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Effects of metabolic blockers on Ca^{2+}-dependent currents in cultured sensory neurones from neonatal rats

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1 The whole cell variant of the patch clamp technique was used to record high voltage-activated Ca^{2+} currents and Ca^{2+}-activated Cl^- tail currents from cultured neonatal rat dorsal root ganglion neurones. The aim of the project was to use these currents as physiological indices of intracellular Ca^{2+} regulation under control conditions and in the presence of metabolic inhibitors.

2 Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (5 μM) and sodium cyanide (1 μM) inhibited Ca^{2+} currents within 20 s, even when ATP was present in the patch pipette solution, suggesting a direct action on Ca^{2+} channels. These metabolic inhibitors did not affect Ca^{2+} current 'run down' or inactivation kinetics.

3 Cultured neonatal dorsal root ganglion neurones of the rat were relatively insensitive to the removal of glucose and ATP from the recording solutions for up to 3 h. These data suggest that the Ca^{2+} homeostatic mechanisms in these cells are highly resistant to metabolic insult.

4 However 2-deoxy-D-glucose (5 mM) in the extracellular recording medium with no ATP or glucose present did prolong the deactivation time of Ca^{2+}-activated Cl^- tail currents and increase the total charge flow following activation of a 500 ms voltage-activated Ca^{2+} current. This effect was prevented by inclusion of D-fructose 1,6-diphosphate (500 μM) in the patch pipette solution.

5 We conclude that some agents used to induce chemical hypoxia, such as carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and sodium cyanide, may interact directly with voltage-activated Ca^{2+} channels and are therefore not appropriate for use in studying disturbed neuronal Ca^{2+} homeostasis. However, the use of 2-deoxy-D-glucose in the absence of glucose and ATP does represent a model of disturbed Ca^{2+} homeostasis in cultured dorsal root ganglion neurones. In this study we have combined the whole cell recording technique with cultured neurones under conditions which produce a degree of metabolic stress as reflected by prolonged Ca^{2+}-activated Cl^- tail currents. The reduced efficiency of handling of intracellular Ca^{2+} loads may be an important factor contributing to the onset of neuronal damage during hypoxia and ischaemia.

Keywords: Cultured neurones; Ca^{2+} currents; Ca^{2+}-activated chloride currents; intracellular Ca^{2+} homeostasis; metabolic inhibition; cyanide; 2-deoxyglucose; fructose 1,6-diphosphate

Introduction

The damaging effects of hypoxia and ischaemia on certain neuronal populations appears to be the result of a failure of intracellular Ca^{2+} ($[Ca^{2+}]_i$) homeostasis, with the subsequent loss of control of numerous essential intracellular Ca^{2+}-dependent processes (Siesjö, 1981; Choi, 1988).

The maintenance of appropriately low free $[Ca^{2+}]_i$ is achieved by complex intracellular buffering mechanisms sited in the mitochondria, the endoplasmic reticulum and via Ca^{2+} binding proteins (Miller, 1991). Nevertheless, ultimately Ca^{2+} ions must be extruded across the plasma membrane to the external environment and this is achieved by several mechanisms including the Na^{2+}-Ca^{2+} exchanger (Blaustein, 1988) and the membrane bound Ca^{2+}-ATPase (McBurney & Neering, 1987; Benham *et al.*, 1992). These systems require ATP either directly, as in the case of the Ca^{2+}-ATPase, or indirectly due to the dependence on ATP of the Na^+-K^+ exchange required for normal functioning of the Na^+-Ca^{2+} exchange process and possibly for phosphorylation of the Na^+-Ca^{2+} exchange protein itself (DiPolo & Beaugé, 1983; 1987; Lagnado & McNaughton, 1990). An adequate supply of ATP is therefore essential for the maintenance of Ca^{2+} homeostasis in neurones and any impairment of this supply as occurs during hypoxia or ischaemia may lead to potentially harmful rises in $[Ca^{2+}]_i$. Maintained depolarization with prolonged activity of voltage-activated Ca^{2+} channels and activation of glutamate receptors, particularly the N-methyl-D-aspartate receptor ion channel complex, result in Ca^{2+} loads which pose a challenge to compromised Ca^{2+} homeostatic mechanisms (Meldrum *et al.*, 1985; Choi, 1988).

The whole cell recording configuration of the patch clamp technique is a powerful tool for the investigation of neuronal Ca^{2+} currents and Ca^{2+}-activated currents. Measurements of both Ca^{2+} induced inactivation of high voltage-activated Ca^{2+} currents (Chad & Eckert, 1986) and Ca^{2+}-activated Cl^- tail currents ($I_{Cl(Ca)}$) (Owen *et al.*, 1984; Mayer, 1985; Bader *et al.*, 1987; Currie & Scott, 1992) act as physiological indices of $[Ca^{2+}]_i$ close to the cell plasma membrane. We have studied the effects of various metabolic insults on these currents used as indicators of the efficiency of neuronal Ca^{2+} homeostasis. Previously we have shown that the lipid metabolite palmitoyl-DL-carnitine slows the rate of decay of $I_{Cl(Ca)}$, reflecting a change in the efficiency of cellular Ca^{2+} homeostasis (Stapleton *et al.*, 1992) and the possible involvement of caffeine-sensitive intracellular Ca^{2+} stores. A simple *in vitro* model of neuronal ischaemia/hypoxia, suitable for application to the whole cell configuration of the patch clamp technique would allow greater understanding of the processes involved in the onset of neuronal damage produced by such insults and may prove useful as an 'assay' system for the evaluation of agents with potential neuroprotective properties.

We have taken two approaches to simulate metabolic stress in our system: (1) the inhibition of oxidative phosphorylation by use of cyanide and the proton ionophore, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP)

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(McLaughlin & Dilger, 1980); (2) the abolition of glucose uptake and metabolism with 2-deoxy-D-glucose (2-DG) and by removing ATP and glucose from the experimental recording conditions.

