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# Internal $Ca^{2+}$ stores involved in anoxic responses of rat hippocampal neurons

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1. During whole-cell recordings from CA1 neurons of rat brain slices with electrodes containing only $KMeSO_4$ and Hepes, brief anoxia (2–3 min) consistently evoked a hyperpolarization ($\Delta V \approx -4$ mV) and reduction in input resistance ($\Delta R \approx -20\%$).

2. As in previous intracellular recordings, Dantrolene sodium (10 $\mu$M) suppressed the anoxic $\Delta V$ and $\Delta R$, confirming that the release of internal $Ca^{2+}$ is a major component of the anoxic response.

3. To identify the relevant intracellular $Ca^{2+}$ store, other blockers of $Ca^{2+}$ release were applied either externally (in the bath) or internally, by addition to the contents of the recording electrode.

4. The anoxic hyperpolarization was abolished or much reduced by heparin (10–20 $\mu g\ ml^{-1}$, internal), thapsigargin (10 $\mu$M, external), Ruthenium Red (50 $\mu$M, internal) and *external* procaine (0·5–2 mM), but not by *internal* procaine (0·5–1 mM) or ryanodine (10 $\mu$M, external).

5. The anoxic fall in resistance was also abolished or reduced by heparin, thapsigargin and *external* procaine, but not by ryanodine, *internal* procaine or Ruthenium Red.

6. In addition, external procaine (0·5–2 mM) eliminated the early (transient) depolarization and reduced the post-anoxic hyperpolarization by $60 \pm 22\%$.

7. None of these agents consistently changed the resting potential, but the input resistance was significantly increased by Dantrolene and external procaine.

8. In view of the marked effects of heparin and thapsigargin, but not ryanodine and internal procaine, we conclude that the anoxic response seen in such whole-cell recordings is initiated predominantly by $Ca^{2+}$ release from an internal store that is $InsP_3$ sensitive rather than $Ca^{2+}$ sensitive.

9. Comparable but less pronounced effects of external procaine were seen during intracellular recordings with 3 M KCl-containing electrodes. The dose-dependent suppression of various features of the anoxic response by external procaine ($EC_{50} \approx 0{\cdot}2$ mM) is presumed to be mediated by a superficial membrane trigger or modulating site.

In many types of cells, the increase in cytosolic free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) caused by $Ca^{2+}$ influx is greatly magnified by $Ca^{2+}$ release from internal stores, either from sarcoplasmic reticulum in muscle (Endo, 1977) or endoplasmic reticulum in other tissues (Tsien & Tsien, 1990; Henzi & MacDermott, 1992). In addition to this well-established mechanism of $Ca^{2+}$-induced $Ca^{2+}$ release (CICR), $Ca^{2+}$ may be released by the action of inositol 1,4,5-trisphosphate ($InsP_3$): $InsP_3$-induced $Ca^{2+}$ release (IICR). Typically, $InsP_3$ is formed as a result of a ligand-triggered, G-protein-mediated activation of phospholipase C (Berridge & Irvine, 1984; Tsien & Tsien, 1990; Henzi & MacDermott, 1992). These two distinct (but perhaps not wholly independent) internal sources of $Ca^{2+}$ can generate or potentiate cytosolic $Ca^{2+}$ signals and various $Ca^{2+}$-dependent processes. There is evidence of both kinds of $Ca^{2+}$ pools in neurons (Henzi & MacDermott, 1992). Neurons in some peripheral ganglia seem to be particularly rich in CICR-type stores, judging by the marked effects of caffeine and their suppression by procaine.

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In the brain, both IICR and CICR stores are indicated by the widespread, though uneven, distribution of Ins$P_3$- and ryanodine-binding sites (Nakanishi, Kuwajima & Mikoshiba, 1992; Smith & Nahorski, 1993). In the hippocampus, both types are evident, but with Ins$P_3$ receptors predominating in CA1 and ryanodine receptors in CA3 (Nakanishi, Maeda & Mikoshiba, 1991; Nakanishi *et al.* 1992; but cf. Smith & Nahorski, 1993). This uneven distribution may account for the very different effects of caffeine on cultured hippocampal neurons reported by Murphy & Miller (1989) on the one hand and Dubinsky & Rothman (1991) on the other.

Our interest in the $Ca^{2+}$ stores of hippocampal neurons arose from an earlier finding (Krnjević & Xu, 1989) that Dantrolene sodium eliminates the hyperpolarization and conductance increase which characterize the response of hippocampal neurons to brief anoxia (Hansen, Hounsgaard & Jahnsen, 1982; Leblond & Krnjević, 1989). It has long been known that Dantrolene uncouples excitation from contraction in skeletal muscle by preventing $Ca^{2+}$ release from sarcoplasmic reticulum (Desmedt & Hainaut, 1977), though its precise site of action remains controversial (Endo, 1977; Danko, Kim, Sreter & Ikemoto, 1985).

Dantrolene can also suppress $Ca^{2+}$ release inside nerve cells, including hippocampal neurons (Dubinsky & Rothman, 1991; Segal & Manor, 1992). Though generally believed to act by suppressing CICR (Berridge & Irvine, 1984; Palade, Dettbarn, Alderson & Volpe, 1989; Henzi & MacDermott, 1992), Dantrolene also depresses IICR, for example in adrenal (Kojima, Kojima, Kreutter & Rasmussen, 1984) and hepatic cells (Mine, Kojima, Kimura & Ogata, 1987), as well as neocortical neurons (Frandsen & Schousboe, 1993).

