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Temperature dependencies of Ca^{2+} current, Ca^{2+}-activated Cl^- current and Ca^{2+} transients in sensory neurones

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Summary We recorded Ca^{2+} current (I_{Ca}) and Ca^{2+}-activated Cl^- current ($I_{Cl(Ca)}$) in isolated chick dorsal root ganglion neurons. At room temperature, $I_{Cl(Ca)}$ is activated by Ca^{2+} influx (e.g. I_{Ca}) or by caffeine-stimulated release of Ca^{2+} via ryanodine receptors. Warming from room temperature to 37°C increased the amplitude of I_{Ca} as well as the amplitude and rate of deactivation of $I_{Cl(Ca)}$ activated by Ca^{2+} influx. In contrast, the activation of $I_{Cl(Ca)}$ by caffeine-stimulated release of Ca^{2+} from intracellular stores abruptly failed between 19 and 28°C. Warming from 22 to 37°C reduced the amplitude of $[Ca^{2+}]_i$ transients (measured with Indo–1) in chick neurons by more than 50% and reduced $[Ca^{2+}]_i$ transients in mouse neurons by more than 40%. We investigated the role of mitochondria in these phenomena using carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP) to inhibit mitochondrial Ca^{2+} uptake. 1–4 μM FCCP slowed the deactivation of I_{Ca}-activated $I_{Cl(Ca)}$ at 20°C and at 36°C, having a greater effect at the higher temperature. In the presence of FCCP, the rate of deactivation of $I_{Cl(Ca)}$ was relatively insensitive to temperature in this protocol. In contrast, FCCP had little effect on $I_{Cl(Ca)}$ activated by caffeine at warmer temperatures (> 22°C) but prolonged $I_{Cl(Ca)}$ at cooler temperatures (< 22°C). Thus, we find that warming reduces the ability of Ca^{2+} release to raise $[Ca^{2+}]_i$, increases the effect of mitochondria on the deactivation of $I_{Cl(Ca)}$ if $I_{Cl(Ca)}$ is activated by Ca^{2+} influx, and reduces the effect of mitochondria if $I_{Cl(Ca)}$ is activated by caffeine-stimulated Ca^{2+} release.

INTRODUCTION

Fluorescence measurements of intracellular Ca^{2+} ($[Ca^{2+}]_i$) in neuronal cell bodies reveal that opening voltage-gated Ca^{2+} channels in the plasma membrane or caffeine-activated channels in the endoplasmic reticulum raises $[Ca^{2+}]_i$. When spatially averaged $[Ca^{2+}]_i$ is measured, the amplitudes and kinetics of the $[Ca^{2+}]_i$ transients mediated by either set of channels are similar: $[Ca^{2+}]_i$ increases from near 0.1 μM to a peak between 0.5 and 1 μM before returning to resting levels over several seconds [1–5]. When $[Ca^{2+}]_i$ near the plasma membrane is considered, the amplitude and time course of $[Ca^{2+}]_i$ is thought to depend on which set of channels is activated. Following depolarization, Ca^{2+} entering via the Ca^{2+} channels accumulates near the plasma membrane, raising $[Ca^{2+}]_i$ there into the micromolar range [6,7], before the Ca^{2+} diffuses into the bulk cytoplasm. In contrast, following application of caffeine, $[Ca^{2+}]_i$ was reported to rise earliest in the center of the neuron [4] or at the part of the cell where the caffeine first arrived [5] and to spread to the rest of the cell.

The details of the $[Ca^{2+}]_i$ responses following these stimuli are studied because of the importance of Ca^{2+} as a regulator of cellular processes, including the specific

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regulation of ion channels, exocytosis, and possible triggering of Ca^{2+}-induced Ca^{2+} release (CICR) by $[Ca^{2+}]_i$ near the plasma membrane. Although some studies have been done at warmer temperatures [8–11], most of the information about the control of $[Ca^{2+}]_i$ has come from experiments done at room temperature. This raises the question of whether information obtained at room temperature can be used to understand how $[Ca^{2+}]_i$ is controlled at physiological temperature. If a single mechanism determines $[Ca^{2+}]_i$, and one has a value for the Q_{10} of that process, one can extrapolate room temperature data to estimate function at physiological temperature. However, extrapolation breaks down if multiple mechanisms are at work and one does not know the quantitative temperature-dependencies of the individual mechanisms contributing to the regulation of $[Ca^{2+}]_i$. For example, one might consider how increasing temperature will effect the ability of I_{Ca} to raise $[Ca^{2+}]_i$. Warming will increase I_{Ca} and Ca^{2+} influx [12] and this will tend to increase the amplitude of Ca^{2+} transients. However, studies in a variety of cell types find that the rates of sequestration and extrusion of Ca^{2+} will be increased [13–15] and there may be changes in cytoplasmic Ca^{2+} buffering. These mechanisms will reduce the ability of the larger I_{Ca} to raise $[Ca^{2+}]_i$. Thus, when multiple mechanisms are acting in opposite directions on $[Ca^{2+}]_i$, it is difficult to predict the answer to even this relatively simple problem.

Given that an understanding of the control of $[Ca^{2+}]_i$ under physiological conditions is important and that such understanding is difficult to obtain by extrapolation of results obtained at room temperature, there is need for direct experimental observation. In the present work, we have investigated the temperature-dependencies of three mechanisms that influence $[Ca^{2+}]_i$ in sensory neurons: the N-type Ca^{2+} current (I_{Ca}), the ability of the endoplasmic reticulum to raise $[Ca^{2+}]_i$, and the ability of the mitochondria to sequester Ca^{2+}. We find that the ability of the intracellular stores to raise $[Ca^{2+}]_i$ near the plasma membrane and spatially averaged $[Ca^{2+}]_i$ is markedly reduced as temperature is increased. In addition, we found that the temperature-dependence of the deactivation of $I_{Cl(Ca)}$ by mitochondrial Ca^{2+} uptake depends on the source of activating Ca^{2+}. Although the mechanisms of these effects are unclear, our observations point out the limitations of studies of Ca^{2+} handling that are done under unphysiological conditions. Some of these results have been reported in an abstract [16].

MATERIALS AND METHODS

Preparation of cultured neurons

Spinal ganglia were collected from E10 chick embryos and neonatal (1 or 2 day old) mice. Embryonic chicks were removed from eggs and decapitated. Neonatal mice were decapitated. We dissociated the neurons by incubating them in collagenase solutions followed by mechanical agitation (for details see [17]). Dissociated neurons were plated onto collagen coated tissue culture dishes (Corning Falcon 3001, Becton Dickinson, NJ, USA) for voltage-clamp experiments, or onto poly-ornithine and laminin coated glass coverslips (Biophysica Technologies, Baltimore, MD, USA) for fluorescence measurements. Cultures were maintained in Earle's minimum essential medium (MEM, Gibco, Grand Isle, NY, USA: #410-1100EB) augmented with 2 mM L-glutamine (Gibco), 10% heat inactivated fetal bovine serum (Gibco), 100 ng/ml nerve growth factor (Collaborative Research Inc., Bedford, MA, USA), 50 units/ml penicillin and 50 µg/ml streptomycin, and 5% chick embryo extract (Sigma Chemical Co., St Louis, MO, USA). They were used for voltage-clamp experiments on the day of plating or within 3 days for the fluorescence measurements.

Voltage-clamp and fluorescence methods

We used the amphotericin perforated patch-clamp technique to record membrane currents under conditions where the predominant currents are N-type Ca^{2+} currents [18] and $I_{Cl(Ca)}$ [17]. The superfusate used in the voltage-clamp experiments summarized in Figures 1–4 contained (mM): NaCl 117, $CaCl_2$ 2, $MgSO_4$ 0.5, KCl 5.0, KH_2PO_4 0.4, NaH_2PO_4 0.6, $NaHCO_3$ 3, tetraethylammonium chloride (TEA) 20, glucose 5.6, HEPES 20 (pH = 7.4). In the experiments summarized in Figures 7–9, TEA was replaced by Na^+ without effect on the membrane currents. 300 nM tetrodotoxin was added to block Na^+ currents in the voltage-clamp experiments. The pipette solution contained (mM): cesium aspartate 90, CsCl 60, EGTA 0.5, HEPES 5.0 (pH = 7.2) plus 0.24 mg/ml amphotericin. Electrode resistances ranged from 2 to 4 MΩ and the tips of the pipettes were dipped in pipette solution containing 1 mM gentamicin to facilitate seal formation. Linear capacity and leakage currents were subtracted using scaled currents obtained from hyperpolarizing steps in a P/5 protocol. Voltages reported in the text and figures have been corrected for junction potential. In control experiments, we estimated access resistance from the time constant of the decay of the capacity current elicited by small depolarizations and found that the access resistance was < 10 MΩ following successful perforation: 50–70% of this series resistance was compensated electronically. Access resistance declined as temperature increased facilitating the recording of the larger currents at higher temperatures.