Methods

Cell culture

Primary cultures of dorsal root ganglion (DRG) neurones were prepared from 2 day old Wistar rats. DRG neurones were enzymatically and mechanically dissociated, plated on laminin-polyornithine coated cover-slips and maintained in culture for 2–3 weeks at 37°C in humidified air with 5% CO_2. F14 culture medium (Imperial Laboratories) supplemented with 10% horse serum (Gibco) was used with added penicillin (5000 i.u. ml^{-1}), streptomycin (5000 $\mu g\ ml^{-1}$), $NaHCO_3$ (14 mM) and nerve growth factor (20 ng ml^{-1}, Sigma). Cell cultures were refed with F14/horse serum mixture containing nerve growth factor (10 ng ml^{-1}) every 7 days.

Electrophysiology

The whole cell variant of the patch clamp technique was used (Hamill *et al.*, 1981). DRG neurones were voltage clamped by use of an Axoclamp 2A switching amplifier operated at a sampling rate of 28–35 kHz, with low resistance (3–7 MΩ) micropipettes. A pulse generator (Digitimer D4030) provided the timing of voltage command protocols. All experiments were performed at room temperature (approx. 23°C).

Standard recording medium contained (in mmol l^{-1}): choline chloride 130, KCl 3.0, $MgCl_2$ 0.6, $NaHCO_3$ 1.0, HEPES 10.0, tetraethylammonium bromide 25.0, D-glucose 4.0, $CaCl_2$ 2.0 and tetrodotoxin 0.0025. The pH was adjusted to 7.4 with NaOH and the osmolarity to 320 mOsmol l^{-1} with sucrose. The patch pipette solution contained (in mmol l^{-1}): CsCl 140, EGTA 1.1, $MgCl_2$ 2.0, $CaCl_2$ 0.1, ATP 2.0, HEPES 10.0, the pH was corrected to 7.2 with Tris (1 mM) and the osmolarity to 310 mOsmol l^{-1} again with sucrose. The estimated free Ca^{2+} concentration in the patch pipette solution was 16 nM. A modest amount of EGTA (1.1 mM) was used to buffer the Ca^{2+} in the patch pipette solution; this was present to ensure stability of Ca^{2+} currents and Ca^{2+}-activated currents under control conditions. The concentration of EGTA used was not sufficient to inhibit Ca^{2+}-activated Cl^- tail currents or currents activated by release of Ca^{2+} from intracellular stores. For several experiments both glucose and ATP were excluded from the recording and patch solutions respectively. In these cases the osmolarity was made up to the appropriate value with mannitol.

Drugs, dissolved in recording medium and after adjustment of pH, were applied extracellularly by low pressure ejection via a micropipette (tip diameter approximately 10 μm) positioned within 100 μm of the cell being recorded. Alternatively, DRG neurones were bathed in the recording medium containing the drug or incubated in culture medium containing the drug for varying periods prior to an experiment. Intracellular drug application was performed by including the drug in the patch pipette solution after suitable adjustment of the pH.

FCCP and sodium cyanide were obtained from Sigma; fructose 1,6-diphosphate (F1,6-DP) and 2DG from ICN Biochemicals.

Analysis

Net Ca^{2+} currents were evaluated following scaled linear subtraction of leakage and capacitative currents. Ca^{2+}-activated currents ($I_{Cl(Ca)}$) were assessed by measurement of the current amplitude 20 ms after the end of the voltage step command and by the time to reach 63% decay of the maximal inward current amplitude ($t_{63\%}$). An estimation of the total charge flow (Q) for each tail current was also made by measurement of the area under the tail current decay curve using an integration programme for tail currents of less than 4 s while for longer tail currents the traces were weighed. Since there was a wide distribution of tail current total charge flow these values have also been normalised (Q%) to give values as percentages of the response to the first voltage step command in each series.

All data are given as mean ± standard error of the mean (s.e.mean) and statistical significance has been assessed by a two-tailed Student's *t* test.

Results

Inhibition of oxidative phosphorylation

FCCP Extracellular application of the proton ionophore FCCP (5 μM) for 3 to 5 min by low pressure ejection caused a reduction in the steady state peak Ca^{2+} current amplitude (I_{max}) and the amplitude of the current measured at the end of a 100 ms voltage step command (I_{end}) when DRG neurones were activated from a holding potential (V_h) of −90 mV by voltage step commands to a clamp potential (V_c) of 0 mV (Table 1). This represents a 47% and 50% reduction in I_{max} and I_{end} respectively. The degree of inactivation of the currents was unaffected by FCCP (5 μM), with 54% inactivation of control currents and 51% inactivation occurring in the presence of FCCP, measured during a 100 ms voltage step command ($n = 8$). In five cells, incomplete recovery was seen 5 to 10 min after removal of the pipette containing FCCP (Table 1). The I_{max} recovered to 66% of its original value while I_{end} recovered to 81% of its original value.

Brief application (10–20 s) of FCCP (5 μM) reduced both I_{max} and I_{end} to the same extent as when applied until a steady state value had been reached as described above, I_{max} being reduced by 43% and I_{end} by 49% (Table 1). The recovery in all three cells was to 77% and 70% of the original value for I_{max} and I_{end} respectively. Hence the reduction in current amplitude occurs rapidly with no change in the degree of inactivation.

Low-voltage-activated Ca^{2+} currents, produced by stepping to a V_c of −30 mV, were not specifically studied in this investigation but on the occasions when they were seen, the

Table 1 Action of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP, 5 μM) on voltage-activated Ca^{2+} currents

Conditions	(n)	I_{max} (nA)	I_{end} (nA)
Control	8	−2.39 ± 0.27	−1.31 ± 0.23
3–5 min application	8	−1.27 ± 0.36**	−0.65 ± 0.26*
recovery	5	−1.59 ± 0.45	−1.06 ± 0.33
Control	3	−1.97 ± 0.24	−0.91 ± 0.16
10–20 s application	3	−1.14 ± 0.35	−0.46 ± 0.06

I_{max} and I_{end} were the Ca^{2+} current amplitude measured at the peak and end of 100 ms voltage step commands to 0 mV respectively. **$P < 0.005$; *$P < 0.02$ when compared with control values.

low voltage-activated component was also inhibited (Figure 1).

