Photolytic manipulation of Ca^{2+} and the time course of slow, Ca^{2+}-activated K^+ current in rat hippocampal neurones

B. Lancaster* and R. S. Zucker

Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720, USA

1. Experiments were performed on hippocampal CA1 pyramidal cells to investigate the time course of a slow, Ca^{2+}-activated K^+ current that follows a burst of action potentials. At a temperature of 27–30 °C, this current rises to a peak 200–400 ms following the cessation of Ca^{2+} entry before decaying to baseline in 4–8 s.

2. Intracellular recordings were made using electrodes containing the photolabile calcium buffers nitr-5 or DM-nitrophen loaded appropriately with Ca^{2+}. Under these conditions, photolysis of the compound using an ultraviolet flashlamp caused an instantaneous increase in cytoplasmic Ca^{2+} throughout the cell. The response to flash photolysis was a membrane hyperpolarization with an onset limited by the membrane time constant. Multiple (up to twenty) flash responses could be generated.

3. The postspike slow after-hyperpolarization (AHP) and flash-induced hyperpolarizations showed a common sensitivity to the β-adrenergic receptor agonist isoprenaline.

4. Following a burst of spikes, the current underlying an AHP in progress could be terminated or reduced by photolysis-induced production of calcium buffer from diazo-4 within the cell. This action was rapid (within the settling time of the flash artifact, i.e. < 10 ms) despite the fact that the manipulation occurred 400–500 ms following the end of Ca^{2+} entry.

5. Partial block of the slow AHP by buffer production was accompanied by an increase in the time to peak of the event.

6. The time to peak of the slow AHP could also be manipulated by experiments which altered the spatial distribution of Ca^{2+} entry, such as production of calcium spikes or dendritic depolarization by glutamate in the presence of tetrodotoxin.

7. The Ca^{2+}-dependent K^+ current responsible for the slow AHP responds immediately to increases or decreases in cytoplasmic Ca^{2+}. It seems likely, therefore, that the slow AHP is controlled solely by changes in free Ca^{2+} and that the time course is governed by the redistribution of cytoplasmic Ca^{2+} following activity-induced entry through voltage- or receptor-operated channels.

Pyramidal neurones of the rat hippocampus contain two Ca^{2+}-dependent K^+ currents with clear and separate roles in the regulation of excitability. Following entry of Ca^{2+} during an action potential, a Ca^{2+}-dependent K^+ current is activated sufficiently rapidly to take part in repolarization of sodium spikes (Lancaster & Nicoll, 1987; Storm, 1987). This current is sensitive to charybdotoxin and submillimolar TEA and probably represents activity of the large-conductance, voltage-sensitive channel (variously termed I_c or B_k) observed in many cell types.

A second Ca^{2+}-activated K^+ current is responsible for a slow after-hyperpolarization (AHP) that is prominent following a short burst of action potentials and is responsible for spike frequency adaptation. Although obviously dependent on Ca^{2+} entry, this current has the intriguing property of rising to a peak amplitude up to 0·5 s following the end of Ca^{2+} entry during action potentials. Since this time course is also observed for the reversed current in elevated extracellular K^+, it is unlikely to be caused by a superimposed inward current (Lancaster & Adams, 1986). Suggested reasons for the delay include slow diffusion (Lancaster & Nicoll, 1987), an enzymatic cascade (Schwindt, Spain & Crill, 1992) and Ca^{2+}-induced Ca^{2+} release (Sah & McLachlan, 1991).

*To whom correspondence should be sent at: Department of Pharmacology, The Wellcome Research Laboratories, South Eden Park Road, Beckenham, Kent BR3 3BS, UK.

To investigate the time course of the slow AHP we have used the properties of photolabile calcium buffers to control Ca^{2+}-dependent hyperpolarizations within single pyramidal neurones. Using these compounds, free Ca^{2+} or calcium buffer can be generated, effectively instantaneously, within a cell at a given time. Furthermore, the change in Ca^{2+} levels is uniform throughout the cell. In contrast, entry of Ca^{2+} through voltage-gated channels may not be uniform, because certain types of calcium channels are more concentrated over certain areas of the cell (Westenbroek, Ahlijanian & Catterall, 1990). In addition, the degree of somatic and dendritic depolarization is not uniform during action potentials and this will also serve to localize Ca^{2+} entry through voltage-dependent channels to particular areas of the cell (Jaffe, Johnston, Lasser-Ross, Lisman, Miyakawa & Ross, 1992.). Complementary information regarding this question was obtained by further experiments in which the degree of somatic or dendritic Ca^{2+} entry was manipulated and shown to affect the time course of the slow AHP.

Some of this material has been published in abstract form (Lancaster & Zucker, 1991).

METHODS

Experiments were performed on adult rat hippocampal slices prepared according to conventional methods. Following Halothane anaesthesia and decapitation, slices (300–400 μm thick) were cut on a vibratome (Oxford Instruments) and placed in a holding chamber for at least 1 h. Individual slices were transferred to a recording chamber and held submerged in a continuously superfusing medium at a temperature of 27–30 °C. The medium was bubbled with 95 % oxygen, 5 % carbon dioxide to give a pH of 7·4. The medium composition was (mM): NaCl, 119; KCl, 2·5; $MgSO_4$ or $MgCl_2$, 1·3; $CaCl_2$, 2·5; NaH_2PO_4, 1·0; $NaHCO_3$, 26 and glucose 11. Drugs were obtained from the Sigma Chemical Company unless specified otherwise. Other compounds used in this study were tetrodotoxin (TTX), tetraethylammonium chloride (TEA, Aldrich), L-glutamate, isoprenaline, DM-nitrophen (Calbiochem) and nitr-5 (Calbiochem). Drs Roger Tsien and Steven Adams of the University of California, San Diego, generously supplied diazo-4.