For fluorescence measurement of $[Ca^{2+}]_i$, ncurons were loaded with the AM ester of Indo–1 by incubation at

37°C for 60 min in MEM plus 5 μM Indo–1/AM ester (Molecular Probes, Eugene, OR, USA), 0.025% cremophore, and 0.5% DMSO (Sigma). The cells were washed in the solution used for the fluorescence measurements (mM): NaCl 137, $CaCl_2$ 2, $MgSO_4$ 0.5, KCl 5.0, KH_2PO_4 0.4, NaH_2PO_4 0.6, $NaHCO_3$ 3, glucose 5.6, HEPES 20 (pH = 7.4). Indo–1 fluorescence was measured with a microfluorometer (Sycamore, Santa Barbara, CA, USA) attached to a Nikon inverted microscope. Fluorescence at 400 and 500 nm was recorded with a TL-1 interface and pClamp (version 5, Axon Instruments, Foster City, CA, USA). The ratio was calculated off-line using SigmaPlot (Jandel Scientific, San Raphael, CA, USA) or Origin (Microcal, Northampton, MA, USA).

We did not calibrate our Indo–1 ratios in terms of Ca^{2+} activities because of uncertainty in the quantitative effects of temperature on the affinity of Indo–1 for Ca^{2+}, the difficulty in obtaining *in situ* calibrations over temperatures ranging from 18 to 40°C, and our interest in the relative changes in $[Ca^{2+}]_i$ responses. With regard to the first issue, the following K_d values have been obtained using in vitro measurements: 441 nM at 25°C and 395 nM at 35°C [19], ≈330 nM at 22°C and ≈260 nM at 37°C [8], and 191 nM at 22°C and 179 nM at 37°C [20]. Although there is variation in both the absolute values for the affinity of Indo–1 for Ca^{2+} and the magnitude of the effect of temperature, there is agreement that the affinity increases with warming.

We found it essential to take scrupulous care with regard to the control of temperature in the culture dishes. We prechilled the superfusate in a small ice bath located just before the experimental chamber and used a feedback temperature control system (Cell Microsystems, Virginia Beach, VA, USA) to drive a preheater and either a circular heating element under the dish (Cell Microsystems) for the voltage-clamp experiments or a teflon coated heating wire placed in the dish for the fluorescence experiments. The flow rate was kept above 1 ml/min; at this rate, the temperature detected with an independent thermistor was stable within ± 1°C of the temperature detected by the feedback thermistor. The temperature changes described below typically took 2 min to stabilize. Further, in order to avoid heat loss to the microscope objective, we did not use immersion oil.

Carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) was dissolved in ethanol before dilution into the superfusate. In preliminary experiments, we found that 1–4 μM FCCP reduced I_{Ca} and $I_{Cl(Ca)}$ elicited by voltage-clamp depolarization as shown previously in rat sensory neurons [21]. We attempted to reduce this effect by raising the Ca^{2+} in solutions containing FCCP to 4 mM in most experiments. Clear effects of FCCP on the kinetics of deactivation of $I_{Cl(Ca)}$ were apparent within 1 min and exposures to this compound were limited to 5 min.

All test compounds were applied to the cells via a puffer pipette. Over the range 18–37°C, pH was constant in our bath and pipette solutions to within 0.05 pH units. Q_{10} values were determined from linear fits to Arrhenius plots. Results are summarized as the mean and standard error of the mean (SEM).

RESULTS

$I_{Cl(Ca)}$ is activated by influx of Ca^{2+} across the plasma membrane or the release of Ca^{2+} from the endoplasmic reticulum

$I_{Cl(Ca)}$ is a large current (> 0.5 nA) in most E10 chick DRG neurons (see Fig. 2 of Ivanenko et al. [17]). It is activated following the opening of Ca^{2+} channels in the plasma membrane by depolarization or the opening of ryanodine-receptor channels in the endoplasmic reticulum by caffeine [17]. We refer to $I_{Cl(Ca)}$ activated in these protocols as 'I_{Ca}-activated $I_{Cl(Ca)}$' or 'Ca^{2+}-release-activated $I_{Cl(Ca)}$'.

We have shown previously that the voltage-dependencies of the activation of I_{Ca} and $I_{Cl(Ca)}$ are similar [17]. However, this observation does not establish that all of the Ca^{2+} that activates $I_{Cl(Ca)}$ is supplied by influx. Specifically, the entry of Ca^{2+} might be amplified by CICR. In order to examine this possibility, we tested the effect of ryanodine inhibition of CICR on I_{Ca}-activated $I_{Cl(Ca)}$ with results shown in Figure 1. Figure 1A shows membrane currents elicited from a chick DRG neuron at room temperature (about 23°C) by a 20 ms step depolarization. The peak and decline of the net inward current during the depolarization is due to the sequential activation of inward I_{Ca} and outward $I_{Cl(Ca)}$ carried by influxes of Ca^{2+} and Cl^-, respectively. Upon repolarization, I_{Ca} rapidly deactivates and the inward current is dominated by $I_{Cl(Ca)}$ [17] that deactivates over several hundred milliseconds as the activating $[Ca^{2+}]_i$ falls [22]. Figure 1B shows that a large inward $I_{Cl(Ca)}$ was activated during the first exposure of this neuron to 10 mM caffeine plus 10 μM ryanodine. This observation implies that the neuron studied in this experiment had intracellular Ca^{2+} stores, that these stores could be activated to release Ca^{2+}, and that this release could activate $I_{Cl(Ca)}$. Specifically, we conclude that the record in Figure 1A shows currents from a cell with functioning intracellular Ca^{2+} stores. Following the exposure to caffeine plus ryanodine, the cell was depolarized in a series of 20 ms steps to potentials between –75 mV and +25 mV in 10 mV increments with 15 s between each depolarization (not shown). The currents elicited in this protocol allowed us to monitor the stability of the neuron and provided Ca^{2+} influx to refill the intracellular stores. The neuron was then exposed to caffeine plus ryanodine for a second time with the resulting current shown in Figure 1C. As reported

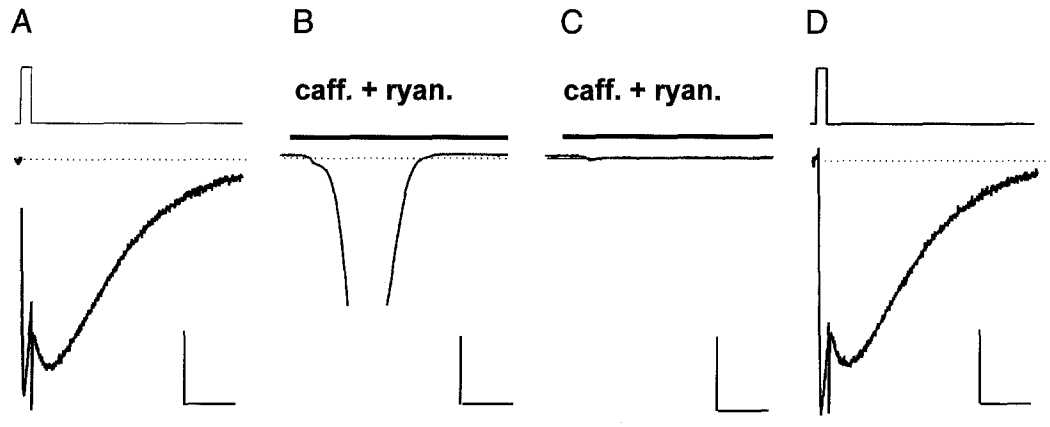

Fig. 1 Membrane currents from a chick DRG neuron in response to depolarization or to application of caffeine plus ryanodine. (**A**) and (**D**) show responses to 20 ms step depolarizations from –85 to –5 mV (upper traces). (**B**) and (**C**) show responses to 10 mM caffeine plus 10 μM ryanodine applied during intervals marked by horizontal lines above the records. Holding potential was –70 mV in (B) and (C). Calibration bars are 0.5 nA, 100 ms for (A) and (D); 1 nA, 2 s for (B) and (C).The current in (B) saturated the A/D converter.

previously, exposure to ryanodine inhibited subsequent responses to caffeine by irreversibly disrupting the function of the ryanodine-receptor Ca^{2+} release channels [17]. Membrane currents subsequently activated by a depolarization are shown in Figure 1D. If Ca^{2+} influx via I_{Ca} were triggering CICR and if this release of Ca^{2+} contributed to the activation of $I_{Cl(Ca)}$, one would expect the amplitude and kinetics of $I_{Cl(Ca)}$ in Figure 1D to differ from those in Figure 1A. However, in this and 4 other chick DRG neurons studied with this protocol, inhibition of Ca^{2+} release caused no significant change in I_{Ca}-activated $I_{Cl(Ca)}$.

Thus, we do not find evidence that ryanodine-sensitive CICR contributes to the activation of $I_{Cl(Ca)}$ at room temperature. Similar observations have been made in chick and rat neurons using Fura–2 to monitor $[Ca^{2+}]_i$ [1,4]. However, amplification of Ca^{2+} influx has been demonstrated in mammalian neurons at warmer temperatures [10,11,23,24] suggesting that the regulation of $[Ca^{2+}]_i$ in neurons is different at physiological temperature compared with room temperature.