To learn more about the internal store from which $Ca^{2+}$ is evidently released during anoxia, we examined the responses to anoxia during applications of some other blockers of $Ca^{2+}$ release: heparin, which binds to the Ins$P_3$ receptors and in this way prevents IICR, both in non-neuronal cells (Hill, Berggren & Boynton, 1987) and in central neurons (Palade *et al.* 1989; Markram & Segal, 1992); ryanodine and procaine, which block CICR (Endo, 1977; Henzi & MacDermott, 1992; McPherson & Campbell, 1993); thapsigargin, which empties various $Ca^{2+}$ stores by blocking $Ca^{2+}$ uptake (Thastrup, Cullen, Drobak, Hanley & Dawson, 1990); and Ruthenium Red, which prevents $Ca^{2+}$ uptake by mitochondria and suppresses CICR (Carafoli, 1987; Galione, 1993).

Most of these results have been summarized in an abstract (Belousov, Godfraind & Krnjević, 1994).

## METHODS

All experiments were performed on hippocampal slices from mature Sprague–Dawley rats (mostly 4–6 weeks old, weighing 100–150 g). After decapitation under full anaesthesia by an overdose of halothane, the brain was rapidly removed and placed in ice-cold, aerated saline (described below). Both hippocampi were dissected out, and 400–450 $\mu$m thick transverse slices were cut with a McIlwain tissue chopper or a vibroslicer (Campden Instruments, London). The slices were kept for at least 1 h at room temperature, in artificial cerebrospinal fluid (ACSF) containing the following (mM): 124 NaCl, 3·0 KCl, 2·0 $CaCl_2$, 1·25 $NaH_2PO_4$, 26 $NaHCO_3$ and 10 glucose, continuously aerated with 95% $O_2$–5% $CO_2$, at pH 7·3. A slice was then transferred to the experimental chamber; for whole-cell recordings, the slice adhered to a piece of lens paper, just submerged under flowing ACSF; for conventional intracellular recordings, the chamber was of the Haas interface type.

The patch electrodes had short shanks and tips of 2·5–3 $\mu$m diameter. The standard filling solution contained only 150 mM potassium methylsulphate ($KMeSO_4$) and 10 mM Hepes; the pH was adjusted to 7·25 with KOH. The osmolality was verified with a Precision Systems (Natick, MA, USA) micro-osmometer and kept between 287 and 300 mosmol $kg^{-1}$. After filling, the patch electrodes had resistances of 5–7 MΩ. They were inserted blindly into the CA1 stratum pyramidale, at an angle of ~45 deg. 'Giga-seal' cell contacts were readily obtained by appropriate suction; when sufficiently stable, they could usually be converted by further suction to satisfactory whole-cell recording, easily recognized by a large drop in time constant and resistance, the appearance of a substantial negative membrane potential and a large action potential (typically $\geqslant$100 mV). For intracellular recordings, the conventional microelectrodes were filled with 3 M KCl and had tip resistances of 60–80 MΩ.

All experiments were performed at 32·5–33 °C. The following drugs (all from Sigma) were applied in the bath: Dantrolene sodium (10 $\mu$M); procaine (0·05–2·0 mM); ryanodine (10 $\mu$M); and thapsigargin (1–10 $\mu$M). For internal applications, procaine (0·5–1·0 mM), heparin (5–20 $\mu$g $ml^{-1}$) or Ruthenium Red (50 $\mu$M) was added to the patch-electrode solution.

All these agents were tested on CA1 neurons recorded in the whole-cell mode; in addition, external procaine was also tested on eleven cells recorded with intracellular 3 M KCl electrodes. Bath applications were usually for 15–25 min. 'Anoxia' was induced by switching the aerating gas to 95% $N_2$–5% $CO_2$ for a period of 2–3 min. Such anoxic tests were repeated at intervals of at least 10 min. Data are given as means ± S.E.M. In the case of bath applications, mean control and test anoxic data were obtained from the same cell for paired comparisons. When drugs were added to the electrode contents, the control anoxic tests were performed on cells recorded with drug-free electrodes, either in the same slice or in experiments adjacent in time. Data were compared by Student's $t$ test, using paired data wherever possible.

## RESULTS

### Control data

Resting membrane properties of CA1 neurons and changes during brief anoxia (2–3 min) were recorded with patch electrodes containing only $KMeSO_4$ and Hepes.

#### Resting properties

As in a previous study with patch electrodes (Zhang & Krnjević, 1993), the whole-cell recordings were characterized by large action potentials (typically >100 mV), relatively high input resistances ($R_N$) and 'poor'

**Table 1. Applications of Dantrolene sodium (10 μM, in the bath), heparin (10–20 μg ml$^{-1}$, internal) and thapsigargin (10 μM, in the bath) reduce or abolish changes in potential and/or resistance evoked by brief anoxia**

| | $n$ | $\Delta V_m$ (mV) | $\Delta R_N$ (%) |
|---|---|---|---|
| Control | 3 | −3·8 ± 1·13 | −16·1 ± 3·80 |
| Dantrolene | 3 | −1·5 ± 1·04 (n.s.) | −4·1 ± 2·12 (n.s.) |
| Difference | | 2·3 ± 0·14* | −65·7 ± 23·00 |
| Control | 9 | −3·8 ± 0·72 | −20·5 ± 3·14 |
| Heparin | 9 | −0·27 ± 2·33 (n.s.) | −6·2 ± 3·69 (n.s.) |
| Control | 5 | −4·1 ± 0·96 | −24·2 ± 5·47 |
| Thapsigargin | 5 | −1·7 ± 1·43 (n.s.) | −10·9 ± 3·47 |
| Difference | | 2·4 ± 1·37 | 60·1 ± 7·88* |