The results presented were obtained with conventional intracellular recording techniques using electrodes filled with potassium methylsulphate (ICN Pharmaceuticals) or potassium acetate (resistance 60–100 MΩ without photolabile compounds). Since the aim of the experiments was to investigate the slow time course of a previously described Ca^{2+}-activated K^+ conductance, the whole-cell recording method was avoided because of the possibility of perturbing native calcium buffers and thereby introducing interpretational vagaries. Intracellular recording has the disadvantage that the amount of compound introduced into the cell and the precise changes in intracellular Ca^{2+} are not known. This was considered to be the lesser problem since interpretation of the data relies upon the fact that Ca^{2+} concentration changes rapidly upon photolysis, rather than relying upon knowledge of the exact size of the change.

To load cells with nitr-5, DM-nitrophen or diazo-4, these compounds were included in the pipette tip diluted with the principal electrolyte. The exact concentrations and degree of Ca^{2+} loading are indicated in the relevant figure legends. Photolysis was achieved using a xenon arc lamp and power supply (Chadwick-Helmuth), which discharged a known, variable amount of electrical energy (20–200 J) in a 1·6 ms flash. The flashlamp was focused onto the recording area prior to the experiment; the focused spot had a diameter of approximately 4 mm and covered the whole slice.

The characteristics of the photolabile compounds used are given in Table 1. When photolysed, nitr-5 and DM-nitrophen convert from high affinity to low affinity buffers and release bound Ca^{2+}. Nitr-5 is a 1,2-bis(*O*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA) derivative (Adams, Kao, Grynkiewicz, Minta & Tsien, 1988) and as such is highly selective for Ca^{2+} over Mg^{2+}. However, technical difficulties made this compound less than ideal for study of Ca^{2+}-dependent K^+ currents (see Results). DM-nitrophen (Kaplan & Ellis-Davies, 1988; McCray, Fidler-Lim, Ellis-Davies & Kaplan, 1992) has the advantage of initially high affinity, which allows a high percentage loading without buffering Ca^{2+} to unphysiologically high starting levels. DM-nitrophen is based upon EDTA and so has poor specificity for Ca^{2+} over Mg^{2+}. This creates the problem that if cells are only lightly filled with DM-nitrophen, Mg^{2+} that is free or weakly bound to ATP (a few millimolar total) is likely to displace much of the bound Ca^{2+}. This is likely to be actively extruded from the cell, but the remaining DM-nitrophen will in any case be unable to elicit much flash-induced Ca^{2+} release. The dissociation constant (K_d) of photolysed DM-nitrophen given in Table 1 is taken from a recently published measurement (Neher & Zucker, 1993), which differs from earlier estimates (Kaplan & Ellis-Davies, 1988). Diazo-4 converts to a high affinity calcium buffer upon exposure to UV light. This compound is a BAPTA derivative and has two photolabile groups, so a flash produces a mixture of species: singly photolysed (K_d 2·2 μM), doubly photolysed (K_d 55 nM) plus some fraction unphotolysed (Adams, Kao & Tsien, 1989).

Ionophoresis was carried out using a WPI ionophoresis unit model 160 connected to an electrode containing 0·5 M sodium glutamate, pH 8·0, giving a resistance of about 60 MΩ. The ejection current used was constant at 70 nA. This electrode was advanced through stratum radiatum of the CA1 area until a burst of action potentials could be produced from short applications of glutamate (5–20 ms) with short latency. These sensitive areas of the apical dendrites were highly localized and sometimes required several tracks to locate. The area of dendritic glutamate application varied from 40 to 60 % of the distance from the pyramidal cell body layer to the hippocampal

Table 1. Calcium binding affinities before and after photolysis of photolabile compounds

	Before (μM)	After (μM)
Nitr-5†	0·145	6·3
DM-nitrophen‡§	0·005*	250
Diazo-2‖	2·2	0·073
Diazo-4‖	89	0·055

*This value is very sensitive to the presence of magnesium; for example, in the presence of equimolar magnesium, 4 mM DM-nitrophen half-bound to calcium buffers calcium to 5 μM. References: †Adams *et al.* 1988; ‡Kaplan & Ellis-Davies, 1988; §Neher & Zucker, 1993; ‖Adams *et al.* 1989.

fissure bordering the dentate gyrus. Thus the region of glutamate application was at least 200 μm from the soma. The results in this paper were obtained using a Dagan 8800 total clamp in bridge mode or single electrode voltage clamp mode for recording the AHP current (Lancaster & Adams, 1986). Data were stored on digital oscilloscope floppy disks (Nicolet 4094) and subsequently transferred to an Intel 80-486-based personal computer for analysis.