I_{Ca} and I_{Ca}-activated $I_{Cl(Ca)}$ are increased at higher temperature

Although the biophysical effects of temperature on Ca^{2+} currents of chick neurons have been documented [12], there is little information about how temperature effects I_{Ca}, $I_{Cl(Ca)}$, and $[Ca^{2+}]_i$ under conditions where $[Ca^{2+}]_i$ is controlled by the neuron and not the experimenter. In order to obtain this information, we used the amphotericin-perforated patch technique to monitor I_{Ca} and $I_{Cl(Ca)}$ over a range of temperatures. The results in Figure 2 are typical of those seen in 6 neurons in which we were able to record currents at least over the range 20–30°C. Figure 2A–C shows membrane currents elicited by 20 ms step depolarizations from –90 mV to -10 mV at 19, 27, and 38°C. The maximum net inward current during the depolarization increased as temperature increased over this range, consistent with an increase in I_{Ca} [12]. We also found that the decline in the net inward current during the depolarization increased at higher temperatures. Although some of this decline in the current can be attributed to the enhanced inactivation of I_{Ca} [12], the observation that the initial amplitude of inward $I_{Cl(Ca)}$ following repolarization is larger at higher temperatures implies that increased $I_{Cl(Ca)}$ also contributes to this effect.

Figure 2D–F shows the current–voltage relationships from this neuron at 19, 27, and 38°C, respectively. The circles are the maximum inward current as a function of step potential. At 19°C the form of this relationship is similar to that expected for the N-type Ca^{2+} current in these neurons [18] supporting the suggestion that $I_{Cl(Ca)}$ is relatively small at times < 10 ms under this condition [17]. However, at warmer temperatures, the maximum inward current shifts to more negative potentials while $I_{Cl(Ca)}$ following repolarization (squares) continues to peak near 0 mV (Fig. 2E,F). The latter observation implies that the maximum Ca^{2+} influx occurs near 0 mV at all temperatures and that the voltage-dependence of I_{Ca} does not change as temperature and inward current are increased. Because the chloride equilibrium potential is –23 mV, $I_{Cl(Ca)}$ will contribute inward current at –30 mV and outward current positive to –10 mV. Thus, the shift in the voltage-dependence of the peak inward current with warming is consistent with an increased $I_{Cl(Ca)}$ consequent to an increased Ca^{2+} influx. Figure 2F also shows that, at higher temperatures, the initial amplitude of $I_{Cl(Ca)}$ following repolarization is relatively insensitive to step potential over the range –20 mV to +20 mV, i.e. potentials where I_{Ca} and Ca^{2+} influx are expected to

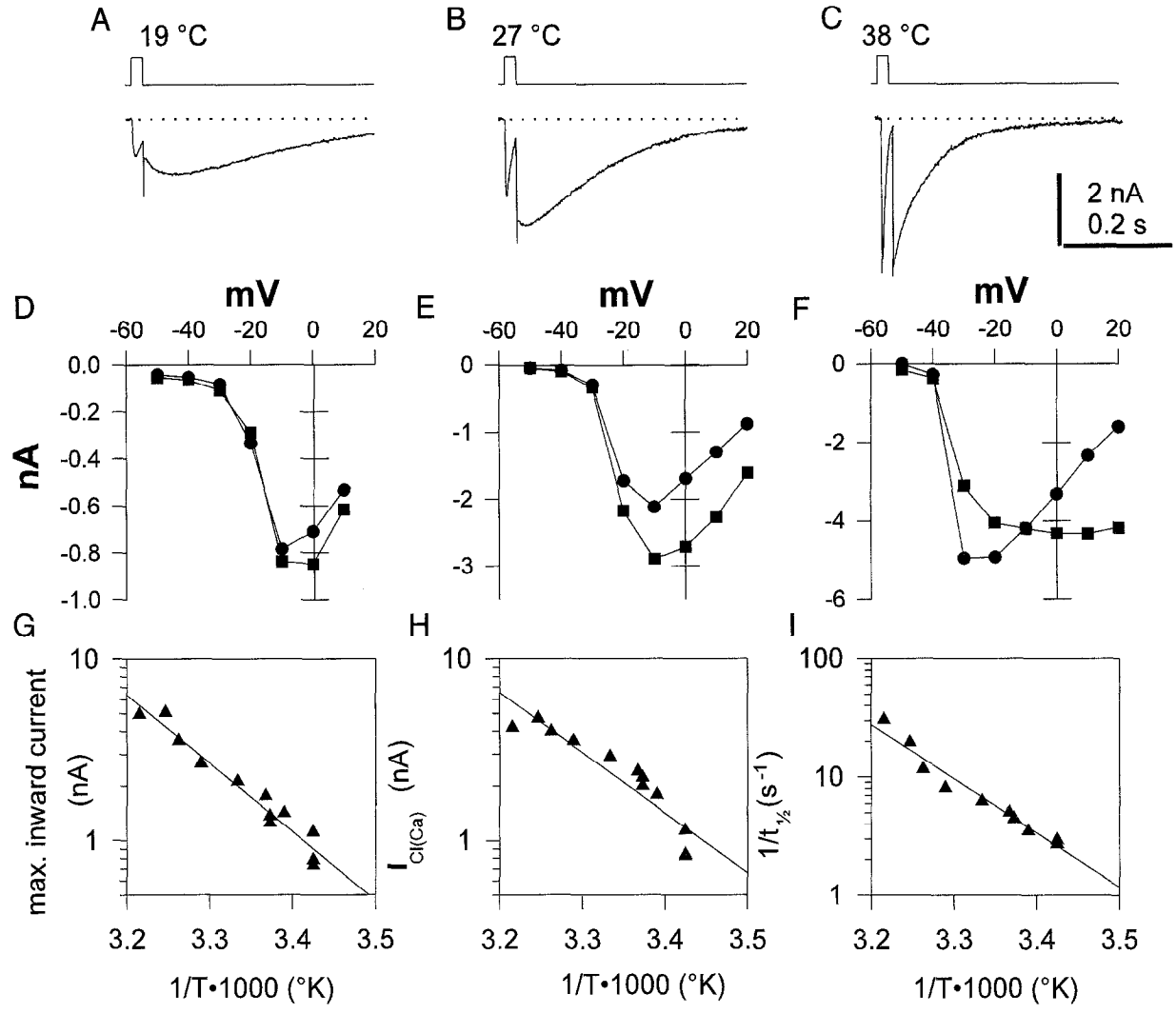

Fig. 2 The effect of temperature on I_{Ca} and I_{Ca}-activated $I_{Cl(Ca)}$ in a chick DRG neuron. (**A–C**) show membrane currents elicited by 20 ms step depolarizations from −90 to −10 mV at temperatures listed above each panel. (**D–F**) plot the current–voltage relationship for the maximum inward current during the step (circles) and the initial amplitude of $I_{Cl(Ca)}$ after repolarization (squares) obtained at the temperature at the top of the column. The bottom row of the figure shows Arrhenius plots for the maximum inward current (**G**), the initial value of $I_{Cl(Ca)}$ following repolarization (**H**), and $1/t_{1/2}$ for the deactivation of $I_{Cl(Ca)}$ (**I**). The straight lines are least squares fits to the data and correspond to Q_{10} values of 2.6, 2.3, and 2.5, respectively. The r^2 values for the fits are 0.95, 0.86, and 0.96, respectively. Dotted lines in (A–C) are 0 nA.

change significantly. We considered the possibility that increased Ca^{2+} influx at higher temperatures saturates the Ca^{2+}-activated Cl^- channels. However, in this case, one would not expect $I_{Cl(Ca)}$ to deactivate until $[Ca^{2+}]_i$ fell to a level that did not saturate the channels. Instead, we find that $I_{Cl(Ca)}$ declines immediately upon repolarization at higher temperatures.

In addition to the increased initial amplitude of $I_{Cl(Ca)}$ following repolarization, increasing temperature also increased the rate of decline of $I_{Cl(Ca)}$ (compare Fig. 2A and Fig. 2C). Because there is no evidence for inactivation of Ca^{2+}-activated Cl^- channels [22], we suggest that the increased rate of decline of $I_{Cl(Ca)}$ results from an increased rate of removal of Ca^{2+} from the Ca^{2+}-activated Cl^- channels.

We used Arrhenius plots to summarize the effects of temperature on the maximum inward current (measured during steps to −10 mV, Fig. 2G), the initial amplitude of $I_{Cl(Ca)}$ following repolarization to the holding potential (Fig. 2H), and a measure of the rate of deactivation of $I_{Cl(Ca)}$ ($1/t_{1/2}$, the inverse of the time for the current to fall to half of its initial value, Fig. 2I). In this and the other cells examined, the Arrhenius plots for the maximum inward current and the deactivation of $I_{Cl(Ca)}$ were well described by single straight lines. This result is somewhat surprising given the multiple mechanisms that underlie these parameters (inward plus outward currents and multiple mechanisms controlling $[Ca^{2+}]_i$) and may be fortuitous. Thus, we do not ascribe any theoretical significance to this observation. The Arrhenius plots for