Data are means ± S.E.M.; $n$, number of cells tested; $\Delta V_m$, changes in resting potential, and $\Delta R_N$ changes in input resistance, both during anoxia. For $\Delta V_m$, Difference is test − control values paired; for $\Delta R_N$, Difference is test values as a percentage of controls (paired); * $P < 0{\cdot}01$; n.s., not significantly different from 0.

resting potentials ($V_m$): in control recordings from thirty-four cells, mean $R_N$ (measured with hyperpolarizing pulses, as illustrated in Figs 1–4) was 73 ± 2·8 MΩ and mean $V_m$ was −57 ± 1·1 mV.

## Changes during brief anoxia

In contrast to the observations with gluconate-containing electrodes (Zhang & Krnjević, 1993), anoxia elicited in most cells (Figs 1–4) the characteristic sustained hyperpolarization, accompanied by a fall in input resistance ($R_N$), described previously (Hansen *et al.* 1982; Leblond & Krnjević, 1989). Most cells also showed an initial transient depolarization (TD) (Figs 3 and 4), as well as a delayed postanoxic hyperpolarization (PAH), seen immediately after the return of oxygen. In various control series, the resistance consistently fell by close to 20% (Tables 1 and 2), in keeping with the previous whole-cell recordings (Zhang & Krnjević, 1993). This is a much smaller change than the ~40% reduction in resistance commonly seen during similar anoxia with intracellular electrodes (Hansen *et al.* 1982; Leblond & Krnjević, 1989).

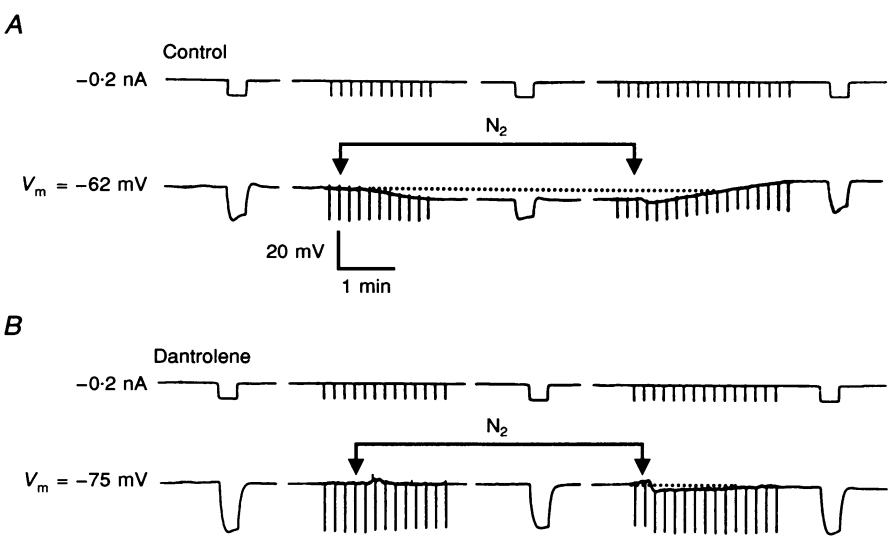


**Figure 1. Dantrolene suppresses anoxic hyperpolarization and associated fall in input resistance ($R_N$)**

Whole-cell recording from CA1 neurons, with electrodes containing only $KMeSO_4$ and Hepes. Lower traces in *A* and *B* record membrane potential (initial values are indicated at left) and upper traces, 200 ms current pulses applied at 10 s intervals to monitor $R_N$. Pulses are displayed on accelerated traces before, during and after 2·5 min period of anoxia (labelled $N_2$). *A* is a control run; note 6 mV hyperpolarization and $R_N$ drops from 90 to 48 MΩ (by 47%). In *B*, from another cell, during bath application of Dantrolene, there is no anoxic hyperpolarization and $R_N$ is reduced from 133 to 118 MΩ (by only 11%).

**Table 2. Minimal effects of ryanodine (10 μM, in the bath), procaine (0·5–1 mM, internal) and Ruthenium Red (50 μM, internal) on changes in potential and/or resistance evoked by brief anoxia**

| | $n$ | $\Delta V_m$ (mV) | $\Delta R_N$ (%) |
|---|---|---|---|
| Control | 6 | −3·8 ± 1.06 | −22·4 ± 4·31 |
| Ryanodine | 6 | −4·7 ± 1·44 | −15·9 ± 4·53 |
| Difference | | 0·88 ± 1·63 | −36·5 ± 18·4 |
| Control | 15 | −3·9 ± 1·46 | −16·2 ± 3·09 |
| Procaine | 16 | −4·1 ± 1·10 | −20·3 ± 3·85 |
| Control | 11 | −5·0 ± 2·08 | −20·3 ± 5·38 |
| Ruthenium Red | 28 | −1·2 ± 0·88 (n.s.) | −16·0 ± 3·06 |

Data are means ± S.E.M.; $n$, number of cells tested; $\Delta V_m$, changes in resting potential and $\Delta R_N$, changes in input resistance, both during anoxia. For $\Delta V_m$, Difference is test − control values paired; For $\Delta R_N$, Difference is test values as a percentage of controls (paired); n.s., not significantly different from 0.