The current-voltage relationship for a cell under control conditions and after application of FCCP (5 μM) is shown in Figure 1. There was no shift in the voltage-dependence of activation and no change in the null potential (the potential at which there is no net current flow through the membrane).

To ensure that the effect seen with FCCP was not due to an increase in Ca^{2+} mediated inactivation we increased the EGTA concentration of the patch pipette solution to 20 mM. Under these conditions FCCP (5 μM) applied for 3 min inhibited I_{max} and I_{end} by 32% and 41% respectively ($n = 7$, $P < 0.02$). Additionally no FCCP-induced currents were seen during any of the experiments. These findings suggest that the major action of FCCP in reducing I_{Ca} is not by a Ca^{2+}-induced Ca^{2+} release mechanism.

In three cells to which FCCP (5 μM) was applied, Ca^{2+}-activated Cl^- tail currents were seen. The mean amplitude of the $I_{Cl(Ca)}$ measured 20 ms after the 100 ms voltage step command was -1.76 ± 0.54 nA under control conditions and -0.61 ± 0.16 nA after application of FCCP (5 μM) ($n = 3$). FCCP had no significant action on the decay of $I_{Cl(Ca)}$. However, this action was associated with a reduction in mean I_{max} of 58% and therefore much less Ca^{2+} was available to activate $I_{Cl(Ca)}$.

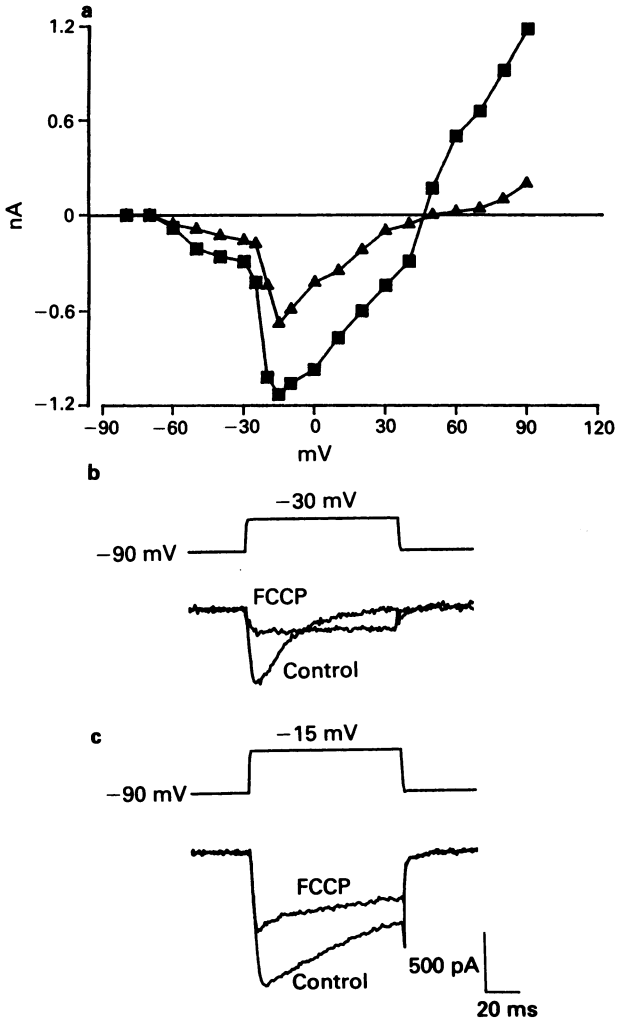

Figure 1 The actions of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) on the peak voltage activated Ca^{2+} currents. (a) The current-voltage relationship for a neurone recorded under control conditions (■) and after 3 to 5 min application of 5 μM FCCP (▲). The maximum peak inward current is plotted after leak subtraction for each voltage step. Note that FCCP reduces Ca^{2+} channel currents over a wide voltage range without producing any shift in voltage dependence. Inset traces show, (b) low voltage-activated, T-type current and (c) high voltage-activated current activated from a holding potential of −90 mV by step depolarizations to −30 mV and −15 mV respectively. FCCP appears to affect the inactivation properties of the low threshold current as well as reduce the peak amplitude of high threshold Ca^{2+} currents.

In three cells the steady state inactivation kinetics were studied for both I_{max} and I_{end}. The potentials at which 50% steady-state inactivation occurred ($V_{0.5}$) for I_{max} and I_{end} after application of FCCP (5 μM) were not significantly different from values obtained under control conditions.

Similar results to those described above were obtained in three cells in which the concentration of FCCP used was 10 μM. I_{max} was reduced from -2.85 ± 0.47 nA to -1.47 ± 0.51 nA and I_{end} was reduced from -1.69 ± 0.44 nA to -1.05 ± 0.54 nA (48% reduction in I_{max} and 38% reduction in I_{end} with 65% and 81% recovery at 5 min respectively, $n = 3$).

More prolonged exposure to FCCP (5 μM) was studied by pretreating the cells with FCCP in the recording medium for 10 or 30 min or by incubation in the culture medium for 18 h. The cells were washed prior to an experiment and recording of Ca^{2+} currents made in normal recording medium in the absence of FCCP. After 10 or 30 min pretreatment I_{max} was measured every minute for 5 min and at 3 min intervals thereafter. There was no significant change in I_{max} nor in the degree of inactivation over the course of 8 min recording (Figure 2).

Eighteen hours incubation of cells with FCCP (5 μM) in the culture medium again did not give rise to any significant change in I_{max} nor in the degree of inactivation when currents were activated using the same protocol as above (inactivation of 44% and 45% at 1 and 5 min respectively, $n = 7$; Figure 2). However, after 18 h in culture with FCCP (5 μM) four out of seven cells were unable to tolerate the patch clamp conditions for more than 5 min. In the remaining three cells 'run-down' of the current over the course of 15 min recording occurred at a similar rate to that which would be expected under control conditions.

Cyanide The effects of sodium cyanide (1 μM) were studied in DRG neurones by both intra- and extracellular application.