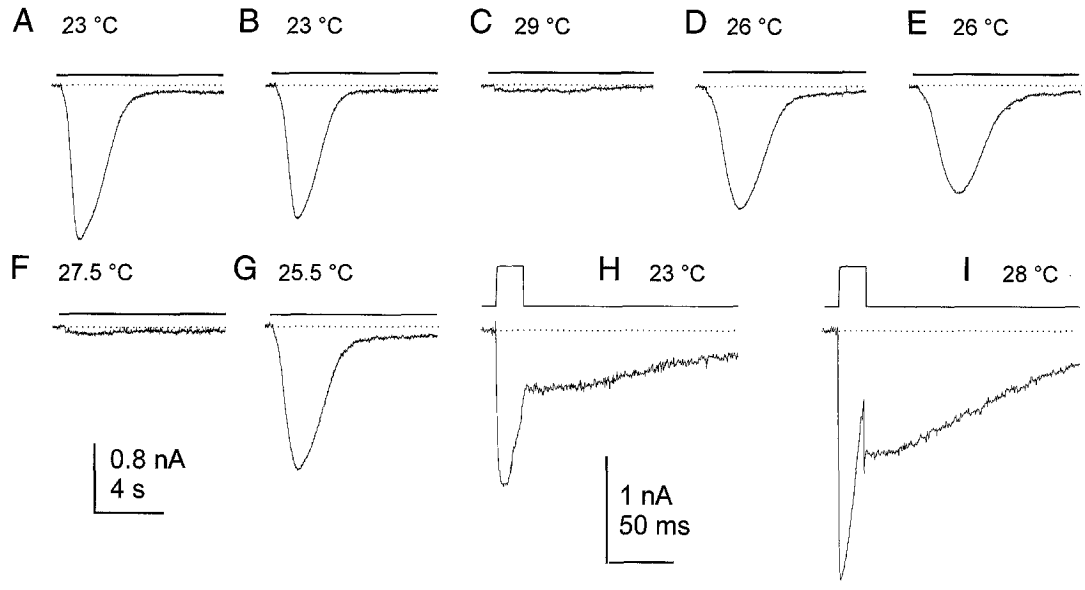

Fig. 3 Effect of temperature on Ca^{2+}-release-activated $I_{Cl(Ca)}$. Membrane currents from a chick DRG neuron in response to caffeine (panels **A–G**) or 20 ms step depolarizations from –74 to –4 mV (H,I) at temperatures listed at the top of each panel are shown. Caffeine was applied during the solid bar above the appropriate traces. Left calibration bars are for (**A–G**), right calibration bars are for (**H,I**). Dotted lines are 0 nA.

the initial amplitude of $I_{Cl(Ca)}$ following repolarization were often curved (Fig. 2H) and the r^2 values for the least squares fits were smaller than those for the maximum inward current and rate of deactivation of $I_{Cl(Ca)}$. Nevertheless, the slopes of straight lines fit to the Arrhenius plots provide useful summaries of our results. In a series of experiments, the average Q_{10} for the maximum inward current activated by depolarization to the potential eliciting the largest inward current at the lowest temperature tested was 2.3 ± 0.08 ($n = 9$ cells), Q_{10} for the initial amplitude of $I_{Cl(Ca)}$ following repolarization to the holding potential was 3.3 ± 0.30 ($n = 7$ cells), and Q_{10} for $1/t_{1/2}$ of the deactivation of $I_{Cl(Ca)}$ was 2.6 ± 0.13 ($n = 7$ cells).

As noted, we attribute the decline of $I_{Cl(Ca)}$ following repolarization to the deactivation of the current by mechanisms that lower $[Ca^{2+}]_i$. The overall increase in the rate of deactivation of $I_{Cl(Ca)}$ at higher temperatures is consistent with an increased rate of activity of these mechanisms and the relatively high Q_{10} implies an enzymatic mechanism. Interestingly, $I_{Cl(Ca)}$ following repolarization typically increased before deactivating at lower temperatures (Figs 1 & 2). The initial increase in $I_{Cl(Ca)}$ cannot be attributed to CICR because biphasic $I_{Cl(Ca)}$ is observed after treatment with ryanodine (Fig. 1D). However, the biphasic time course of $I_{Cl(Ca)}$ is abolished at higher temperature (Fig. 2C). We considered the possibility that the loss of the biphasic waveform might not be a direct effect of increased temperature but was instead caused by the increased Ca^{2+} load. Specifically, the larger Ca^{2+} influx at higher temperatures might recruit Ca^{2+} buffering mechanisms with different affinities and kinetics compared with those that dominate when the Ca^{2+} loads are smaller (e.g. Herrington et al. [25]). We investigated this possibility in two experiments in which both extracellular Ca^{2+} and temperature were manipulated such that the Ca^{2+} load was nearly constant as temperature was changed. Again, we found that warming abolished the biphasic waveform of $I_{Cl(Ca)}$. Accordingly, we conclude that the temperature-dependent changes in the deactivation of $I_{Cl(Ca)}$ are due to alterations in the rates of the mechanisms that lower $[Ca^{2+}]_i$ near the plasma membrane and not to the recruitment of new mechanisms.

The activation of $I_{Cl(Ca)}$ by caffeine is abolished by increasing the temperature

Although I_{Ca}-activated $I_{Cl(Ca)}$ is increased by warming, Ca^{2+}-release-activated $I_{Cl(Ca)}$ is much smaller in cells studied at 35–37°C compared with currents from cells tested at room temperature [17]. If the Ca^{2+}-activated Cl^- channels are sensitive only to the Ca^{2+} delivered by the stimulus, the implication is that the two methods for delivering Ca^{2+} to the channels have opposite temperature-dependencies. In order to investigate this issue further, we compared the temperature-dependencies of the activation of $I_{Cl(Ca)}$ by depolarization and by caffeine in individual cells by monitoring responses over a temperature range of 18–38°C.

In these experiments, exposures to caffeine were separated by 2–4 min intervals during which the temperature was changed and the cells were depolarized (20 ms steps to potentials from –50 mV to +20 mV in 10 mV

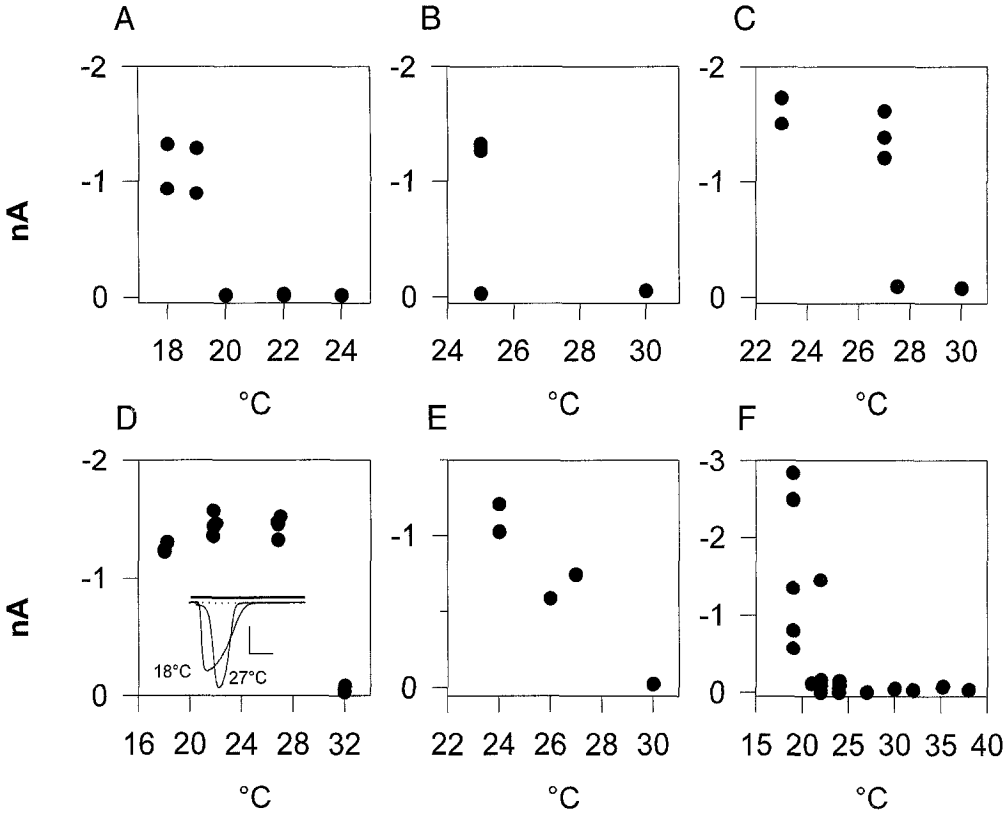

Fig. 4 Summary of Ca^{2+}-release-activated $I_{Cl(Ca)}$ in 6 neurons as a function of temperature. Each symbol represents the amplitude of Ca^{2+}-release-activated $I_{Cl(Ca)}$ in response to an application of caffeine. The cells were depolarized to facilitate filling of the stores between caffeine challenges. Data in (C) are from the cell in Figure 3. Data in (F) are from the cell in Figure 2. The cell in (D) gave data over the widest temperature range and the inset shows superimposed currents in response to caffeine (applied during the solid bar above the traces) at 18 and 27°C. The dashed line is 0 nA, the calibration bars are 0.5 nA and 2 s.

increments) to facilitate refilling of the stores and to monitor I_{Ca} and $I_{Cl(Ca)}$. The results from a typical experiment are shown in Figure 3. Figure 3A,B is the first two caffeine responses and illustrates the reproducibility of Ca^{2+}-release-activated $I_{Cl(Ca)}$ at room temperature. Figure 3C shows that warming the cell to 29°C abolished the ability to activate $I_{Cl(Ca)}$ with caffeine. The response returned on cooling to 26°C (Fig. 3D,E) but was again abolished on warming 1.5°C (Fig. 3F). Currents elicited by 20 ms step depolarizations are shown in Figure 3H,I, illustrating the increase in I_{Ca} and I_{Ca}-activated $I_{Cl(Ca)}$ in this cell as temperature is increased.