The reversal potential for the anoxic hyperpolarization ($E_{rev}$), given by the intersection of voltage–current lines obtained from series of 200 ms current pulses, was −78·9 ± 2·7 mV, in good agreement with intracellular recordings (Leblond & Krnjević, 1989). The transient depolarization (TD), which often precedes the onset of the hyperpolarization, had a mean amplitude of 1·4 ± 0·15 mV. The post-anoxic hyperpolarization (PAH) – seen immediately after the return of oxygen – was also a very regular feature in the present recordings (−7·9 ± 0·53 mV).

## Effects of external or internal applications of various blockers of $Ca^{2+}$ release

$R_N$ was significantly higher during bath applications of 10 μM Dantrolene sodium (by 39 ± 2·67%; $n = 3$ sets of paired data, $P < 0·01$) and 0·5–1 mM procaine (by 71 ± 15·3%; $n = 8$ paired data, $P < 0·001$), but was unaffected by the other drugs; there were no consistent changes in $V_m$. With the exception of external procaine (as described below), most of the blockers had no significant effect on the transient depolarization, post-anoxic hyperpolarization or reversal level of the anoxic potential shift.

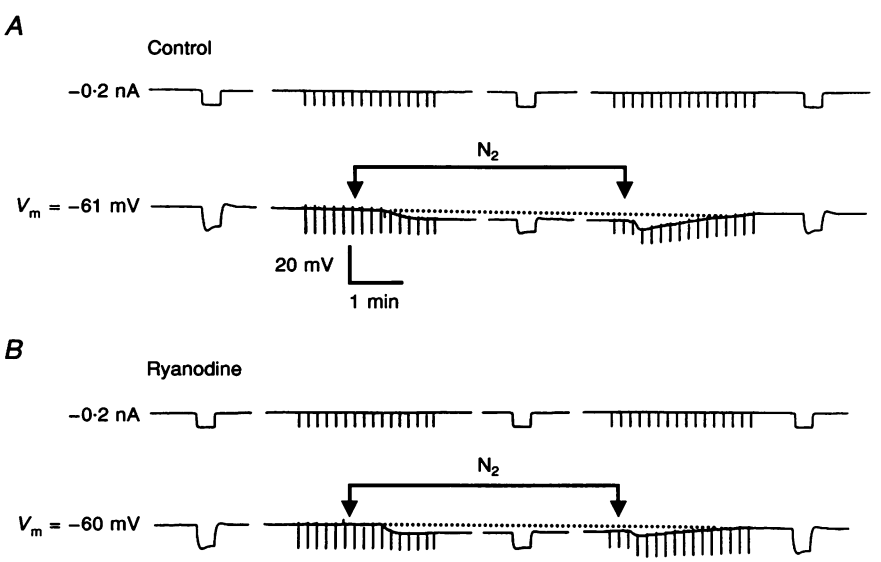


**Figure 2. Ryanodine has no consistent effects on anoxic potential and resistance changes**

Experimental conditions and description as for Fig. 1. In this case, both sets of traces were recorded from same neuron, before (*A*, Control) and during (*B*) ryanodine bath application. Note only marginally different $R_N$ values (68 and 66 MΩ), anoxic hyperpolarizations (6·5 and 5·0 mV) and resistance changes (−41 and −36%) in *A* and *B*.

### Dantrolene sodium

Only a few tests were performed, which confirmed the previous finding (with intracellular electrodes) that Dantrolene suppresses the anoxic response of CA1 neurons (Krnjević & Xu, 1989). As illustrated in Fig. 1, 10 $\mu$M bath applications abolished the anoxic hyperpolarization and resistance drop. These data are summarized in Table 1.

## Blockers of $Ca^{2+}$-induced $Ca^{2+}$ release (CICR)

### Ryanodine

Prolonged bath applications of ryanodine (10 $\mu$M) had no clear effect on the resting properties of six cells, and neither enhanced nor depressed significantly various features of their anoxic responses (Fig. 2 and Table 2). There was no evidence of any use-dependent changes (Shirasaki, Harata & Akaike, 1994): in the presence of ryanodine, similar anoxic $\Delta R$ values were recorded during the first anoxic test ($-15{\cdot}4 \pm 4{\cdot}4\%$) and the second or third ($-16{\cdot}5 \pm 4{\cdot}4\%$, $n = 6$).

### Procaine (internal)

Bath applications of procaine sharply reduced the anoxic responses (Fig. 3*A* and *B*), and had other striking effects, even at quite low concentrations (< 0·5 mM) (see below). By internal application, procaine should be particularly effective in blocking CICR (Endo, 1977). But this was not the case. The anoxic changes were quite unaffected when procaine (0·5–1 mM) was applied internally (Fig. 3*C*); except for a moderate, but significant, positive shift in the reversal potential of the anoxic hyperpolarization (by $25 \pm 9{\cdot}4$ mV; $n = 30$, unpaired, $P < 0{\cdot}05$). None of the other agents altered this reversal potential.