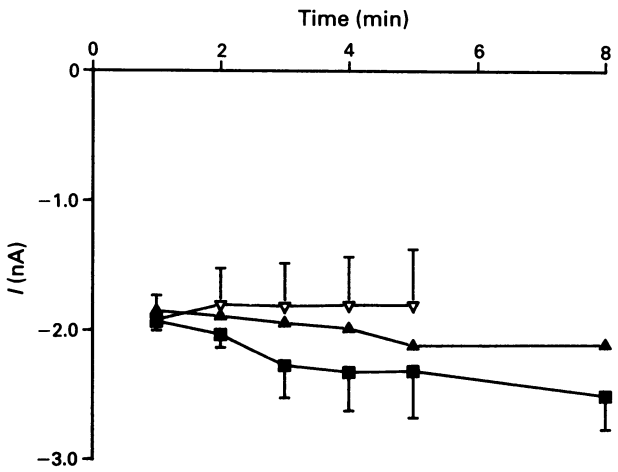

Figure 2 The effects of prolonged application of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) on peak high voltage-activated Ca^{2+} currents. (a) Graph of time plotted against mean peak Ca^{2+} current recorded from cells pretreated with 5 μM FCCP for 10 min (■, $n = 3$), 30 min (▲, $n = 3$) and 18 h (▽, $n = 7$). Surprisingly, long term treatment with FCCP has no significant effect on Ca^{2+} currents recorded from cultured DRG neurones. The apparent increase in current during 8 min recording from cells treated for 10 min with FCCP was not signigicant. However, this may represent some additional recovery from block with FCCP.

Extracellular application of cyanide (1 μM) by low pressure ejection caused a reduction in the mean I_{max} and mean I_{end} of the Ca^{2+} current (Figure 3). The percentage inhibition of I_{max} and I_{end} was 58% ($P<0.01$) and 27% ($P<0.02$) respectively ($n = 8$). The mean degree of inactivation was 59% for control currents and 28% in the presence of cyanide ions (1 μM) which may reflect less inactivation produced by smaller Ca^{2+} currents or some degree of differential sensitivity of different voltage-activated Ca^{2+} channels with distinct inactivation properties. On removing the pressure ejection pipette no significant recovery in 10 min was seen in any component of the Ca^{2+} current ($n = 8$) (Figure 3).

Intracellular application was achieved by inclusion of sodium cyanide (1 μM) in the patch pipette solution. Calcium currents were activated by 100 ms voltage step commands from a V_h of −90 mV to a V_c of 0 mV immediately on entering the whole cell configuration and at 1 min intervals thereafter for 4 min. Over this time I_{max} was reduced from −1.52 ± 0.17 nA to −1.06 ± 0.20 nA, a mean reduction in maximal Ca^{2+} current amplitude of 30% ($n = 12$, $P<0.02$) and I_{end} was reduced from −0.77 ± 0.12 nA to −0.66 ± 0.16 nA, a 14% reduction in mean I_{end} ($n = 12$). The degree of inactivation of the currents on first entering the whole cell configuration was 49% while that after 4 min recording was 38%, suggesting that the inactivation kinetics were unaffected.

The initial results with both cyanide and FCCP demonstrated that, in cultured DRG neurones, Ca^{2+} homeostatic mechanisms were surprisingly resistant to the effects of metabolic blockade with these agents. However, since ATP was present in the patch solution and because of the rapid onset and achievement of a steady-state Ca^{2+} current inhibition and, in the case of FCCP, rapid but partial recovery was seen, we suggest that both drugs may interact with Ca^{2+} channels or membrane constituents which result in inhibition of the current. Both low and high voltage-activated Ca^{2+} currents were attenuated by FCCP and cyanide. Steady state inactivation of the high voltage-activated Ca^{2+} currents was unaffected. These findings indicate that FCCP and cyanide may interact in a nonselective manner rather than with a distinct type of Ca^{2+} channel. For these reasons we changed the approach to producing metabolic stress by inhibiting glycolysis using 2-deoxyglucose with no ATP or glucose in the recording solutions.

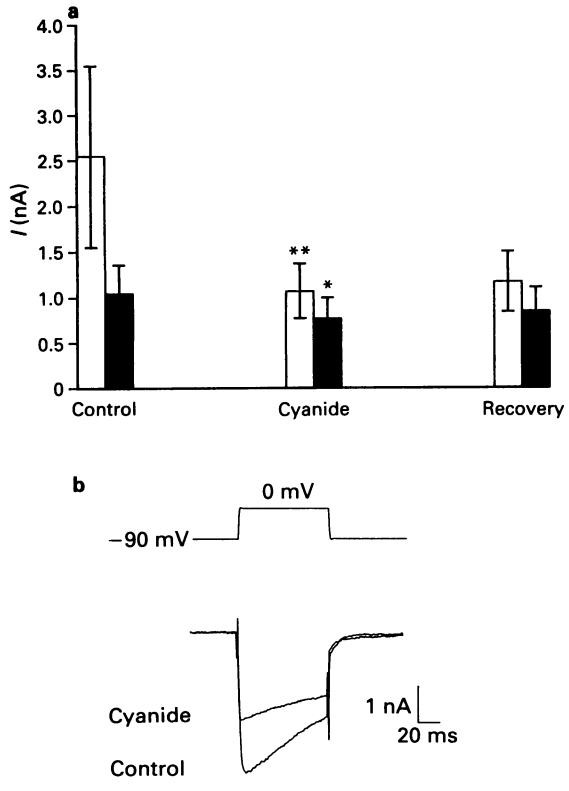

Figure 3 The effect of 1 μM sodium cyanide on high voltage-activated Ca^{2+} currents. (a) Chart showing the inhibitory action of cyanide on high voltage-activated Ca^{2+} currents measured at the peak (I_{max}, open columns) and at the end of the 100 ms voltage step command (I_{end}, solid columns). Data shown are the mean ± s.e.mean from eight experiments. Cyanide (1 μM) was applied extracellularly for 3 to 5 min and no recovery was observed 5 min after removal of the pressure ejection pipette containing cyanide. *$P<0.02$; **$P<0.01$. (b) Traces showing high voltage-activated Ca^{2+} currents recorded under control conditions and following inhibition by cyanide (1 μM). All data was recorded from cells voltage clamped at a holding potential of −90 mV.