RESULTS

In CA1 pyramidal neurones, a short burst of action potentials is followed by a hyperpolarization generated by a voltage-insensitive Ca^{2+}-activated K^+ current (I_{AHP}; Lancaster & Adams, 1986). Following the cessation of Ca^{2+} entry through voltage-dependent channels, this event increases in amplitude for a few hundred milliseconds (Fig. 1*C*). The reason for this slow time course of onset is not obvious, since it might be expected that Ca^{2+}-dependent events would be maximal immediately after Ca^{2+} entry and decay monotonically thereafter. As a method for raising intracellular Ca^{2+}, an alternative to entry through voltage-gated channels is release from a photolabile chelator, which gives a uniform rise in Ca^{2+} throughout the cell, assuming uniform illumination and chelator concentration. Figure 1 shows the effects on membrane potential of Ca^{2+} release from two chemically different photolabile chelators, DM-nitrophen and nitr-5. The BAPTA-based nitr-5 gives a buffered step increase in Ca^{2+} concentration of 1–2 μM (Landò & Zucker, 1989), whereas DM-nitrophen, in addition to a small steady-state increase, is thought also to produce a large initial transient in free Ca^{2+} (Delaney & Zucker, 1990; Zucker, 1993). Regardless of the exact profile of Ca^{2+} release, photolysis of either compound resulted in qualitatively similar responses: a hyperpolarization, which usually peaked in less than 50 ms and which could last up to 1 min for the first flash response.

Most experiments were performed with DM-nitrophen 30–50 % Ca^{2+} loaded, since nitr-5 in the electrode frequently resulted in cells with very negative resting potential (−80 to −90 mV) and low input resistance. This was probably due to a shunt to the equilibrium potential for potassium, E_K; the Ca^{2+} loading of the nitr-5 (K_d for Ca^{2+}, 140 nM; Adams *et al.* 1988) could buffer resting Ca^{2+} to sufficiently high levels to cause tonic activation of the AHP current. Reduction of the Ca^{2+} loading of the nitr-5 to less than 50 % reduces substantially the Ca^{2+} step resulting from photolysis. Flash responses were obtained in only two of fourteen cells recorded with nitr-5 in the electrode compared to a success rate of twenty-five of forty-three cells with DM-nitrophen. The failures seen with DM-nitrophen may result from lightly filled cells having the Ca^{2+} displaced by Mg^{2+} (see Methods and below). These were known to be true failures because entry of the compound into the cell was monitored by spike broadening, as reported previously for calcium buffers (Lancaster & Nicoll, 1987). Upon flash exposure the spike broadening was reversed, indicating that photolysis had occurred. These negative results allow us to eliminate the

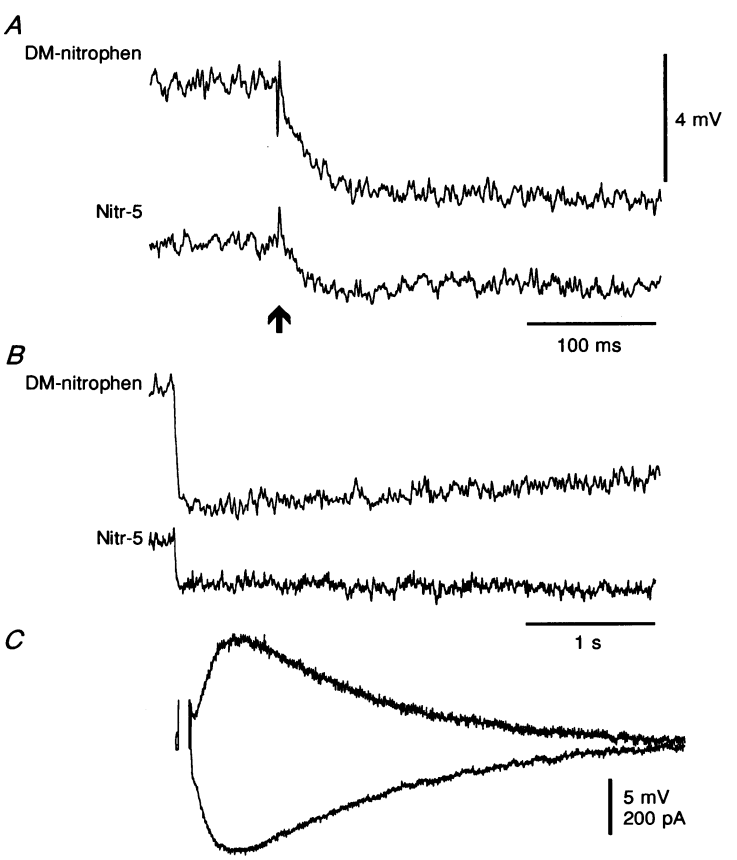

Figure 1. Membrane potential responses to photolysis of DM-nitrophen or nitr-5 in two different cells
A, responses shown on a fast time scale peak within about 50 ms following the flash (arrow). *B*, the slower time scale indicates that the responses decay slowly and could persist for up to 1 min. *C*, the AHP current (top trace) or the potential itself (lower trace) shown for comparison on the same time scale as the traces in *B*. For DM-nitrophen, the recording electrode contained 69 mM DM-nitrophen 30 % loaded with calcium; flash energy, 20 J; membrane potential, −64 mV. The nitr-5 concentration in the electrode was 60 mM, 50 % calcium loaded; flash energy, 100 J; cell membrane potential, −62 mV.

remote possibility that reactive by-products could evoke flash-induced hyperpolarizations.