### Ruthenium Red

Overall, the data obtained from twenty-eight cells with Ruthenium Red-containing pipettes (50 $\mu$M) hardly differed from eleven control recordings (in the same series of slices), except for the suppression of the anoxic hyperpolarization (Table 2). This effect was especially marked during the first anoxic tests (5–10 min after the start of whole-cell recording), when $\Delta V$ values were $0{\cdot}82 \pm 1{\cdot}0$ mV; but in tests performed 10–15 min later, the anoxic hyperpolarizations recovered to $-5{\cdot}0 \pm 1{\cdot}1$ mV ($n = 22$). There was no comparable depression of the anoxic $\Delta R_N$ values, but the post-anoxic hyperpolarizations were also initially small ($-4{\cdot}9 \pm 0{\cdot}96$ mV) and then increased to $-9{\cdot}0 \pm 1{\cdot}1$ mV.

The regular depolarizations (by $6{\cdot}3 \pm 1{\cdot}8$ mV; paired data from 22 cells) and corresponding $R_N$ increases (by $47{\cdot}5 \pm 13{\cdot}0\%$) seen after recovery from the first anoxic test suggest a possible explanation for the curious transient disappearance of anoxic $\Delta V$ values: that block of mitochondrial $Ca^{2+}$ uptake (Carafoli, 1987) may cause a

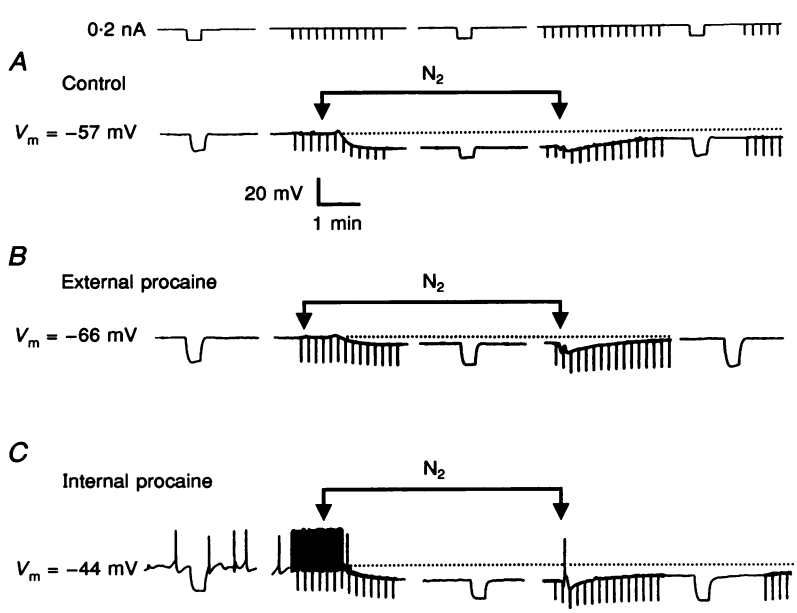


**Figure 3. Unlike external procaine, internally applied procaine has minimal effects on resting cell properties and changes evoked by anoxia**

Experimental conditions and description as for Fig. 1. *A* and *B* are from one cell; *A* is a control run. Note 11 mV anoxic hyperpolarization and $R_N$ drop from 62 to 38 MΩ (by 39%); *B*, after 15 min in procaine (0·5 mM), $R_N$ has risen to 92 MΩ (by 50%) and anoxic changes are much reduced: the hyperpolarization, $R_N$ drop and transient depolarization all by 50–60%, and post-anoxic hyperpolarization (measured from initial base-line) by 27%. In *C*, from another cell, recorded with patch electrode containing 1 mM procaine, anoxic hyperpolarization (−11 mV) and $R_N$ drop (−29%) are comparable to control values in *A* (and other figures).

temporary rise in $[Ca^{2+}]$, which activates $G_K$ sufficiently to occlude the anoxic hyperpolarization. Whatever its mechanism, the temporary suppression of $\Delta V$ values is probably a real phenomenon, as no comparable changes were seen in eleven control recordings, with drug-free electrodes, from the same series of slices – values of $V_m$, $R_N$, and the anoxic $\Delta V$, $\Delta R$, TD, PAH and $E_{rev}$, obtained early and at similar times after the first anoxic test showed no significant difference. Also, no temporary depression was evident during applications of other agents.

## Blockers of Ins$P_3$-induced $Ca^{2+}$ release (IICR)

### Heparin

Both the anoxic hyperpolarization and the fall in $R_N$ were generally reduced in cells recorded with electrodes containing heparin (10–20 $\mu$g ml$^{-1}$) (Fig. 4 and Table 1). The hyperpolarization was indeed not significant. The anoxic $R_N$ drop diminished progressively when anoxic tests were repeated (in 9 cells out of 10, including 3 cells recorded with electrodes containing heparin at only 5 $\mu$g ml$^{-1}$ (Fig. 4)). The mean $\Delta R_N$ values recorded in successive anoxic tests were $-17{\cdot}4 \pm 3{\cdot}4\%$ ($n = 10$) in the first; $-5{\cdot}9 \pm 5{\cdot}1\%$ ($n = 10$) in the second; and $-2{\cdot}0 \pm 3{\cdot}8\%$ ($n = 6$) in the third. Such progressive reductions of $\Delta R_N$ were not a feature of control anoxic tests performed with heparin-free electrodes (in the same or adjacent experiments).