Inhibition of glycolysis

No ATP/glucose Experiments were performed on DRG neurones bathed, for between 30 min and 3 h, in recording medium in which no glucose was present, the osmolarity being corrected with mannitol and with patch pipette solution in which ATP was excluded. Again Ca^{2+} currents were activated on entering the whole cell recording configuration and every 3 min thereafter by 100 ms voltage step commands from a V_h of −90 mV to a V_c of 0 mV. No significant change in either I_{max} or I_{end} was seen in the course of 15 min recording (I_{max} −1.79 ± 0.28 nA, I_{end} −1.08 ± 0.26 nA at 3 min; I_{max} −1.82 ± 0.30 nA, I_{end} −0.98 ± 0.42 nA at 15 min, $n = 6$).

2-Deoxy-D-glucose Neuronal glycolysis was also inhibited by the inclusion of 2DG (5 mM) in the glucose-free recording medium. Calcium currents and Ca^{2+}-activated currents were activated by both 100 ms and 500 ms depolarizing voltage step commands again using patch pipette solution without ATP. For each cell, 100 ms depolarizing voltage steps were applied immediately on entering the whole cell recording configuration and at 15 s intervals for 1 min. This was followed after a further minute by 500 ms depolarizing steps at 1 min intervals. Voltage-activated Ca^{2+} currents were studied in addition to Ca^{2+}-activated Cl^- tail currents (Currie & Scott, 1992) measured 20 ms after the end of the depolarizing step command ($I_{Cl(Ca)}$) and by an assessment of the time to 63% decay of this current ($t_{63\%}$) and of the total charge flow during the tail current (Q). Calcium-activated tail currents were seen in approximately 52% (16/31 cells) of cells studied.

In control cells with normal patch and recording media without 2DG, consecutive 100 ms depolarizing voltage step commands every 15 s caused no significant change in the properties of voltage-activated Ca^{2+} currents produced at a V_c of 0 mV ($n = 11$). Likewise with 2DG present, four 100 ms depolarizing voltage steps had no affect on Ca^{2+} currents ($n = 11$). The amplitude of the $I_{Cl(Ca)}$ measured 20 ms after the end of the 100 ms voltage step command was not significantly altered by the activation of consecutive steps. Under control conditions the mean amplitude of $I_{Cl(Ca)}$ changed from −1.09 ± 0.26 nA to −1.18 ± 0.30 nA, and with 2DG present $I_{Cl(Ca)}$ had values of −1.29 ± 0.18 nA and −1.11 ± 0.19 nA at the 1st and 4th steps respectively ($n = 11$). Additionally the times to 63% decay ($t_{63\%}$) of $I_{Cl(Ca)}$ were not significantly changed by 2DG when the current was activated following a 100 ms Ca^{2+} current. Under control conditions $t_{63\%}$ was 680 ± 180 ms and 860 ± 240 ms ($n = 11$) and with 2DG present the mean values were 850 ± 240 ms and 1090 ± 280 ms ($n = 11$) for the 1st and 4th steps respectively. Again the total charge flow (Q) during the full time course of the tail current activated by a 100 ms voltage step command was not significantly altered for both control cells and those treated with 2DG. These data show that 2DG has no significant action on Ca^{2+} currents activated by a 100 ms voltage step command nor on the accompanying Cl^- tail current. As a result, we decided to increase the intracellular

Table 2 Comparison of Ca^{2+} currents and Ca^{2+}-activated Cl^- currents recorded under control conditions and with 2-deoxy-D-glucose (2DG, 5 mM)

Step No.	I_{max} (nA)	I_{end} (nA)	$I_{Cl(Ca)}$ (nA)	$t_{63\%}$ (ms)
Control $n = 7$				
1	-1.10 ± 0.22	-0.49 ± 0.10	-1.66 ± 0.45	2990 ± 660
2	-1.01 ± 0.20	-0.42 ± 0.09	-1.38 ± 0.36	3140 ± 730
3	-1.00 ± 0.20	-0.43 ± 0.09	-1.31 ± 0.34	3040 ± 750
4	-0.96 ± 0.19	-0.41 ± 0.08	-1.29 ± 0.36	2800 ± 690
2-Deoxyglucose (5 mM) $n = 11$				
1	-1.19 ± 0.19	-0.31 ± 0.10	-1.25 ± 0.18	4000 ± 800
2	-1.10 ± 0.20	-0.25 ± 0.09	-1.14 ± 0.18	5580 ± 1100*
3	-0.99 ± 0.18	-0.18 ± 0.10	-1.03 ± 0.17	7610 ± 1790*
4	-0.94 ± 0.17	-0.14 ± 0.10	-0.99 ± 0.18	7350 ± 2080*

I_{max} and I_{end} were the Ca^{2+} current amplitude measured at the peak and end of 500 ms voltage step commands to 0 mV. $I_{Cl(Ca)}$ was the tail current amplitude measured 20 ms after the end of the voltage step command and $t_{63\%}$ was the time for the tail current to deactivate by 63% of $I_{Cl(Ca)}$.
*$P < 0.05$, values compared with step no. 1 with 2DG.