Figure 4 summarizes the effects of temperature on Ca^{2+}-release-activated $I_{Cl(Ca)}$ in 6 neurons (including the cell in Fig. 3, see figure caption). In four of these neurons (Fig. 4A–D), the peak amplitude of Ca^{2+}-release-activated $I_{Cl(Ca)}$ was not very temperature-sensitive at lower temperatures but the response reversibly switched off when the temperature was raised above a critical temperature that ranged between 19°C and 28°C. In one neuron (Fig. 4E), Ca^{2+}-release-activated $I_{Cl(Ca)}$ was abolished near 28°C but increased as temperature was reduced below 28°C. In two neurons (Fig. 3C,F) the response to caffeine was unstable with some exposures producing large responses while other exposures at the same temperature were ineffective. This behavior may result from the steep temperature-dependence of the phenomenon relative to our ability to measure and control temperature.

Our study of the properties of Ca^{2+}-release-activated $I_{Cl(Ca)}$ over a range of permissive temperatures was limited by our inability to lower the temperature below 18°C. The cell in Figure 4D had a relatively high critical temperature and provided responses over the largest temperature range. The inset in Figure 4D shows superimposed Ca^{2+}-release-activated $I_{Cl(Ca)}$ recorded at 18 and 27°C. $I_{Cl(Ca)}$ peaked earlier but lasted somewhat longer at the lower temperature. As we discuss below (Fig. 9), the temperature-dependence of the time course of Ca^{2+}-release-activated $I_{Cl(Ca)}$ is different when mitochondrial Ca^{2+} uptake is inhibited.

Although the general protocols used to obtain the data in Figure 4 were as described above, the details of the experiments could be varied without obvious effect on

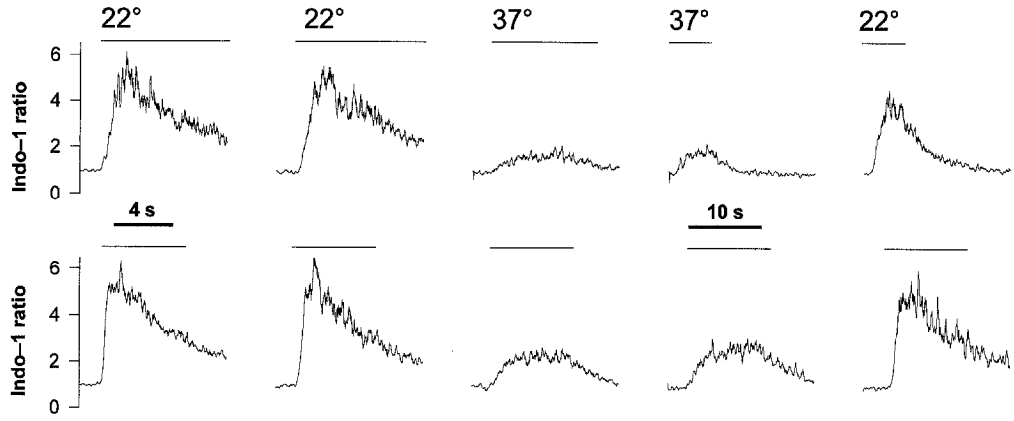

Fig. 5 Effect of temperature on $[Ca^{2+}]_i$ in chick DRG neurons. Indo–1 fluorescence records from two chick DRG neurons (top and bottom rows) in response to 10 mM caffeine at temperatures indicated at the top of the figure. Caffeine was applied during the interval marked by the horizontal line above each record. The 10 s time bar is for the last two records in the top row. The 4 s time bar is for all other records.

the results. That is, some experiments started at high temperatures while others started at low temperatures. The depolarizations between caffeine challenges were given at the lower temperature in some runs while in others they were given at the higher temperature. The constant observation was that $I_{Cl(Ca)}$ was activated if caffeine was applied at a sufficiently low temperature. In this regard, we found one neuron with large I_{Ca}-activated $I_{Cl(Ca)}$ that did not respond to caffeine. We suspect that this was due to our inability to lower the temperature enough for this neuron and that all neurons will respond to caffeine if cooled sufficiently.

The amplitude of Indo–1 signals in response to caffeine is reduced at higher temperature

The abolition of Ca^{2+}-release-activated $I_{Cl(Ca)}$ with warming cannot be attributed to inhibition of Ca^{2+}-activated chloride channels because the amplitude of I_{Ca}-activated $I_{Cl(Ca)}$ is increased at higher temperature. This implies that the failure of caffeine to activate $I_{Cl(Ca)}$ above the critical temperature is due to the inability of the endoplasmic reticulum to raise $[Ca^{2+}]_i$ near the plasma membrane. We investigated the mechanism that underlies this phenomenon by monitoring the effect of caffeine on spatially averaged $[Ca^{2+}]_i$ using Indo–1 in cells that were not voltage-clamped.

Chick DRG neurons loaded with Indo–1 and exposed to 10 mM caffeine at room temperature respond with a phasic increase in Indo–1 fluorescence ratio that can be elicited repeatedly with 2 min rest periods between exposures to caffeine [17]. Indo–1 signals from two chick DRG neurons exposed to caffeine at 22 and 37°C are shown in Figure 5. The amplitude of the Indo–1 response to caffeine was reversibly reduced by warming. We interpret these observations as indicating that caffeine elicits an increase in $[Ca^{2+}]_i$ and that the amplitude of the $[Ca^{2+}]_i$ transient is reduced at higher temperatures. Because the affinity of Indo–1 for Ca^{2+} increases with warming [8,19,20], the $[Ca^{2+}]_i$ transients we observed at higher temperatures were over-estimated and warming is a more potent inhibitor of the response to caffeine than indicated by our records. In 6 chick neurons studied with the protocol used in Figure 5, the resting Indo–1 fluorescence ratio was the same at 22 and 37°C (0.97 ± 0.03 at 22°C versus 0.97 ± 0.02 at 37°C). The peak of the response to caffeine was significantly reduced from 4.5 ± 0.3 at 22°C to 2.0 ± 0.2 at 37°C ($P < 0.005$).

Reports of experiments done on rat sensory neurons suggest that the situation might be different in mammalian cells. In particular, data collected at room temperature [1] and data at 33–35°C [10] indicate that caffeine raises $[Ca^{2+}]_i$ to between 300 to 400 nM suggesting that there is little temperature-sensitivity in these cells. However, fluorescence measurements of $[Ca^{2+}]_i$ from different laboratories, using different indicators at different temperatures, could easily differ by a factor of 2 or more. This could obscure a temperature-dependence similar to that we observed in chick cells. We investigated this possibility by determining the temperature-sensitivity of the ability of caffeine to raise $[Ca^{2+}]_i$ in mouse DRG neurons loaded with Indo–1 with typical results shown in Figure 6. As found with the chick neurons, warming mouse neurons reversibly reduced their response to caffeine. In a total of four mouse neurons studied with this protocol, increasing the temperature significantly reduced the peak response to caffeine from 4.6 ± 0.3 at 22°C to 2.9 ± 0.2 at 37°C ($P < 0.005$, $n = 4$ cells) without affecting the resting Indo–1 fluorescence ratio (1.2 ± 0.04 at 22°C versus 1.1 ± 0.01 at 37°C).

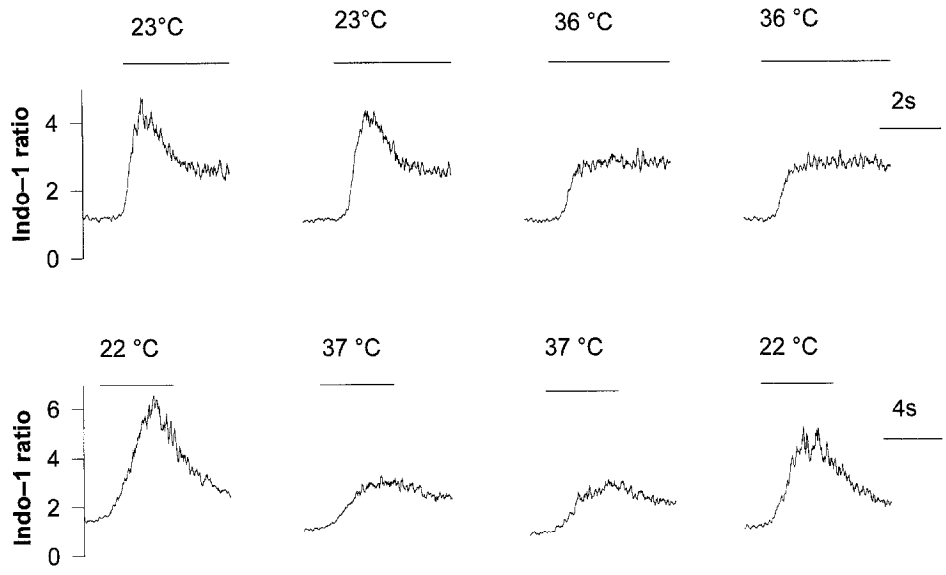

Fig. 6 Effect of temperature on [Ca^{2+}]$_i$ in mouse DRG neurons. Indo–1 fluorescence records from two mouse DRG neurons (top and bottom rows) in response to 10 mM caffeine at temperatures indicated at the top of the figure. Caffeine was applied during the interval marked by the horizontal line above each record.

