartate solution in the pipette (Cyclic AMP, B). Arrows indicate the means: 48.5 pA pF^{-1} for control and 52.1 pA pF^{-1} for cyclic AMP. (C) The percentage of K^+ current density remaining 5 min after the beginning of the CWC clamp (compared with initial measurement). Five cells were studied for each internal solution. Error bars indicate the standard deviations.

densities of the K^+ currents at +60 mV in the two populations of cells were not significantly different, 48.5±26 pA pF^{-1} for the control cells and 52.1±37 pA pF^{-1} for the supplemented cells (Fig. 12A,B). The K^+ currents used to calculate the current densities shown in Fig. 12 were measured within the first minute of breaking the patch membrane. Considering that the effects of the supplements on the internal solution might require more time to become detectable, we remeasured the K^+ currents 5 min later in five cells of each population. The percentage of K^+ current density remaining after 5 min was 99±34 % for the control cells and 112±22 % for the cells perfused with ATP, db-cAMP and theophylline (Fig. 12B). The difference between these two current densities is not significant.

The K^+ current of individual giant neurons studied using the PPWC technique was not affected by the addition of db-cAMP and theophylline to the external solution. In experiments similar to those reported earlier to demonstrate the action of db-cAMP and theophylline on Ba^{2+} currents (Figs 9, 10) and the outward current observed in Mg^{2+} Tris saline (Fig. 11), 1 mmol l^{-1} db-cAMP and 2 mmol l^{-1} theophylline were added to the external bath. Fig. 13 shows that the addition of db-cAMP and theophylline did not affect the K^+ currents measured for one typical neuron. The change in the amplitude of the K^+ current measured at +60 mV following addition of db-cAMP and theophylline was 1.5±1.7 % for the five experiments of this type. K^+ currents were measured every 30 s for 10 min following the solution change. The addition of db-cAMP also caused no change in the holding current or resting resistance of the neurons, suggesting that the cyclic-AMP-activated K^+ channel, which was identified in *Drosophila* muscle (Delgado *et al.* 1991), is not abundant in these neurons.

Discussion

Application of db-cAMP and theophylline to cleavage-arrested embryonic *Drosophila* neurons causes an increase in the magnitude of the voltage-activated inward Ba^{2+} current, indicating an increase in the activity of Ca^{2+} channels. This result is obtained both by comparing the net currents at 0 mV of populations of neurons in solutions that best expose the Ba^{2+} current (Fig. 8) and by monitoring the Ba^{2+} current of individual neurons (Fig. 9). The mean increase in net inward current at 0 mV is approximately 40 pA for the comparison of cell populations and about 20 pA for individual cells. We conclude that db-cAMP and theophylline act on the Ca^{2+} channels and not exclusively on an overlapping background current, because the inward shift of current at 0 mV is larger than the background current at 0 mV and is also larger than the current shift at +60 mV (thus following the voltage-dependence of the Ba^{2+} current and not that of the background current). This action of db-cAMP and theophylline on Ca^{2+} channels is specific, because db-cAMP and theophylline do not

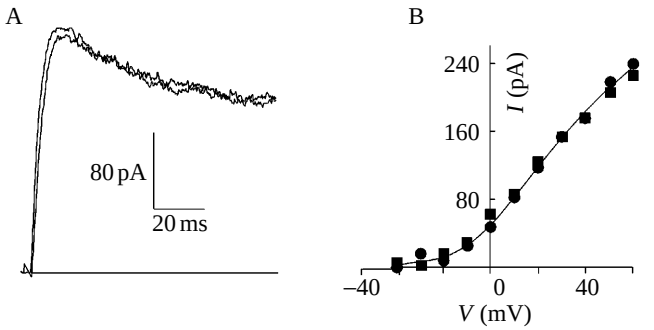

Fig. 13. Addition of db-cAMP and theophylline to the bath solution does not affect the K^+ currents measured in individual neurons using the PPWC technique. The use of the PPWC technique guaranteed that no enzyme critical for the action of cyclic AMP was washed out of the cell. The pipette contained potassium aspartate solution, and the bath solution was *Drosophila* saline. (A) K^+ currents measured at 60 mV, before and after the addition of 1 mmol l^{-1} db-cAMP and 2 mmol l^{-1} theophylline to the bath. (B) Currents measured (30–40 ms after the beginning of the pulse) before (●) and after (■) addition of db-cAMP and theophylline are plotted against membrane potential. Results are for cell no. 2827c38.

affect the voltage-activated K^+ currents when applied in the same way to individual giant neurons using the PPWC technique (Fig. 13) or when applied intracellularly under CWC patch-clamp (Fig. 12). However, db-cAMP and theophylline also suppress the outward background current activated at positive voltages (Figs 8, 10). This background current observed when Ba^{2+} replaces Ca^{2+} is very similar to the even larger outward current obtained when Mg^{2+} replaces Ca^{2+} in the extracellular solution and is thought to be a non-specific current carried by K^+ channels which have altered selectivity as a result of the absence of Ca^{2+} (Armstrong and Lopez-Barneo, 1987; Armstrong and Miller, 1990). The sensitivity to db-cAMP and theophylline of the large outward currents recorded in Mg^{2+} Tris saline (without Ba^{2+} exposure) (Fig. 11) supports the conclusion that db-cAMP also suppresses the outward current that contaminates the recorded Ba^{2+} current. It is not clear whether this second action of db-cAMP would have any role under physiological conditions.