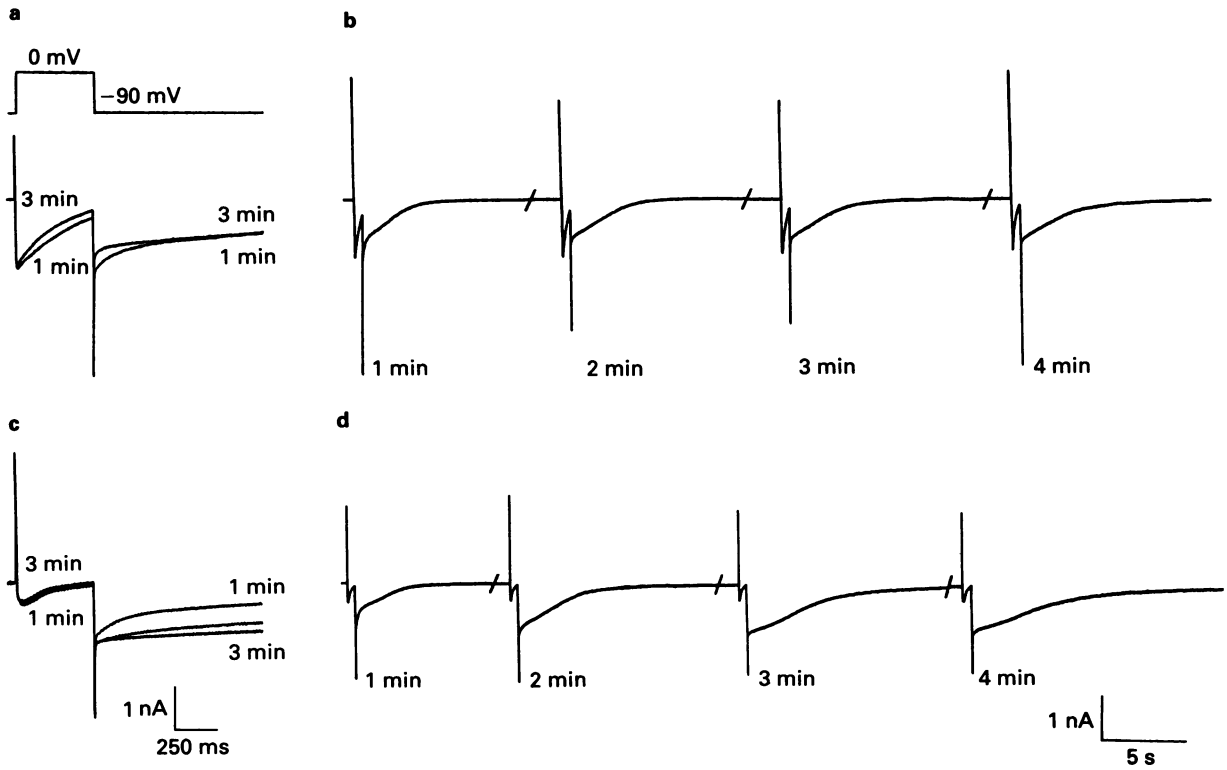

Figure 4 Action of 2-deoxyglucose on voltage-activated Ca^{2+} currents and Ca^{2+}-activated Cl^- tail currents. (a) Control currents at 1 and 3 min expanded to show the Ca^{2+} current activated by a 500 ms voltage step command and the initial part of the Cl^- tail current. (b) Series of four currents activated at a frequency of one per min under control conditions. There was no significant change in the Ca^{2+} current and Cl^- tail current. (c) Expanded current records at 1, 2 and 3 min showing no significant change in the Ca^{2+} current but that the tail current deactivation was prolonged by 5 mM 2-deoxyglucose. The tail current at 2 min is the middle record. (d) Series of four currents activated at a frequency of one per min in the presence of 2-deoxyglucose. The Cl^- tail currents were greatly prolonged but the Ca^{2+} currents were not changed significantly. All Ca^{2+} currents were activated from a holding potential of −90 mV by 500 ms voltage step commands to 0 mV. The records presented are not leak subtracted.

Ca^{2+} load by increasing the duration of the voltage-activated Ca^{2+} current to 500 ms.

When 500 ms voltage step commands were applied to DRG neurones every minute, under both control conditions and in the presence of 2DG (5 mM), again there was no significant difference in the values of I_{max} or I_{end} for high voltage-activated Ca^{2+} currents nor for the amplitude of $I_{Cl(Ca)}$ between the first and fourth events (Table 2, Figure 4). However, the $t_{63\%}$ values for $I_{Cl(Ca)}$, in the presence of 2DG (5 mM) became greatly prolonged when compared with controls which remained almost unchanged. The values of the total charge flow showed a significant increase between the first and second steps from 4.4 ± 1.0 nC to 5.5 ± 0.9 nC in the presence of 2DG (5 mM) ($n = 11$, $P < 0.001$) while control values were not significantly affected (Figures 4 and 5).

Finally, in order to investigate whether protracted exposure to 2DG would enhance this prolongation of the decay time to 63% of Ca^{2+}-activated Cl^- tail currents, DRG neurones were incubated in culture for 18 h with 2DG (5 mM). The same recording conditions and voltage command protocols were used as for the previous experiments with 2DG. Only one small amplitude (720 pA) Ca^{2+}-acti-

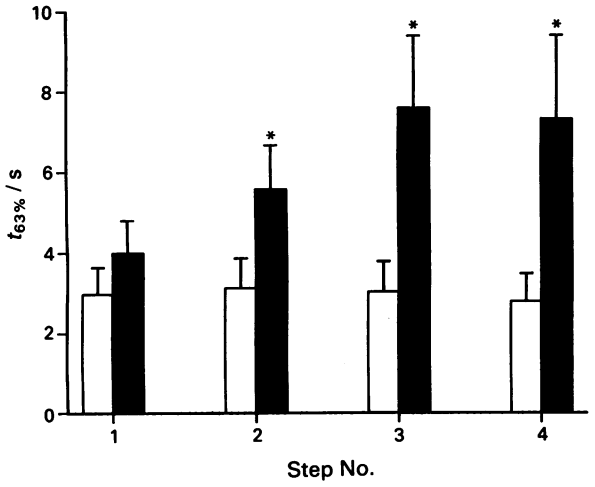

Figure 5 Actions of 2-deoxyglucose on Cl^- tail current decay following activation by a 500 ms high voltage-activated Ca^{2+} current. Chart shows time for Cl^- tail currents to deactivate by 63% ($t_{63\%}$) when activated by four 500 ms Ca^{2+} currents evoked at a rate of one per min. Control data (open columns, $n = 7$) shows no significant change in current decay but in the presence of 5 mM 2-deoxyglucose (solid columns, $n = 11$) Cl^- tail current deactivation is slowed as reflected by the increase in $t_{63\%}$. All data shown are means ± s.e.mean for seven control and 11 2-deoxyglucose experiments. $^*P < 0.05$.