When flash intensity is reduced, this results in lower percentage photolysis of DM-nitrophen per flash and allows a sequence of flash responses to be obtained. Figure 2*A* shows selected, individual records from a total of twenty-one consecutive flash responses that were obtained in this cell using weak (20 J) flashes at 2–3 min intervals. At flash number twenty-two the DM-nitrophen was exhausted; the flash artifact was negligible. The main difference between the responses in this sequence was the much longer duration of the early flash responses. We propose the following explanation for this observation. Initially, the injected DM-nitrophen is only partly (around 50 %) bound to Ca^{2+}. A weak flash photolyses about 15 % of the DM-nitrophen and the resultant equilibrium mixture still has total DM-nitrophen in excess of total calcium. Thus Ca^{2+} is still buffered, but to a new, higher level since the remaining DM-nitrophen is more heavily Ca^{2+} loaded. Using a model of DM-nitrophen photolysis (details in Delaney & Zucker, 1990), we calculate that 6 mM of DM-nitrophen injected with 3 mM Ca^{2+} in the presence of 2 mM MgATP, 2 mM free Mg^{2+} and 0·5 mM of a cytoplasmic calcium buffer with 5 μM affinity will equilibrate with a free Ca^{2+} concentration of 140 nM. After a single flash the remaining, largely magnesium-loaded, DM-nitrophen buffers Ca^{2+} to 400 nM. Successive flashes leave Ca^{2+} buffered to progressively higher levels, and this buffering effect of DM-nitrophen is able to retard Ca^{2+} removal processes, which must pump total Ca^{2+} off the exogenous buffer. Eventually photolysis will cause the total Ca^{2+} in the cytoplasm to exceed the concentration of unphotolysed DM-nitrophen; at that point, further photolysis releases Ca^{2+} only onto native buffers (which have a lower affinity than unphotolysed DM-nitrophen), allowing it to be removed more readily by transport processes.

All flash responses, regardless of duration, consisted of a hyperpolarization with a rapid onset. Figure 2*Ba* shows traces from a different cell from *A*, in which responses 2 and 11 (out of 12) have been normalized to the peak and superimposed. The early responses may be generated by a Ca^{2+} change including an initial spike of Ca^{2+} (large release followed by rebinding), whereas late responses come from a small amount of remaining, but Ca^{2+}-saturated, buffer which produces only a step in Ca^{2+} concentration. Nevertheless, all flash responses showed little delay. This observation, combined with responses to nitr-5 photolysis which only causes a step in Ca^{2+}, indicates that a large Ca^{2+} transient to early DM-nitrophen flashes is not necessary to cause the rapid onset of the responses. The onset of the flash response tracks the hyperpolarization caused by current injection (Fig. 2*Bb*). This indicates that the time course of the flash response was limited only by the membrane time constant.