In comparing the results from the chick and mouse neurons, we found that the peak responses to caffeine at the lower temperature and the resting ratios at both temperatures were not significantly different. However, the reduction of the peak Indo–1 response to caffeine was significantly greater ($P < 0.02$) in chick than in mouse neurons. In addition, the response to caffeine in the mouse neurons often had clear phasic and tonic components (Fig. 6, top row) and warming reduced the phasic component with little effect on the tonic component.

Uptake of Ca^{2+} by mitochondria contributes to deactivation of I_{Ca}-activated $I_{Cl(Ca)}$ at 20 and 36°C

One of the mechanisms that might contribute to the reduced $I_{Cl(Ca)}$ and Indo–1 responses to caffeine at higher temperatures is an enhanced sequestration of Ca^{2+} by the mitochondria. We investigated the role of mitochondrial Ca^{2+} uptake on the activation and deactivation of $I_{Cl(Ca)}$ using FCCP, an ionophore that rapidly depolarizes mitochondria (within 10 s) and abolishes their ability to take up Ca^{2+} actively [26,27].

We first studied the effect of FCCP on the time course of I_{Ca}-activated $I_{Cl(Ca)}$ at 36°C., i.e. at a temperature where the rate of mitochondrial Ca^{2+} uptake is expected to be relatively high. The effects of 1 μM FCCP plus 4 mM Ca^{2+} on membrane currents from a chick DRG neuron at this temperature are shown in Figure 7A,B which shows the currents elicited by 20 and 100 ms depolarizations, respectively. In all cells studied at 36°C, FCCP reduced the maximum inward current and inhibited the development of $I_{Cl(Ca)}$ during depolarization (most apparent in Fig.7B). In addition, FCCP reduced the initial amplitude of $I_{Cl(Ca)}$ following repolarization. These effects were seen whether or not extracellular Ca^{2+} was increased. Following the 20 ms depolarization (Fig. 7A), the initial amplitude of $I_{Cl(Ca)}$ was relatively small, consistent with a small Ca^{2+} influx during the short pulse. Here, the kinetics of deactivation of $I_{Cl(Ca)}$ were not much changed by FCCP. In contrast, following the 100 ms depolarization (Fig. 7B), the initial amplitude of $I_{Cl(Ca)}$ was greater and the deactivation of the current was clearly slowed by FCCP. However, interpretation of these records is complicated by the change in the amplitude of $I_{Cl(Ca)}$. In order to compare currents of similar magnitudes, we superimposed the control record from Figure 7A (20 ms depolarization) with the FCCP record from Figure 7B (100 ms depolarization) in Figure 7C. Here, the longer depolarization makes up for the inhibition of I_{Ca} and the initial amplitudes of $I_{Cl(Ca)}$ following repolarization are similar. The records in Figure 7C show clearly the slower rate of deactivation of $I_{Cl(Ca)}$ in the presence of an inhibitor of mitochondrial Ca^{2+} uptake. We quantified this effect by measuring $t_{1/2}$ for the decline of $I_{Cl(Ca)}$ in 7 neurons exposed to 1, 2, or 4 μM FCCP, taking care to match the initial amplitudes of $I_{Cl(Ca)}$ as done in Figure 7C. In six of these cells, $t_{1/2}$ increased significantly ($P < 0.05$) from 59 ± 3 ms to 627 ± 213 ms. The seventh cell showed a similar change although $t_{1/2}$ was unusually large in control (193 ms, increasing to 396 ms in the presence of FCCP). We interpret these data as indicating that Ca^{2+} uptake by the mitochondria makes a significant contribution to the deactivation of $I_{Cl(Ca)}$ activated by step depolarizations of 100 ms duration at 36°C.

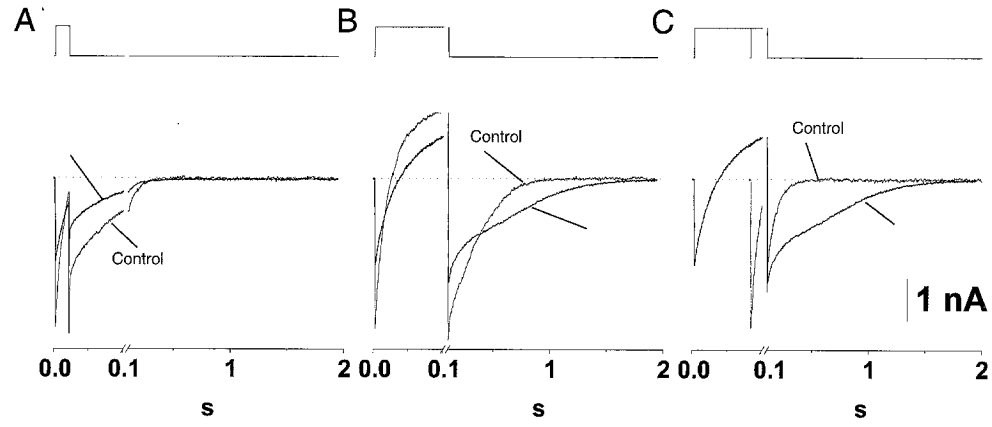

Fig. 7 Effect of FCCP on I_{Ca} and I_{Ca}-activated $I_{Cl(Ca)}$ at 36°C. Membrane currents from a chick DRG neuron recorded in control and in the presence of 1 μM FCCP plus 4 mM Ca^{2+} at 36°C. Membrane potential (uppermost trace in each panel) was stepped from –70 to 0 mV for 20 ms (**A**) or 100 ms (**B**). (**C**) shows the current elicited by the 20 ms step in control and the current elicited by the 100 ms step in the presence of FCCP, aligned so that repolarization and the deactivation of $I_{Cl(Ca)}$ begin at 0.1 s. Although the initial amplitudes of $I_{Cl(Ca)}$ were similar in the two runs, $t_{1/2}$ of the deactivation of $I_{Cl(Ca)}$ increased from 55 to 330 ms in the presence of FCCP. Note the split time bases in the panels. The 1 nA calibration bar is for all panels and the dotted lines are 0 nA.

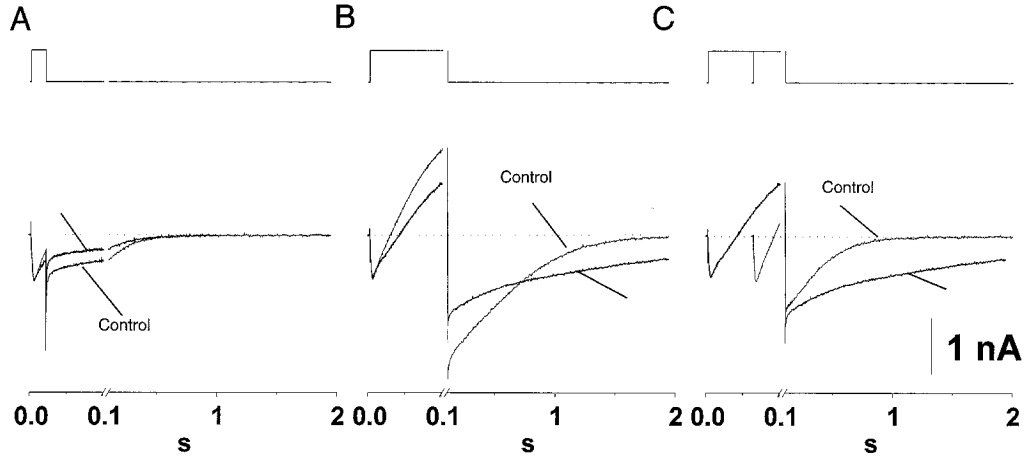

Fig. 8 Effect of FCCP on I_{Ca} and I_{Ca}-activated $I_{Cl(Ca)}$ at 20°C. Membrane currents from a chick DRG neuron recorded in control and in the presence of 2 μM FCCP plus 4 mM Ca^{2+} at 20°C. Membrane potential (uppermost trace in each panel) was stepped from –70 to 0 mV for 20 ms (**A**) or 100 ms (**B**). (**C**) shows the current elicited by a 40 ms step to 0 mV in control and the current elicited by the 100 ms step in the presence of FCCP, aligned so that repolarization and the deactivation of $I_{Cl(Ca)}$ begin at 0.1 s. $t_{1/2}$ of the deactivation of $I_{Cl(Ca)}$ increased from 177 to 670 ms in the presence of FCCP. The 1 nA calibration bar is for all panels and the dotted lines are 0 nA.