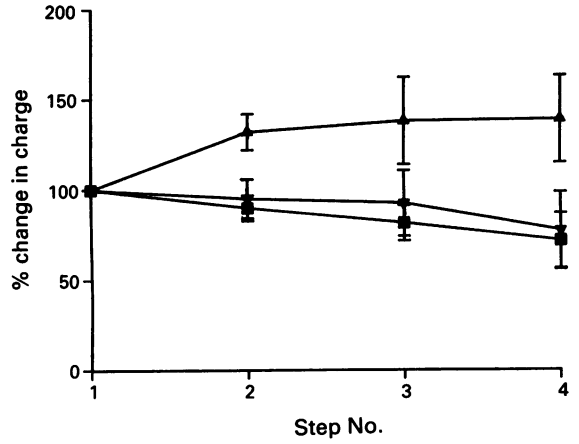

Figure 6 Percentage change in total charge flow during Ca^{2+}-activated Cl^- tail currents following consecutive 500 ms high voltage-activated Ca^{2+} currents. Four 500 ms voltage steps were applied to activate Ca^{2+} currents at a rate of one per min. The step number is plotted against the percentage change in charge relative to the total charge flow during the first Ca^{2+}-activated Cl^- tail current (100%). Under control conditions (■, $n = 7$) and in the presence of fructose 1,6-diphosphate (500 μM) with 2-deoxyglucose (5 mM, $n = 7$) (▼) the mean percentage change in charge declined slightly. In contrast, with 2-deoxyglucose alone (5 mM, $n = 11$, ▲) the percentage change in charge increases relative to both control and fructose 1,6-diphosphate data. Mean percentage change ± s.e.mean are presented.

vated Cl^- tail current was seen in 23 recorded cells. The degree of 'run-down' of I_{max} and I_{end} after consecutive 100 ms voltage step commands was similar to that after brief exposure (12% of I_{max} and 14% of I_{end} after brief exposure, $n = 11$ and 11% of I_{max} and 10% of I_{end} after 18 h exposure, $n = 23$). The degree of inactivation during a 100 ms voltage step command was unaffected by prolonged exposure to 2DG.

Voltage step commands of 500 ms duration were also applied to cells at 1 min intervals after exposure to 2DG (5 mM) for 18 h. Once more, 'run down' in relatively small Ca^{2+} currents was seen with a fall in I_{max} of 37% (from -0.76 ± 0.09 nA to -0.48 ± 0.06 nA, $n = 18$) over the course of 4 min and a reduction of I_{end} by 59% over the same time (-0.22 ± 0.05 nA to -0.09 ± 0.03 nA, $n = 18$). As expected a greater degree of inactivation occurred with the 500 ms depolarizing steps than with 100 ms steps (71% and 81% for the 1st and 4th steps respectively for 500 ms steps compared with 36% and 36% for 100 ms steps).

Fructose 1,6-diphosphate In order to test the ATP dependence of the tail current prolongation and total charge increase seen in the presence of 2DG (5 mM) and absence of glucose or ATP in the patch pipette solution, we performed similar experiments to those described above with the inclusion of fructose, 1,6-diphosphate (500 μM) (F1,6-DP) in the patch pipette solution with 2DG (5 mM) present (again with ATP and glucose excluded). F1,6-DP is a downstream metabolite in the glycolytic pathway, the substrate for aldolase (fructose 1,6-diphosphate: D-glyceraldehyde 3-phosphate lyase) and hence beyond the glycolytic block produced by 2DG. We have used 500 μM F1,6-DP (K_{eq} for this enzyme is approximately 750 μM; Bergmeyer & Bernt, 1974) in order to ensure a suitable supply of substrate and to produce a reasonable diffusion gradient from the patch pipette into the cell. Again 500 ms depolarizing voltage step commands were applied every minute to activate high threshold Ca^{2+} currents and Ca^{2+}-activated Cl^- tail currents. Although some 'run-down' in I_{max} was seen and relatively small total charge flow values for the tail currents were observed (Q at 1 min: 3.9 ± 1.1 nC and 2.1 ± 0.7 nC at 4 min) there was no significant change in I_{end}, $I_{Cl(Ca)}$, $t_{63\%}$ nor in Q ($n = 7$) during the 4 voltage step commands. When the total charge flow was evaluated as a percentage of the initial value for each cell (Q%) the rate of decline in Q% for both controls and F1,6-DP with successive steps was remarkably similar whereas the values for 2DG alone show a substantial increase (Figure 6). These data suggest that in the presence of F1,6-DP the effect of 2DG in prolonging tail current decay time and in increasing total charge flow of the Cl^- tail currents is bypassed, possibly due to the return of the ability to synthesize ATP and hence maintain intracellular Ca^{2+} homeostasis as reflected in the Cl^- tail current parameters.

Discussion

The reduction of an adequate supply of ATP to neurones as occurs clinically in hypoxia, ischaemia or hypoglycaemia may precipitate the loss of intracellular Ca^{2+} homeostasis and the subsequent triggering of a damaging cascade of Ca^{2+}-dependent events leading ultimately to neuronal death (Siesjö, 1981). The two approaches used in this study to induce metabolic stress: inhibition of mitochondrial oxidative phosphorylation and inhibition of glycolysis, demonstrate several important points for the future evaluation of the effects of chemical hypoxia on Ca^{2+} homeostasis. (i) Primary cultures of neonatal rat DRG neurones are remarkably resistant to metabolic stress. (ii) Agents, such as FCCP and cyanide, used to induce chemical hypoxia may themselves interact with Ca^{2+} channels or with intracellular Ca^{2+} homeostatic mechanisms in a reversible manner making them unsuitable agents for the production of chemical hypoxia while studying whole cell Ca^{2+} currents and Ca^{2+}-dependent currents. (iii) Given suitable metabolic stress, such as exposure to 2DG, Ca^{2+} homeostatic mechanisms may be disturbed sufficiently to modulate Ca^{2+}-dependent currents, without directly interacting with either the voltage-activated Ca^{2+} channels or with Ca^{2+}-activated Cl^- channels.