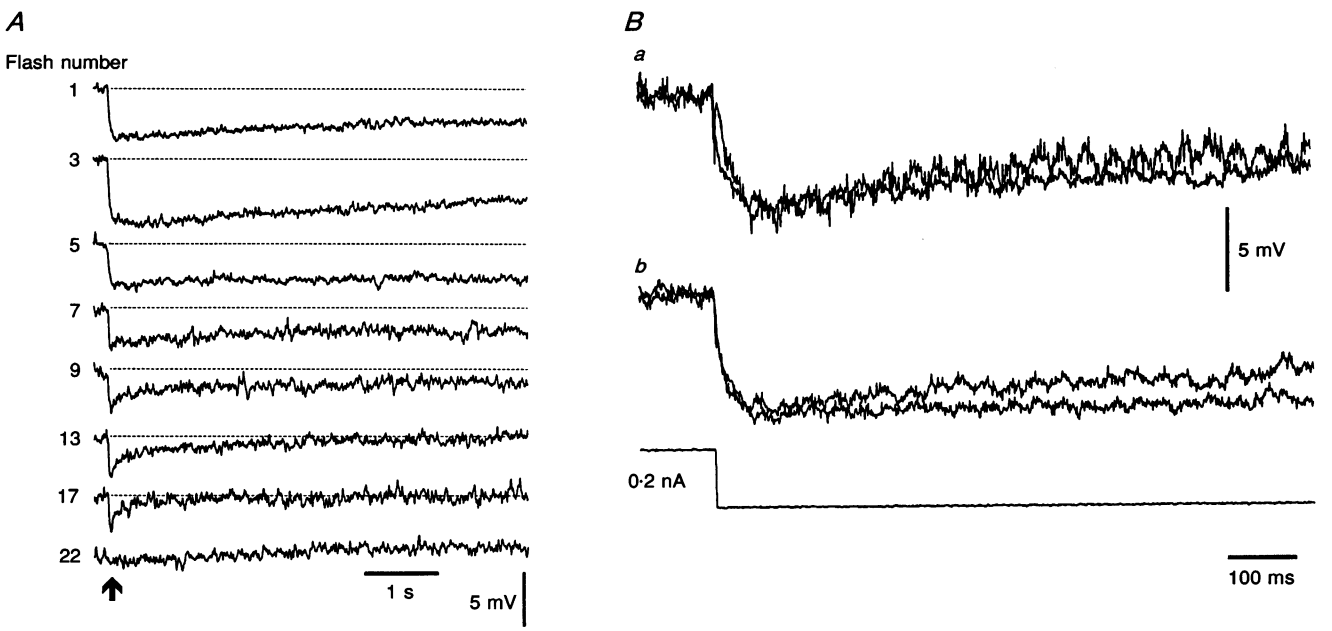

Figure 2. Multiple, rapid-onset responses to DM-nitrophen photolysis
A, same cell as in Fig. 1. Selected traces from 21 consecutive responses to a 20 J flash (arrow) at 2–3 min intervals. The initial membrane potential (−64 mV) is indicated by the dashed line. Flash number 22 produced no response and illustrates that the discharge artifact was minimal and not a component of even the small responses. DM-nitrophen in the electrode was 69 mM, 30 % calcium loaded. *B*, results from a different cell illustrate the consistent, rapid onset of the flash responses. *Ba*, superimposed traces from flash responses 2 and 11 (out of 12) normalized to peak amplitude show a similar onset time course. *Bb*, the onset of a flash response coincides with that produced by current injection. DM-nitrophen in the electrode was 59 mM, 60 % calcium loaded; flash energy, 20 J; membrane potential, −61 mV.

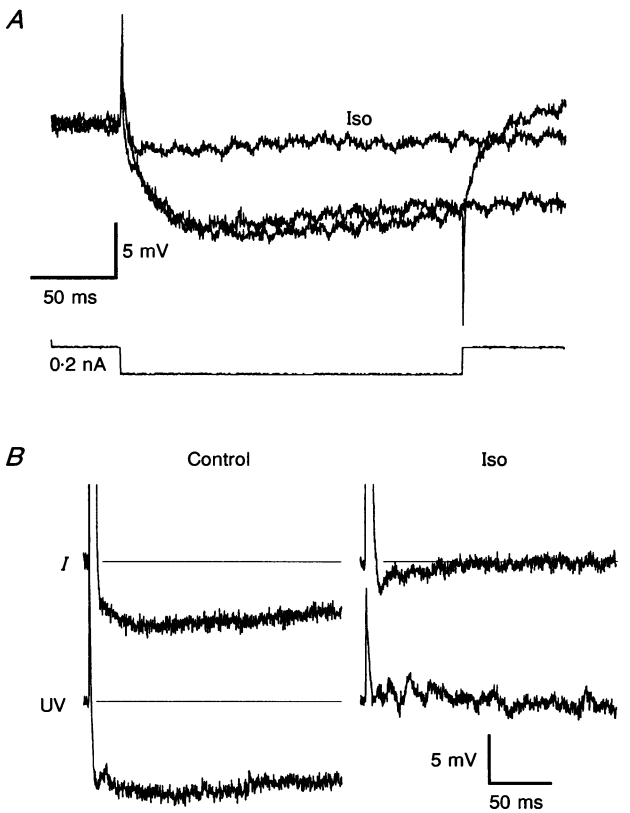

Figure 3. Flash responses are sensitive to isoprenaline
A, three superimposed records showing the response to current injection, a first flash response (50 J) and a second flash response 8 min after application of 5 μM isoprenaline (trace labelled Iso). *B*, a different cell showing a depolarization-evoked AHP (trace *I*) and flash-evoked response (50 J; trace UV) before and 4 min after application of 1 μM isoprenaline. Membrane potential, −70 mV. For both cells the DM-nitrophen in the electrode was 70 mM, 30 % calcium loaded.

If the flash-induced hyperpolarization is the same phenomenon as the slow Ca^{2+}-dependent AHP, they should share pharmacological sensitivity, the best characterized example of which is block of the AHP by noradrenaline acting at β-receptors. Figure 3*A* illustrates a flash response that activated with the time course of the membrane time constant and was sensitive to the β-adrenergic agonist isoprenaline. The trace labelled Iso was the response to a second weak flash after 8 min in the presence of 5 μM isoprenaline; this result was obtained in four of four cells. Both DM-nitrophen and nitr-5 buffer calcium before photolysis sufficiently to prevent activation of the AHP in most cells. Consequently, it took a large number of cells to obtain data of flash responses together with evoked AHPs. Figure 3*B* shows a common sensitivity of the AHP (trace *I*) and the flash response (trace UV) in the same cell. The AHP and the response to a second weak flash were blocked in tandem after 4 min in the presence of 1 μM isoprenaline.

The converse experiment to rapid activation of AHP current is to investigate whether the AHP can be deactivated rapidly following a photolysis-induced increase in calcium buffer within the cell. A step in calcium buffer was achieved by photolysis of diazo-4, which undergoes an affinity increase from a K_d of 89 μM to 55 nM upon complete photolysis (see Methods). The efficacy of this

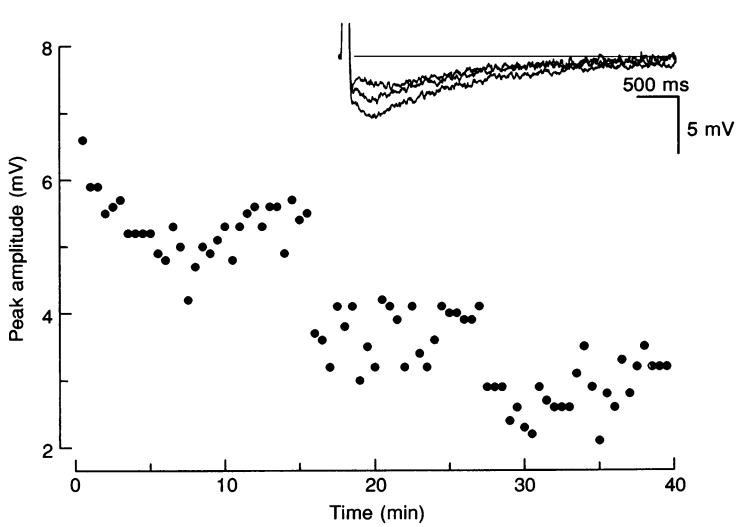

Figure 4. Block of the slow AHP by photolysis of diazo-4
The slow AHP following a burst of action potentials was elicited at 30 s intervals. The preparation was exposed to a 50 J flash at 16·5 and 27·5 min. Inset shows sample records from control, single- and double-flash periods. Diazo-4 in the electrode was 3·5 mM; membrane potential, −65 mV.