We next investigated the role of mitochondrial Ca^{2+} uptake on the deactivation of $I_{Cl(Ca)}$ at 20°C in two DRG neurons. In the experiment illustrated in Figure 8, 2 μM FCCP plus 4 mM Ca^{2+} had no effect on the maximum inward current during the depolarization at 20°C (Fig. 8A,B). However, this treatment reduced the activation of $I_{Cl(Ca)}$ during and immediately following the step suggesting that FCCP reduced I_{Ca} (again in spite of the increased extracellular Ca^{2+}) and that the unchanged maximum inward current is due to equal reductions in inward I_{Ca} and outward $I_{Cl(Ca)}$. As seen at 36°C, FCCP had little if any effect on the kinetics of deactivation of the small $I_{Cl(Ca)}$ following the 20 ms depolarization (Fig. 8A) but slowed the deactivation of the larger $I_{Cl(Ca)}$ following the 100 ms depolarization (Fig. 8B). In order to compare kinetics of currents with similar magnitudes, Figure 8C plots the response to a 40 ms depolarization to 0 mV in control and the response to the 100 ms depolarization in the presence of FCCP. In this experiment, FCCP increased $t_{1/2}$ of the deactivation from 177 to 670 ms by FCCP. A similar result was obtained in the other neuron studied with this protocol ($t_{1/2}$ increased from 199 to 408 ms). We interpret these data as indicating that Ca^{2+} uptake by the mitochondria makes a significant contribution to the deactivation of $I_{Cl(Ca)}$ activated by step depolarizations of 100 ms duration at 20°C. Interestingly, $t_{1/2}$ is similar at 36 and 20°C in the presence of FCCP. In addition, the ability of FCCP to increase $t_{1/2}$ is greater at higher temperatures. This suggests that the mitochondria are more important in the control of I_{Ca}-activated $I_{Cl(Ca)}$ at warmer temperatures.

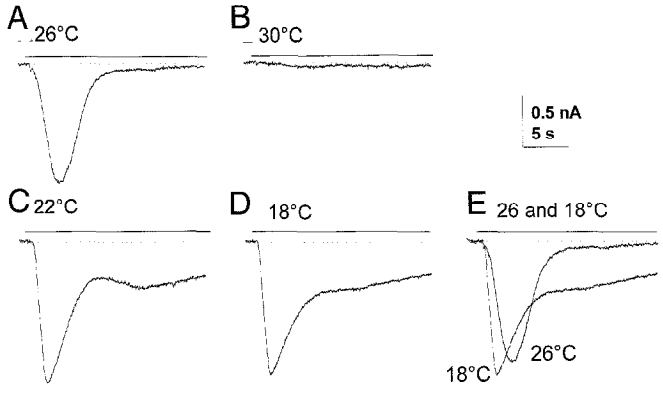

Fig. 9 Effect of FCCP on Ca^{2+}-release-activated $I_{Cl(Ca)}$. Membrane currents from a chick DRG neuron in response to 10 mM caffeine plus 2 μM FCCP applied during the horizontal line above each record. (**E**) shows the records in (**A**) and (**D**) superimposed. The dotted lines are 0 nA.

FCCP prolongs caffeine activated-$I_{Cl(Ca)}$ at low temperature

A different picture emerged when we investigated the temperature-dependence of the effect of mitochondrial uptake of Ca^{2+} on Ca^{2+}-release-activated $I_{Cl(Ca)}$. Figure 9 shows Ca^{2+}-release-activated $I_{Cl(Ca)}$ elicited in a chick DRG neuron by exposures to 10 mM caffeine plus 2 μM FCCP (4 mM Ca^{2+}) at temperatures ranging from 18 to 30°C. At 26°C (Fig. 9A) the activation of $I_{Cl(Ca)}$ by the combination of caffeine plus mitochondrial inhibitor was similar to responses to caffeine alone reported above. In addition, warming reversibly abolished the activation of $I_{Cl(Ca)}$ by caffeine plus FCCP (Fig. 9B). Again, this is similar to the response to caffeine alone. However, at cooler temperatures (Fig. 9C,D), $I_{Cl(Ca)}$ activated by caffeine plus FCCP was greatly prolonged and some cells showed a second activation of $I_{Cl(Ca)}$ late in the exposure to caffeine plus FCCP (Fig. 9C). Records of $I_{Cl(Ca)}$ elicited at 26 and 18°C in this experiment are superimposed in Figure 9E for comparison with currents elicited by caffeine alone at similar temperatures (Fig. 4D). In both experiments, Ca^{2+}-release-activated $I_{Cl(Ca)}$ peaked earlier and lasted longer at the lower temperature. However, in the absence of FCCP (Fig. 4), $I_{Cl(Ca)}$ deactivates within 5 s of the peak current whereas in the presence of FCCP there is substantial $I_{Cl(Ca)}$ flowing 15 s after the peak current (Fig. 9D). Similar results were obtained in 5 other cells.

DISCUSSION

$I_{Cl(Ca)}$ provides information about $[Ca^{2+}]_i$ near the plasma membrane

We used $I_{Cl(Ca)}$ to obtain information about $[Ca^{2+}]_i$ near the plasma membrane of chick DRG neurons. Although the biophysical properties of these channels have not been characterized, Ca^{2+}-activated Cl^- channels in *Xenopus* oocytes [28] and in ventricular myocytes [29] require high micromolar or millimolar Ca^{2+} for activation. This property is of interest because modeling of Ca^{2+} diffusion in neuronal cell bodies predicts that $[Ca^{2+}]_i$ near the plasma membrane rises above 10 μM following 50–100 ms depolarizations and then falls below 1 μM over 100 ms [6]. Thus, the Ca^{2+}-sensitivity of Ca^{2+}-activated Cl^- channels in other cell types and the predicted time course of micromolar $[Ca^{2+}]_i$ near the plasma membrane lead to the prediction that I_{Ca}-activated $I_{Cl(Ca)}$ in chick neurons will deactivate over 100–200 ms and that the current will be completely deactivated by 500 ms. This time course contrasts sharply with that of spatially averaged $[Ca^{2+}]_i$ which typically peaks later near 500–700 nM [1,4,6,10]. Our observations of $I_{Cl(Ca)}$ are consistent with this prediction and we suggest that $I_{Cl(Ca)}$ provides qualitative information about micromolar $[Ca^{2+}]_i$ near the plasma membrane of chick DRG neurons.

Our results with FCCP provide further evidence for this conclusion. That is, mitochondria are a relatively low affinity sink for Ca^{2+} that will be most important when $[Ca^{2+}]_i$ is above 0.5–1 μM [25,26]. Thus, our observation that FCCP prolongs the deactivation of $I_{Cl(Ca)}$ following a depolarization is consistent with the suggestion that $I_{Cl(Ca)}$ is activated by micromolar $[Ca^{2+}]_i$ and that the mitochondria play an important role in reducing $[Ca^{2+}]_i$ near the plasma membrane into the submicromolar range.

Warming increases the amplitudes of I_{Ca} and $I_{Cl(Ca)}$ and speeds the deactivation of I_{Ca}-activated $I_{Cl(Ca)}$

Warming chick neurons increased the amplitude of net inward current with a Q_{10} of 2.3. This differs somewhat from findings by Nobile et al. [12] who reported Q_{10} values of 2.6 between 20 and 30°C and 2.1 above 30°C for I_{Ca} in chick DRG neurons internally perfused with EGTA. Our intermediate Q_{10} and our failure to identify a break in the Arrhenius plot may be related to the lack of exogenous Ca^{2+} buffering in our experiments and our measurements of mixed I_{Ca} plus $I_{Cl(Ca)}$. That is, increased Ca^{2+} influx at higher temperatures will activate larger outward $I_{Cl(Ca)}$ causing the net current to be smaller than I_{Ca}. As a result, our mixed current should be less temperature-sensitive than the pure I_{Ca}.

We also observed that warming increased the rate of deactivation of $I_{Cl(Ca)}$ ($1/t_{1/2}$) with $Q_{10} = 2.6$. This is similar to observations by Sah and McLachlan [11] who found a Q_{10} of 2.9 for the deactivation of Ca^{2+}-activated K^+ currents in guinea pig neurons. The relatively high Q_{10} values for the deactivation of $I_{Cl(Ca)}$, our observation that FCCP slows deactivation of $I_{Cl(Ca)}$, and observations by Stapleton et al. [21] that deactivation of $I_{Cl(Ca)}$ was slowed

by metabolic inhibition (exposure to 2-deoxy-D-glucose) all imply that enzymatic activity lowers $[Ca^{2+}]_i$ near the plasma membrane and deactivates $I_{Cl(Ca)}$. Our observations with FCCP further imply that these mechanisms accumulate Ca^{2+} entering the cytoplasm via voltage-dependent Ca^{2+} channels at times shorter than 1 s, i.e. before the gradient between the plasma membrane and the bulk cytoplasm has collapsed.

Caffeine raises $[Ca^{2+}]_i$ near the plasma membrane into the micromolar range

As noted above, we find that I_{Ca}-activated $I_{Cl(Ca)}$ correlates with predicted micromolar $[Ca^{2+}]_i$ near the plasma membrane and not with measured nanomolar $[Ca^{2+}]_i$ in the bulk cytoplasm. Interestingly, numerous studies have found that caffeine raises spatially averaged $[Ca^{2+}]_i$ to between 200 and 600 nM [3,4,10,24]. That is, the fluorescence data imply that caffeine does not raise $[Ca^{2+}]_i$ sufficiently to activate $I_{Cl(Ca)}$. Yet, caffeine activates robust $I_{Cl(Ca)}$ in chick and rat [30] neurons. One possibility is that caffeine sensitizes Ca^{2+}-activated Cl^- channels enabling activation of $I_{Cl(Ca)}$ at lower $[Ca^{2+}]_i$. However, we find that exposure to caffeine does not change I_{Ca} or I_{Ca}-activated $I_{Cl(Ca)}$ (data not shown). Alternatively, caffeine might create a gradient of Ca^{2+} within the neuron similar to that created by influx across the plasma membrane such that caffeine activates $I_{Cl(Ca)}$ in the same way that depolarization does, i.e. by raising $[Ca^{2+}]_i$ near the plasma membrane into the micromolar range. There are few data available to evaluate this suggestion. Ultrastructural studies find junctions between the endoplasmic reticulum and plasma membrane in neuronal cell bodies resembling those responsible for excitation-contraction coupling in striated muscle cells [31]. Thus, the release machinery might be located near the plasma membrane. In addition, Tse et al. [32] found that IP_3-stimulated exocytosis was consistent with the release of Ca^{2+} near the plasma membrane. However, as noted earlier, Ca^{2+} imaging studies have not described regions of high $[Ca^{2+}]_i$ near the plasma membrane following exposure to caffeine [4,5]. We believe that our results suggest that the localization of Ca^{2+} release in response to caffeine should be reexamined.