### Thapsigargin

As a blocker of $Ca^{2+}$ uptake, this agent depletes internal stores (Thastrup *et al.* 1991), and therefore can be expected to suppress all phenomena generated by $Ca^{2+}$ release. Indeed, heparin and thapsigargin (10 $\mu$M) had very similar effects: as can be seen in Table 1, the anoxic hyperpolarization vanished and $\Delta R_N$ diminished by 60%. This suppression of the anoxic response was not progressive with repeated anoxic tests. Lower doses of thapsigargin (1–3 $\mu$M), tested on five cells, had no detectable effect.

In summary, according to the above data (Tables 1 and 2), anoxic hyperpolarizations and resistance falls were effectively abolished or much reduced only by Dantrolene sodium, heparin and thapsigargin. The early transient depolarization (TD) and the post-anoxic hyperpolarization (PAH) were quite unaffected.

## Procaine bath applications

In the same range of concentration (0·5–1 mM), procaine was much more effective when applied externally than internally (cf. Fig. 3*B* and *C*), producing significant changes in $R_N$ (up by $71 \pm 15{\cdot}3\%$; $n = 8$, $P < 0{\cdot}001$), and nearly abolishing the characteristic anoxic hyperpolarization (down by $4{\cdot}3 \pm 1{\cdot}1$ mV; $P < 0{\cdot}01$), $\Delta R_N$ (down by $60 \pm 15{\cdot}0\%$; $P < 0{\cdot}01$), the transient depolarization (TD; down by $0{\cdot}69 \pm 0{\cdot}19$ mV; $P < 0{\cdot}01$), as well as the post-anoxic hyperpolarization (PAH; down by $7{\cdot}8 \pm 2{\cdot}4$ mV; $P < 0{\cdot}05$) – all values are from paired observations on eight cells, before and during applications of 0·5–1 mM procaine. Only the resting $V_m$ and the reversal potential for the anoxic $\Delta V_m$ were not significantly altered ($-3{\cdot}9 \pm 4{\cdot}2$ and $+16 \pm 8{\cdot}4$ mV, respectively). These effects of procaine were clearly dose dependent, as shown by Fig. 5*A–D*. The respective $EC_{50}$ values, obtained from the best-fitting Hill plots (Fig. 5*F*), ranged from 0·12 mM for the increase in $R_N$ and the suppression of TD to 0·52 mM for the suppression of the PAH. The corresponding Hill coefficients were $< 1{\cdot}0$, except for TD and PAH.

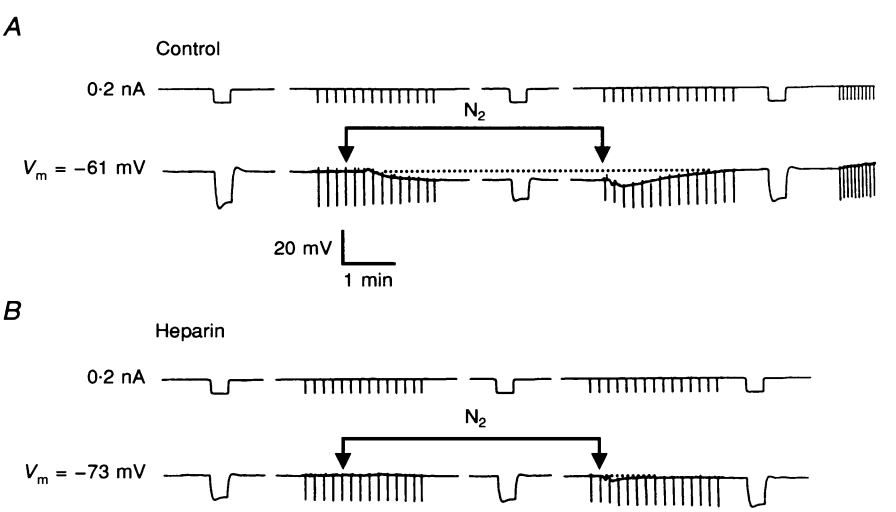


**Figure 4. Heparin can suppress anoxic hyperpolarization and resistance fall**

Experimental conditions and description as for Fig. 1. *A* and *B*, two different neurons in same slice; *A* was recorded with electrode containing standard internal solution, and *B*, with electrode containing heparin (5 $\mu$g ml$^{-1}$, internal). In *A*, note 5 mV hyperpolarization and $R_N$ drop from 110 to 63 MΩ (by 43%). In *B*, there is only a minimal anoxic hyperpolarization (1 mV) and $R_N$ *increases* from 72 to 76 MΩ during anoxia.