The mechanism of action of db-cAMP and theophylline was not determined in this study. Although we undertook these experiments with the assumption that the application of extracellular db-cAMP and theophylline would increase intracellular cyclic AMP levels, we do not have independent evidence that intracellular cyclic AMP levels are increased or that such an increase is involved in the observed actions on membrane currents. The lack of reversal of the enhancement of Ba^{2+} current when db-cAMP and theophylline are removed from the bath supports the assumption that these compounds are acting intracellularly. However, the action of these compounds might be mediated by some mechanism not involving intracellular cyclic AMP. Even if the actions on membrane currents are mediated by increases in intracellular cyclic AMP levels, we have no evidence to distinguish between direct binding of cyclic AMP or involvement of phosphorylation. The necessity of using the PPWC technique to observe stable Ca^{2+} channel currents makes it difficult to examine the mechanism involved directly. The application of extracellular membrane-permeant kinase blockers would be a useful test for the involvement of phosphorylation in the changes of membrane currents observed in this study.

The action of extracellularly applied db-cAMP and theophylline in increasing the Ca^{2+} channel current is consistent with the results of several previous studies of the effects of cyclic AMP on the excitability of *Drosophila* neurons and muscle cells. Byerly and Leung (1988) found that the addition of ATP, db-cAMP and theophylline to the intracellular solution slowed the wash-out of Ca^{2+} currents in normal *Drosophila* neurons under CWC clamp. Corfas and Dudai (1990*a*) found that a mechanosensory neuron in the *rutabaga* mutant fatigues less rapidly in response to repeated stimulation than does the same type of neuron in wild-type flies. In *dunce* mutants, the neuron fatigues more rapidly than in wild-type flies. Forskolin, an activator of adenyl cyclase in both wild-type and *rutabaga* flies, increased the rate of fatigue of the mechanosensory response; and the protein kinase inhibitor H7 reduced sensory fatigue (Corfas and Dudai, 1990*b*). These studies suggest that cyclic AMP tends to suppress the excitability of the mechanosensory neuron, possibly through a cyclic-AMP-dependent phosphorylation. Our result could account for the observations of Corfas and Dudai (1990*a*,*b*) if the sensory neurons contain a Ca^{2+}-activated K^+ current, the activation of which causes firing fatigue. With higher cyclic AMP levels, each action potential would bring more Ca^{2+} into the neurons, activating more K^+ channels and hyperpolarizing the cell more quickly. Two studies have reported evidence for Ca^{2+}-activated K^+ currents in *Drosophila* neurons (Byerly and Leung, 1988; Saito and Wu, 1991), although such currents were weak in the neurons of this study. Studies of synaptic transmission reported by Zhong and Wu (1991) indicated that the larval motor neuron releases more transmitter in the presence of higher levels of cyclic AMP. The amplitude of excitatory junctional current (EJC) was enhanced in *dunce* mutants, although spontaneous miniature EJCs were unchanged. Application of db-cAMP to wild-type neuromuscular junctions enhanced the amplitude of the EJC, mimicking the effects of the *dunce* mutation. The increased release of transmitter by larval motor neurons in the presence of higher cyclic AMP levels could result from enhancement of the voltage-activated Ca^{2+} current. Zhong and Wu (1989) report an increase in the amplitude of both the transient K^+ current and the Ca^{2+} current of *dunce* muscle membrane. Since *dunce* larval muscle presumably has an elevated intracellular cyclic AMP level, this increased Ca^{2+} current is consistent with the action of db-cAMP and theophylline reported here. However, our result suggesting that cyclic AMP does not affect the K^+ currents of these *Drosophila* neurons contrasts with the activation of K^+ currents by cyclic AMP in muscle (Zhong and Wu, 1989; Delgado *et al.* 1991). Single-channel recordings show that cyclic AMP activates a K^+ channel in *Drosophila* larval muscle (Delgado *et al.* 1991).

Although cleavage-arrested *Drosophila* neurons have considerable advantages for electrophysiological investigations (including PPWC study), a number of results from this study cause us to question their suitability as a model for *Drosophila* neuronal membrane currents. The densities of Ca^{2+} and K^+ channels are lower than expected. The K^+ channel density of the cleavage-arrested cells is about half that of normal embryonic neurons, and the density of Ca^{2+} channels appears to be even more reduced. The net current measured at 0 mV in 18 randomly selected giant neurons exposed to the standard solutions for measuring Ba^{2+} currents was actually outward, +2.7±26 pA (mean ± S.D., *N*=18) (Fig. 8). In contrast, the mean Ca^{2+} current measured in normal embryonic *Drosophila* cells was −41±33 pA (*N*=266, Byerly and Leung, 1988). Since Ba^{2+} currents are more than twice as large as Ca^{2+} currents in embryonic *Drosophila* neurons, there seem to be a reduced number of functional Ca^{2+} channels in the giant neurons. The cytochalasin-B used in the cleavage-arrested cultures may be affecting channel function or expression. If Ca^{2+} channels are primarily expressed in neurites and these are suppressed by cytochalasin-B, then Ba^{2+} currrents would be reduced. Johnson and Byerly (1993) have found that cytochalasin-B has a rapid inhibiting effect on Ca^{2+} channel function. Although the Ba^{2+} current of the cleavage-arrested cells was studied in solutions free of cytochalasin-B, the Ca^{2+} channels of these cells may be in an abnormal state. Since these giant cells have highly variable currents and the choice of cells to study is somewhat arbitrary, results obtained from such studies may be poorly reproducible, probably accounting for some of the discrepancies between our observations and those of Saito and Wu (1991). These cleavage-arrested cultures do not seem to have any of the muscle cells that are always present in normal cultures (see Fig. 1). A disturbing possibility is that the muscle cells may be incorporated into some of the giant cells that we study.

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