buffer transition is illustrated in Fig. 4. The AHP was elicited once per 30 s in a cell recorded with a diazo-4-containing electrode. A 50 J flash was given at 16·5 and 27·5 min. Following each flash there was an irreversible reduction in the peak amplitude of the Ca^{2+}-dependent AHP. Diazo-4 was used in twenty-seven experiments. In fifteen cells a single flash resulted in complete block of the AHP and partial block was observed in the remaining twelve cells. No failures were observed.

Having established that diazo-4 behaved as expected, the question of how rapidly this effect took place is addressed in Fig. 5*A* for the AHP and Fig. 5*B* for I_{AHP} (two different cells). The top of Fig. 5*A* shows three superimposed records of membrane potential following a burst of three action potentials before, during and after a 50 J flash. The flash discharge followed current injection with a latency of 400 ms and caused a step decrease in the AHP. The lower trace illustrates for comparison the discharge artifact in isolation at the same membrane potential. Electrodes which contained diazo-4 (unlike DM-nitrophen) had resistances sufficiently low to enable single electrode voltage clamp to be performed, which gave time resolution of the photolysis-induced change beyond the limitation of the membrane time constant. In Fig. 5*B* the flash response is a step decrease in I_{AHP} 400 ms following the burst of spikes. The time course is shown at high resolution on the right. The discharge artifact breaks through the clamp, giving a large biphasic signal, following which separation of the current traces is apparent in less than 10 ms. The flash blocked the ability of the cell subsequently to generate an AHP (bottom trace).

When photolysis of diazo-4 resulted in a reduction of AHP peak amplitude as in Fig. 4, this was accompanied by alterations in peak latency. Production of intracellular buffer by photolysis increased the AHP peak latency. The data in Fig. 6*A* (same experiment as Fig. 4) show a tendency to longer peak latencies as the AHP gets smaller, i.e. as photolysis proceeds. The correlation coefficient for the best fit shown is only modest at 0·6, but scatter is present in the small amplitude events due to measuring error. The first twenty AHPs (before any photolysis) had a mean peak latency measured from the end of the depolarizing stimulus of 232 ± 19 ms (S.D.; mean peak amplitude $5·3 \pm 0·5$ mV). The final twenty AHPs after two 50 J flashes had a mean peak latency of 365 ± 43 ms (mean peak amplitude $2·9 \pm 0·4$ mV). In contrast, a range of different amplitude slow AHPs evoked by varying numbers of action potentials showed no correlation between peak amplitude and latency (Fig. 6*B*). Thus over the range of five spikes or less, fewer spikes produce less Ca^{2+} entry and smaller AHPs without changes in latency. On the other hand, if Ca^{2+} entry is constant but intracellular buffering is increased (as in Fig. 6*A*) there is an additional effect on the latency of the response.

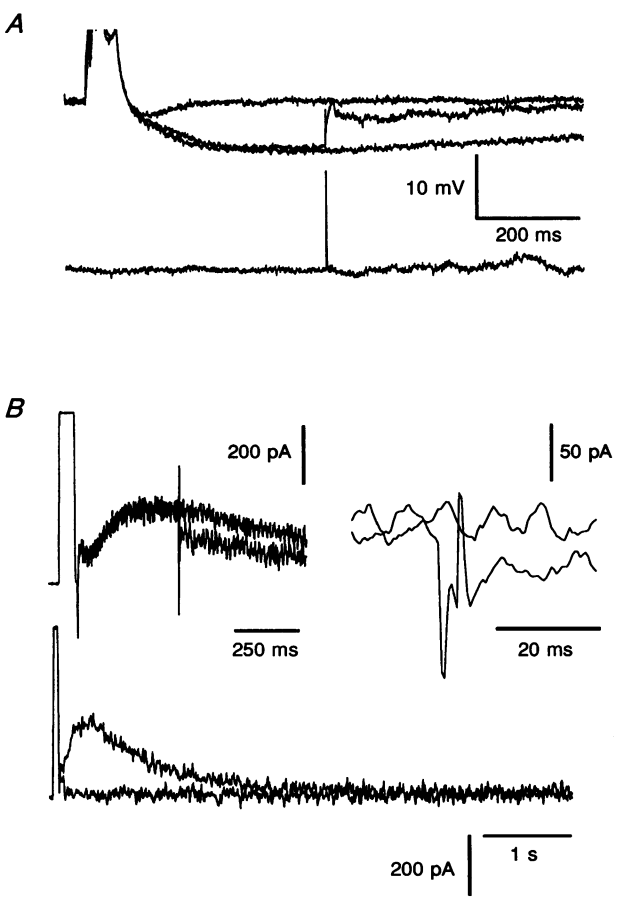

Figure 5. The slow AHP or AHP current are blocked rapidly by photolysis of diazo-4
A, top: three superimposed records of membrane potential following a burst of three action potentials before, during and after a 50 J flash triggered 400 ms after the end of depolarizing current injection. The lower trace in *A* shows the flash artifact in isolation at the resting membrane potential of −66 mV. Diazo-4 in the electrode was 21 mM. *B*, the top sets of records illustrate the step reduction of AHP current caused by photolysis of diazo-4 and the time course on an expanded time scale. Bottom traces illustrate the postspike membrane current before and after the flash. Diazo-4 in the electrode was 4 mM; 150 J flash; membrane potential, −67 mV.