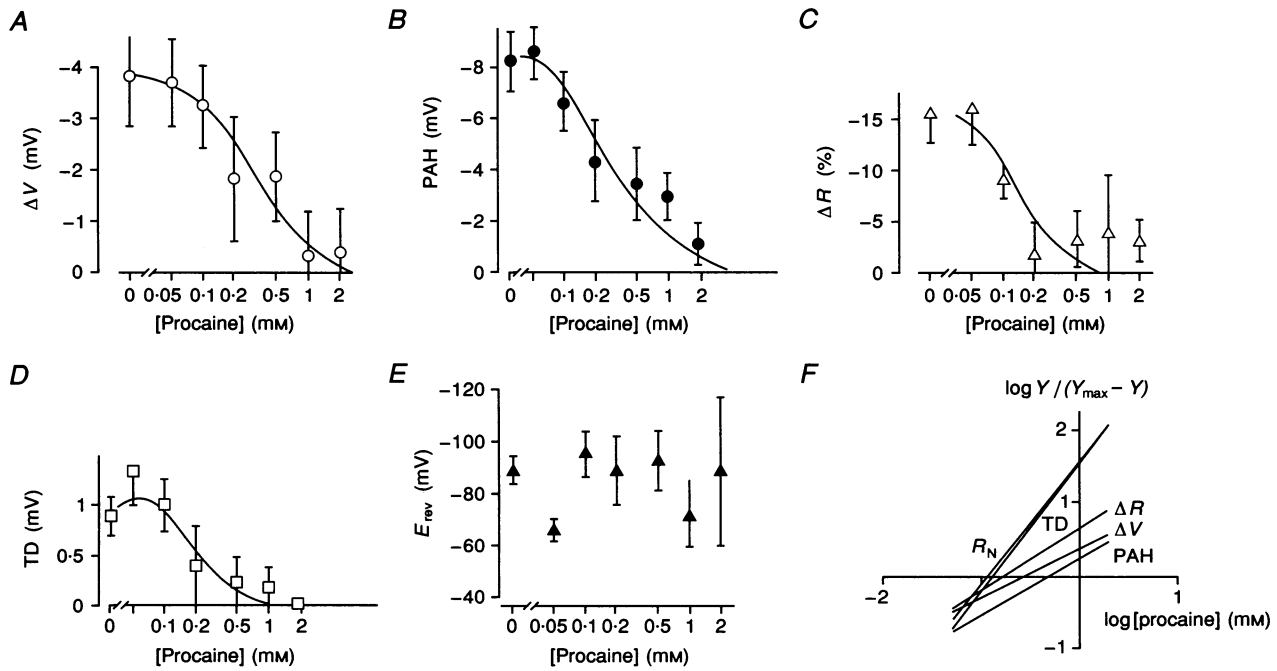


**Figure 5. Dose dependence of effects of procaine bath applications**

*A–E*, linear plots of mean amplitude (± S.E.M.) of peak anoxic changes as function of procaine concentration (on log scale). $\Delta V$, membrane hyperpolarization; $E_{rev}$, reversal potential; $\Delta R$, change in input resistance; TD, transient depolarization; and PAH, post-anoxic hyperpolarization. *F*, lines of best fit for Hill plots ($\log[Y/(Y_{max} - Y)]$ *vs.* log[procaine]), where $Y$ is percentage of maximal change produced by procaine, e.g. increase in $R_N$ or suppression of $\Delta V$, etc. $EC_{50}$ values (mM) were 0·12 for $R_N$, 0·30 for $\Delta V$, 0·18 for $\Delta R$, 0·52 for PAH and 0·12 for TD (all obtained from the Hill plots).

### Intracellular recordings of effects of external procaine

Bath applications of procaine (mostly 1 mM) were also made while recording from eleven cells with high resistance 3 M KCl electrodes. As usual in such recordings, the resting potentials were more negative (−67·2 ± 2·6 mV), the input resistances much lower (35 ± 5·4 MΩ) and the anoxic changes in $R_N$ greater (−35 ± 2·7%, $n = 11$).

The effects of procaine (1 mM) were less pronounced than in the whole-cell recordings: increases in $R_N$ (by 11·5 ± 6·1%) were not significant; the anoxic $\Delta R_N$ diminished by 51 ± 6·8% ($P < 0·001$); but neither the TD (+0·78 ± 0·49 mV) nor the PAH changed significantly (+3·3 ± 2·1 mV).

## DISCUSSION

Intracellular $Ca^{2+}$ stores appear to be of at least two types – one is sensitive to $InsP_3$ (hence IICR store) and the other to $Ca^{2+}$ itself (hence CICR store) (Tsien & Tsien, 1990; Henzi & MacDermott, 1992). The latter has been identified with ryanodine binding sites, and is generally held to be the principal site of action of caffeine.

Is this relatively simple scheme applicable to hippocampal neurons? Murphy & Miller (1989) found that both caffeine and quisqualate could induce $Ca^{2+}$ release in cultured hippocampal neurons (presumably by CICR and IICR, respectively). The absence of any interaction between the two types of release seemed to indicate independent stores. But Dubinsky & Rothman (1991) failed to detect any action of caffeine in cultured hippocampal cells – more in keeping with other evidence (Palade *et al.* 1989) that brain microsomes are predominantly $InsP_3$ sensitive and lack CICR mechanisms (the reverse of the properties of sarcoplasmic reticulum). In a recent paper, Shirasaki *et al.* (1994) reported that blockers of either CICR or IICR can prevent $Ca^{2+}$ release in acutely dissociated cells.

These apparently contradictory findings can be explained in part by the sampling of different types of neurons: CA1 is especially rich in $InsP_3$ receptors and CA3 in ryanodine receptors – though both may be present in the same cell (Palade *et al.* 1989; Nakanishi *et al.* 1991, 1992). Another variable is the developmental state, which has been shown to influence $Ca^{2+}$ mobilization by quisqualate (Murphy & Miller, 1989). Perhaps even more significant are recent reports that neither caffeine nor heparin is as specific as is generally believed (Ehrlich, Kaftan, Bezprozvannaya & Bezprozvanny, 1994).

Thus, both IICR and CICR may occur in hippocampal neurons; but they probably interact functionally (Dubinsky & Rothman, 1991; Shirasaki *et al.* 1994).