Calcium-activated Cl^- tail currents are observed as slowly decaying inward currents in about 50% of cultured dorsal root ganglion (DRG) neurones loaded with chloride. The duration of the Cl^- tail current is in part determined by the

amount of Ca^{2+} entry through voltage-activated Ca^{2+} channels (Mayer, 1985; Bader *et al.*, 1987; Akasu *et al.*, 1990; Currie & Scott, 1992) but is also modulated by the efficiency with which Ca^{2+} homeostatic mechanisms handle the intracellular Ca^{2+} load (Currie & Scott, 1992; Stapleton *et al.*, 1992). Calcium-activated Cl^- conductances can also be observed following very modest Ca^{2+} entry during a single action potential recorded in the absence of K^+ channel blockers (Mayer, 1985). Furthermore, $I_{Cl(Ca)}$ can be used to identify changes in intracellular Ca^{2+} produced by release of Ca^{2+} from intracellular stores (Currie & Scott, 1992) and to detect photoreleased Ca^{2+} from DM-nitrophen (Currie & Scott, 1993). Recording $I_{Cl(Ca)}$ has a number of benefits including the fact that experiments can be conducted in the presence of K^+ channel blockers so that voltage-activated Ca^{2+} currents can be measured free from contamination by voltage- and Ca^{2+}-activated K^+ conductances which would partially mask the Ca^{2+} currents. Additionally, the reversal potential for $I_{Cl(Ca)}$ is 0 mV under our recording conditions, so high voltage-activated Ca^{2+} currents (activated on stepping to 0 mV) are not contaminated by Cl^- current, which is not observed until repolarization. On repolarization, at the end of the voltage step command, there is a substantial driving force of 90 mV for Cl^- to leave the cell. Therefore Ca^{2+} currents, which reflect the Ca^{2+} load, can be accurately measured under control and experimental conditions and compared with the properties of $I_{Cl(Ca)}$.

Primary cultures of neonatal rat DRG neurones appear to be highly resistant to metabolic stress as reflected by their ability to survive even after long term (up to 18 h) exposure to FCCP and by the apparent lack of disturbance in Ca^{2+} homeostasis as assessed by the degree of inactivation of Ca^{2+} current (Chad & Eckert, 1986). Our data suggest that primary cultures of DRG neurones function on low levels of ATP and can be independent of oxidative phosphorylation. This may not apply to freshly dissociated cells as studied by Duchen and colleagues (Duchen, 1990; Duchen *et al.*, 1990) who found altered intracellular Ca^{2+} homeostasis with FCCP (5 μM) and cyanide (2 mM). It is probable also that central neurones with selective vulnerability to hypoxia such as hippocampal CA1 neurones have a greater sensitivity to such metabolic insults (Simon *et al.*, 1984). Another possibility for the apparent insensitivity of these cultures to chemical hypoxia is that they are neonatal in origin and as such might be expected to withstand more prolonged exposure to hypoxic conditions (Kass & Lipton, 1989; Krnjevic *et al.*, 1989; Friedman & Haddad, 1993). However Kostyuk and colleagues (1993) have recently demonstrated that run-down of Ca^{2+} currents in rat neonatal DRG neurones appears to be more ATP-sensitive than in those seen in adult and aging neurones.

Single electrode voltage clamp studies on hippocampal slice preparations (Krnjevic & Leblond, 1989), dissociated mouse sensory neurones (Duchen, 1990) and in guinea-pig ventricular myocytes (Goldhaber *et al.*, 1991) confirm a reduction in Ca^{2+} current in response to chemical hypoxia and nitrogen perfusion as seen in our experiments. However, care must be taken in the interpretation of changes in Ca^{2+} current due to induced chemical hypoxia since the agents used may themselves interact with Ca^{2+} channels as seems probable with FCCP, since the effects of this substance on Ca^{2+} currents in our system have a rapid onset of action (10–20 s) and are readily reversible even after 18 h exposure. Additionally FCCP rapidly inhibited Ca^{2+} currents even when ATP and HEPES were present in the patch solution, suggesting that the action was not due to reduced intracellular ATP or pH.

A Ca^{2+}-dependent K^+ conductance has been demonstrated during hypoxia in hippocampal slice preparations (Fujiwara *et al.*, 1987; Krnjevic & Leblond, 1989) and in dissociated mouse sensory neurones (Duchen, 1990). Duchen (1990) also described an increased Ca^{2+}-dependent Cl^- conductance in response to metabolic blockade. We have demonstrated a prolongation of a Ca^{2+}-dependent Cl^- current following inhibition of glycolysis with 2DG in rat DRG neurones. This effect suggests that given a suitably severe metabolic insult, Ca^{2+} homeostatic mechanisms begin to fail, possibly due to the lack of ATP. This concept is supported by the finding that in the presence of F1,6-DP, a glycolytic intermediary which bypasses the 2DG-induced block, the tail current changes are no longer seen, suggesting a restoration of ATP synthesis and hence of Ca^{2+} homeostasis. However, tail current amplitudes and decay time (and hence total charge) were highly variable between different cells, possibly reflecting variations in the number of Ca^{2+}-dependent Cl^- channels present, in channel distribution or in their threshold for activation. These channels may also require phosphorylation for proper function by analogy with Ca^{2+} channels themselves (Armstrong & Eckert, 1987) and differences in resting $[Ca^{2+}]_i$ may affect the degree to which this occurs.

Another interesting finding is the virtual absence of tail currents, after prolonged (18 h) exposure to 2DG in culture. This may reflect down-regulation of Ca^{2+}-activated Cl^- channels or a change in their properties resulting in reduced availability, and may be analogous to the dependence of Ca^{2+} channels on phosphorylation for their normal function (Armstrong & Eckert, 1987) but may also be due to an increase in the activity of a Ca^{2+}-dependent phosphatase. Our data suggest that Ca^{2+}-activated Cl^- tail currents are more sensitive to metabolic stress than Ca^{2+} currents themselves.

This study has demonstrated that in primary cultures of neonatal rat DRG neurones, prolongation of the decay time of Ca^{2+}-activated Cl^- tail currents and an increase of the total charge flow occurs in the presence of 2DG after removal of both glucose and ATP from the recording solutions. We believe that the use of $I_{Cl(Ca)}$ and 2DG may prove useful in the study of changes in intracellular Ca^{2+} produced by metabolic stress. Further study will be directed at cells with a greater sensitivity to metabolic stress.

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