Warming reduces the ability of the endoplasmic reticulum to elevate $[Ca^{2+}]_i$ and to activate Ca^{2+}-release-activated $I_{Cl(Ca)}$

As chick DRG neurons are warmed from 18°C, Ca^{2+}-release-activated $I_{Cl(Ca)}$ peaks later, shortens in duration, and abruptly fails at a temperature between 19 and 28°C. We also found that warming reduced the caffeine-stimulated $[Ca^{2+}]_i$ transient in these cells by about half. These observations raise two questions. First, what is the mechanism by which warming reduces the amplitude of the caffeine-stimulated $[Ca^{2+}]_i$ transient? Second, how is it that warming abolishes Ca^{2+}-release-activated $I_{Cl(Ca)}$ while it only reduces the caffeine-stimulated $[Ca^{2+}]_i$ transient by about half?

We have considered three general mechanisms to account for the reduction of the caffeine-stimulated $[Ca^{2+}]_i$ transient. First, the activity of the ryanodine-receptor Ca^{2+} release channels might be reduced by warming. This possibility is consistent with the conclusion by Sitsapesan et al. [33] that the net effect of warming is to reduce Ca^{2+} flux through ryanodine-receptor release channels. Second, the Ca^{2+} content of the endoplasmic reticulum might be reduced at higher temperatures. This possibility is interesting in light of the report by Reichling and Levine [34] that rapid warming of rat DRG neurons elicited a Ca^{2+}-activated cation current and that the activation of this current did not depend on Ca^{2+} entry. This raises the possibility that warming causes the intracellular stores to dump their Ca^{2+}, rendering them unable to respond to caffeine. Third, increased rates of Ca^{2+} buffering, sequestration, and extrusion are expected to blunt the effect of Ca^{2+} release on $[Ca^{2+}]_i$ at warmer temperatures as suggested by Puglisi et al. [19] in the heart. Likely mechanisms here include increased activities by the mitochondria [1], Ca^{2+}-ATPases of the endoplasmic reticulum [35], and possibly Na^+/Ca^{2+}-exchange. Available data suggest that they are all temperature-dependent (mitochondria [13], Na^+/Ca^{2+}-exchange [15], endoplasmic reticulum Ca^{2+} pump [14]).

With regard to the abolition of Ca^{2+}-release-activated $I_{Cl(Ca)}$, we note that Sadoshima and Akaike [36] reported changes in the kinetics of Ca^{2+}-release-activated K^+ current (activated by caffeine) in frog sympathetic neurons warmed from 12 to 32°C that resemble our observations of $I_{Cl(Ca)}$. However, while the amplitude of the K^+ currents were reduced by about half, they were not abolished. We also note that the neurons were not voltage-clamped in our Indo–1 experiments. Thus exposure to caffeine might depolarize them and activate Ca^{2+} influx in addition to release. In this case, the Indo–1 responses at warmer temperatures would be attributed to Ca^{2+} influx. However, this seems unlikely as Indo–1 responses recorded in zero Ca^{2+} and Cd^{2+}-containing solutions were similar to those obtained in normal solution suggesting that influx does not contribute to the response to caffeine. Other potential mechanisms include a highly co-operative Ca^{2+} dependence for activation of the Ca^{2+}-activated Cl^- channels and the possibility that the mechanisms that raise and lower $[Ca^{2+}]_i$ near the plasma membrane might have a stronger temperature-dependence than those working to control $[Ca^{2+}]_i$ in the center of the neuron.

Mitochondrial Ca^{2+} uptake deactivates $I_{Cl(Ca)}$

We investigated the role of the mitochondria in the regulation of $[Ca^{2+}]_i$ by examining the action of FCCP on the time course of the deactivation of $I_{Cl(Ca)}$ following step depolarization and on $I_{Cl(Ca)}$ activated by the release of Ca^{2+} from caffeine- and ryanodine-sensitive stores. Although the effects of this compound, and other mitochondrial inhibitors, on $[Ca^{2+}]_i$ and Ca^{2+}-activated currents are commonly attributed to an inhibition of Ca^{2+} uptake [1,21,25,27,37] other modes of action are possible. In particular, cytoplasmic ATP levels may be reduced and acidification of the cytoplasm by the release of H^+ from the mitochondria is expected. We do not think that levels of ATP are changing significantly in our experiments based on the observation that ATP levels in cultured central neurons are maintained over long periods of mitochondrial inhibition [38] and the relatively short exposures used in our experiments (< 5 min in Figures 7 and 8, 10 s in Fig. 9). With regard to acidification, Wang and coworkers [39] reported that FCCP caused an acidification of less than 0.3 pH units in cultured central neurons and we expect a similar response in our sensory neurons. Lastly, we note that FCCP-sensitive, non-mitochondrial stores have been described in neurons (see discussion in Friel and Tsien [40]).

1–4 μM FCCP slowed the deactivation of I_{Ca}-activated $I_{Cl(Ca)}$ at 20 and at 36°C. This effect was greater at the higher temperature and we observed that $t_{1/2}$ in the presence of FCCP is similar at 36 and 20°C (mean $t_{1/2}$ was 627 ± 213 ms ($n = 6$) at 36°C versus 539 ms ($n = 2$) at 20°C). These observations suggest that the relative contribution of the mitochondria to the buffering of Ca^{2+} following activation of I_{Ca} increases with warming and that this increase is responsible for the increased rate of deactivation of $I_{Cl(Ca)}$ at higher temperatures. They further imply that, when I_{Ca} is the source of the Ca^{2+}, the function of non-mitochondrial Ca^{2+}-uptake mechanisms is not highly temperature-dependent.

The effect of FCCP on Ca^{2+}-release-activated $I_{Cl(Ca)}$ was different. FCCP had little or no effect on Ca^{2+}-release-activated $I_{Cl(Ca)}$ at temperatures just below the critical temperature for the activation of this current. However, FCCP prolonged the activation of $I_{Cl(Ca)}$ at lower temperatures. This suggests that, although the rate of Ca^{2+} uptake by mitochondria is increased with warming, the relative contribution of this mechanism to the buffering of Ca^{2+} released from the endoplasmic reticulum is reduced at higher temperature. Presumably, the activity of the non-mitochondrial regulatory mechanisms is more steeply temperature-dependent than that of the mitochondria, when the endoplasmic reticulum is the source of the Ca^{2+}.

These observations and conclusions contrast with those of Puglisi et al. [19] who found, in cardiac myocytes, that the relative contribution of the mitochondria to the control of $[Ca^{2+}]_i$ during and following a depolarization was the same at 25 and 35°C. While the mechanisms that underlie this difference are not clear, we note that we used $I_{Cl(Ca)}$ as an indicator of $[Ca^{2+}]_i$ whereas Puglisi et al. [19] monitored spatially averaged $[Ca^{2+}]_i$ using Indo–1 fluorescence. In addition, the control of $[Ca^{2+}]_i$ is different in cardiac myocytes and neurons. Activation of cardiac contraction is mediated by L-type Ca^{2+} channels, CICR, and Na^+/Ca^{2+}-exchange whereas $[Ca^{2+}]_i$ transients in sensory neurons are determined largely by N-type Ca^{2+} channels, plasma membrane Ca^{2+}-ATPases, and mitochondria.

It is impossible to know how Ca^{2+} regulatory mechanisms will contribute to the control of $[Ca^{2+}]_i$ at physiological temperature using data obtained at room temperature

We discussed reasons why one might expect this to be so in the Introduction and we believe that our results on the activation of Ca^{2+}-release-activated $I_{Cl(Ca)}$ by caffeine and the effects of FCCP provide instructive examples. In particular, we expected that an increased rate of Ca^{2+} uptake by the endoplasmic reticulum at higher temperatures would result in a large supply of Ca^{2+} available for release, enhancing the ability of caffeine to raise $[Ca^{2+}]_i$. With regard to FCCP, we expected that inhibition of mitochondrial Ca^{2+} uptake by FCCP would enable the activation of $I_{Cl(Ca)}$ by caffeine at higher temperatures. Neither of these expectations was supported by experimental results illustrating the difficulty in predicting the temperature-dependent behavior of complex systems. In addition, our results imply that the temperature-dependence of the relative contribution of the mitochondria to the control of $[Ca^{2+}]_i$ depends on the source of the Ca^{2+}. We conclude that in order to understand the regulation of $[Ca^{2+}]_i$ one must study cells at physiological temperature.

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