How do our results fit into this picture? Our attention was drawn to $Ca^{2+}$ release by the observation that Dantrolene sodium can suppress the anoxic response (Krnjević & Xu, 1989). Dantrolene is widely supposed to suppress CICR (Henzi & MacDermott, 1992), but there is strong evidence against this notion (Endo, 1977; Danko *et al.* 1985), and although Dantrolene has been reported to have no effect on IICR (Berridge & Irvine, 1984; Palade *et al.* 1989), it does block IICR in some cells (Kojima *et al.* 1984; Mine *et al.* 1987), including hippocampal neurons (Segal & Manor, 1992). The previous results do not exclude the possibility that Dantrolene depresses both CICR and IICR, as well as perhaps having some quite distinct mechanism of action (Danko *et al.* 1985).

### $Ca^{2+}$-sensitive $Ca^{2+}$ store

$Ca^{2+}$-induced $Ca^{2+}$ release is mediated by ryanodine-receptor $Ca^{2+}$ channels (Endo, 1977; Tsien & Tsien, 1990; Henzi & MacDermott, 1992; Irving, Collingridge & Schofield, 1992; Galione 1993; McPherson & Campbell, 1993). The lack of a significant effect of ryanodine in the present experiments is in keeping with the earlier finding that caffeine does not suppress the anoxic response (Leblond & Krnjević, 1989). Thus, CICR is unlikely to be a major feature of anoxia, and indeed may be relatively unimportant in hippocampal neurons (Murphy & Miller, 1989; Palade *et al.* 1990; Dubinsky & Rothman, 1991; but cf. Shirasaki *et al.* 1994).

Procaine being a well-established blocker of caffeine- and $Ca^{2+}$-induced $Ca^{2+}$ release (Endo, 1977; Tsien & Tsien, 1990; Galione, 1993), our inability to detect any depression of the anoxic response by internally applied procaine is further evidence against a major involvement of CICR. The only apparent effect of internal procaine, a more positive reversal potential for the anoxic $\Delta V_m$, may be ascribed to a reduction in $K^+$ conductance (Butterworth & Strichartz, 1990; Cook & Quast, 1990).

Although it has some other actions (it blocks mitochondrial $Ca^{2+}$ uptake; Carafoli, 1987), Ruthenium Red generally inhibits CICR (Carafoli, 1987; Henzi & MacDermott, 1992; Galione, 1993). The lack of any sustained effect in the present experiments therefore also argues against a major involvement of CICR.

### Ins$P_3$-sensitive $Ca^{2+}$ store

Bearing in mind the ineffectiveness of CICR blockers, the finding that heparin abolishes the anoxic hyperpolarization and significantly reduces the associated $R_N$ drop is good evidence that IICR is the predominant mechanism of $Ca^{2+}$ release during anoxia.

By preventing $Ca^{2+}$ uptake into internal stores (Thastrup *et al.* 1990; Irving *et al.* 1992), thapsigargin depletes the stores indiscriminately and so would tend to suppress both CICR and IICR. Though non-specific, this action is in keeping with the observed suppression of the anoxic hyperpolarization.

How would hypoxia activate Ins$P_3$ formation and so cause an early release of $Ca^{2+}$? One can envisage two main possible mechanisms of stimulation of phospholipase C: by a more-or-less direct effect of a lack of $O_2$, a drop in pH or some other intrinsic messenger; or indirectly via a receptor for a ligand such a glutamate, which is likely to be released during hypoxia (Nicholls, 1989). Further studies should provide an answer to this question.

### External procaine

Ion channel block presumably accounts for the rise in $R_N$ produced by procaine (Butterworth & Strichartz, 1990; Cook & Quast, 1990). In view of the lack of effect of several-fold higher concentrations of internal procaine, the marked depression of the anoxic responses by external procaine was unexpected. As a local anaesthetic, procaine acts mainly after intracellular penetration (Butterworth & Strichartz, 1990). But its critical site(s) of action for the suppression of the anoxic response must be superficial – in or on the membrane – and thus easily accessible to external but not internal procaine: a site perhaps analogous to that responsible for the 'slow inactivation' of $Na^+$ current, which is also sensitive to external but not internal procaine (Khodorov, Shishkova, Peganov & Revenko, 1976).

By suppressing $Ca^{2+}$ and $K^+$ currents (Butterworth & Strichartz, 1990; Cook & Quast, 1990) procaine could block the slow-AHP-type, $Ca^{2+}$-dependent $K^+$ current that appears to mediate the anoxic hyperpolarization and $R_N$ fall (Erdemli & Krnjević, 1994). On the other hand, procaine interferes with ligand-gated excitations, such as glutamate-evoked firing (Krnjević & Phillis, 1963) – which may account for a previous report that local anaesthetics depress synaptic responses in hippocampal slices (Butterworth & Cole, 1990) and protects them against long-term anoxic damage (Lucas, West, Rigor & Schurr, 1989). In addition, like other local anaesthetics, procaine may affect some relevant enzymes, such as $Na^+$–$K^+$-ATPase (Henn & Sperelakis, 1968), which could account for the depression of the PAH, and phospholipase (Waite & Sisson, 1972; Irvine, Hemington & Dawson, 1978), which would reduce Ins$P_3$ formation and suppress IICR.

In conclusion, the finding that anoxic changes in potential and input resistance were suppressed by heparin and thapsigargin, but not by ryanodine, internal procaine or Ruthenium Red, strongly suggests that – at least in these whole-cell recordings – $Ca^{2+}$ release from internal stores (most likely of the Ins$P_3$-sensitive type) plays a major role in the anoxic activation of $K^+$ conductance.

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