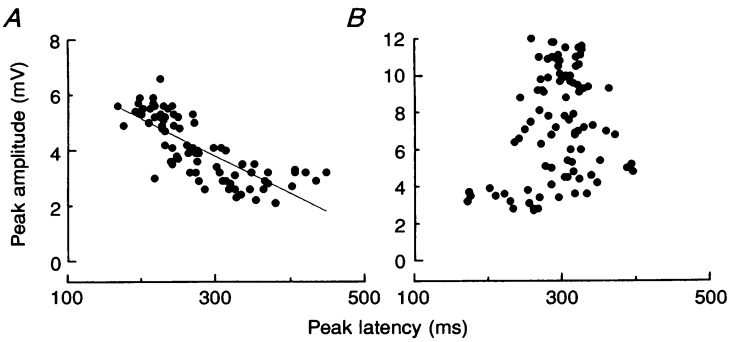

Figure 6. Increased calcium buffering prolongs the peak latency of the AHP
A, AHP peak amplitude plotted against peak latency for the experiment in Fig. 4. *B*, a similar plot for a control cell in which different numbers of evoked spikes were used to change AHP amplitude. Peak latencies were measured from the end of the depolarization following current injection. Cell in *A*: temperature, 28·5 °C; membrane potential, −65 mV. Cell in *B*: temperature, 29 °C; membrane potential, −59 mV.

A series of experiments was designed to assess the possible involvement of Ca^{2+}-induced Ca^{2+} release from internal stores and also to shed some light on the relation of Ca^{2+} influx to the AHP time course. The reasoning for the following experiments was that if the AHP time course is a result of an intracellular Ca^{2+} release process, the peak latency should not be modifiable by altering the nature or distribution of Ca^{2+} entry. This was tested in two ways. The first was to induce depolarization of the middle to distal apical dendrites by brief, ionophoretic pulses of glutamate with the aim of causing dendritic Ca^{2+} entry. The second method was to elicit AHPs in TTX plus TEA by calcium spikes. Under these circumstances Ca^{2+} entry occurs over a greater fraction of the dendritic tree than with sodium spikes because of reduced cable filtering of the longer duration calcium spike depolarization (Jaffe *et al.* 1992).

An example of dendritic glutamate application is illustrated in Fig. 7*A* and *B*. In normal Ringer solution, glutamate pulses to the apical dendrites (Fig. 7*A b*; 15 ms pulse, 60 % of the distance from the cell soma layer to the hippocampal fissure) evoked a burst of sodium spikes similar to those elicited by current injection at the soma (Figure 7*A a*). These events were followed by slow AHPs of similar magnitude and time course (Fig. 7*B a*). This was the case for eight of ten cells; in two cells the glutamate application produced an AHP slightly larger by 10–20 %. The ionophoretic electrode was left in place during TTX application. In the absence of sodium spikes, longer duration glutamate applications (Fig. 7*A c*) were required to elicit the slow AHPs shown in Fig. 7*B b*. In a TTX-containing Ringer solution, dendritic glutamate pulses evoked an AHP in six of eight cells. In the cells which

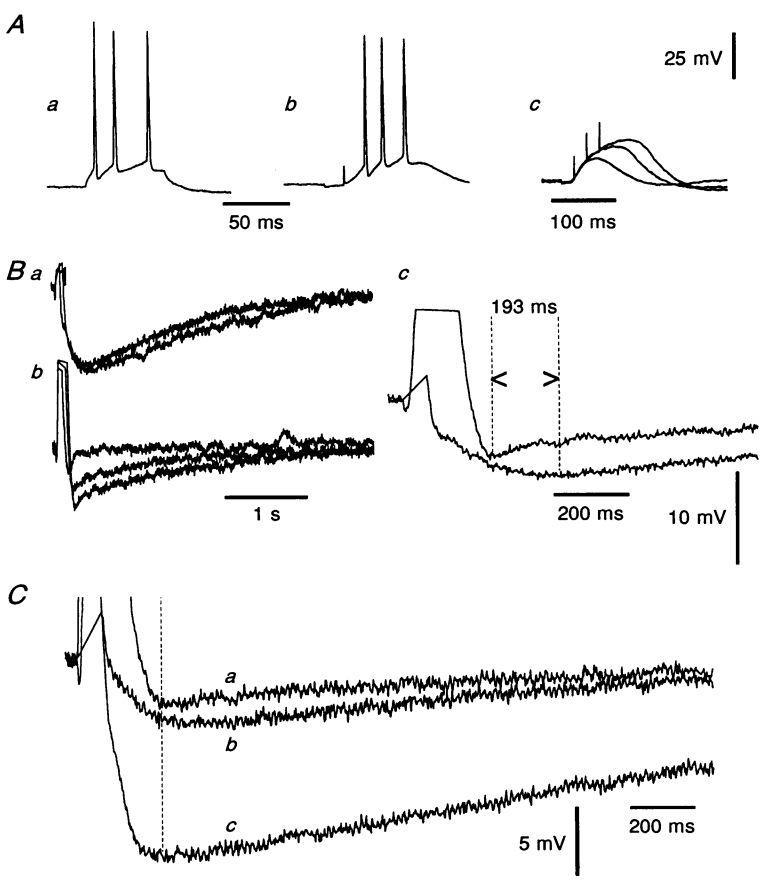

Figure 7. Manipulation of Ca^{2+} entry can alter the time course of the AHP
A a, action potentials evoked by current injection at the soma. *A b*, action potentials evoked by 15 ms ionophoretic application of glutamate to apical dendrites, 60 % of the distance from stratum pyramidale to the hippocampal fissure. *A c*, responses to longer applications of glutamate (20, 40 and 60 ms) in the presence of TTX; same location as *A b*. *B a*, slow AHPs following the current- or glutamate-elicited sodium spikes (shown in *A a* and *b*) are very similar in time course and amplitude. *B b*, AHPs following the longer glutamate applications (shown in *A c*) have a shorter peak latency. This difference is clear in the overlaid records of *B c*. Membrane potential −73 mV. *C*, results from a different cell. Trace *b* is the control slow AHP in response to three sodium spikes. Trace *a* is the response following glutamate ionophoresis to the apical dendrites in 1 μM TTX, 4 mM TEA (10 ms ionophoretic pulse, 45 % of the distance from stratum pyramidale to the fissure). Trace *c* is the AHP following a calcium spike in TTX and TEA as above. Membrane potential, −66 mV.

showed an AHP, five of six cells had an earlier AHP peak latency following glutamate pulses than following sodium spikes, e.g. Fig. 7*Bc*. In the cell shown in Fig. 7*C*, AHPs were evoked under three different conditions: injected current to produce sodium spikes (trace *b*); injected current in TTX and TEA Ringer solution, which produced a calcium spike (trace *c*); and dendritic glutamate application (trace *a*). A calcium spike (four of five cells) and dendritic glutamate pulses elicited AHPs which peaked during the rising phase of the AHP evoked by sodium spikes. It should be noted that the changes in time course are unlikely to be influenced by electrotonic characteristics of the cell, since even the fastest AHP is at least tenfold slower than the limit imposed by passive membrane properties. The results in Fig. 7 indicate that the time course of the Ca^{2+}-dependent AHP is not invariant and can be manipulated in ways that should not interfere with Ca^{2+}-induced Ca^{2+} release processes.

DISCUSSION

The photolysis of nitr-5 or DM-nitrophen evoked hyperpolarizations with very similar characteristics. The differences between the two buffers acted as a control for factors other than release of Ca^{2+}, since they are derived from different parent compounds and produce different photoproducts. In addition, DM-nitrophen, unlike nitr-5, can bind Mg^{2+} as well as Ca^{2+}. However, since photolysis of either compound produced hyperpolarizations, these are likely to be due to the Ca^{2+} release, which is the only property in common, rather than to any effect of Mg^{2+} release or photolysis by-products, which would be unlikely to activate rapidly and specifically just the AHP current. It is noteworthy that a variety of Ca^{2+}-sensitive proteins are sensitive to release of caged Ca^{2+}, including K^+ channels (Gurney, Tsien & Lester, 1987), non-specific cation channels (Kimitsuki & Ohmori, 1992), Ca^{2+} channels (Morad, Davies, Kaplan & Lux, 1988; Fryer & Zucker, 1993), vesicle fusion proteins (Delaney & Zucker, 1990; Neher & Zucker, 1993) and contractile proteins (Lea, Fenton, Potter & Ashley, 1990). Again, this is interpreted most sensibly as due to release of Ca^{2+} rather than other products.

The nature of Ca^{2+} release produced by nitr-5 and DM-nitrophen is somewhat different. Nitr-5 produces a small amplitude step in Ca^{2+} concentration (Landò & Zucker, 1989), whereas partly loaded DM-nitrophen additionally produces a large but short-lasting (1–2 ms) transient of Ca^{2+} immediately after a flash (Delaney & Zucker, 1990; Zucker, 1993). Estimates of the Ca^{2+} concentration spike produced by DM-nitrophen photolysis are in the region of 100 μM (Zucker, 1993). It is clear that the Ca^{2+} release from DM-nitrophen must be large, since it can evoke transmitter release (Delaney & Zucker, 1990) and calcium channel inactivation (Morad *et al.* 1988; Fryer & Zucker 1993), processes which seem to require the high Ca^{2+} levels associated with domains (Imredy & Yue, 1992; Llinás, Sugimori & Silver, 1992). In the experiments described here, a rapid-onset hyperpolarization was observed with both calcium chelators, implying that the speed of Ca^{2+} release was the important variable rather than the concentration of free Ca^{2+}. Also, DM-nitrophen photolysis at the end of a sequence of responses (when a Ca^{2+} transient cannot be produced by the small quantity of saturated buffer remaining) still produced a rapid-onset hyperpolarization.

The variable rate of success with nitr-5 and DM-nitrophen is indicative of some of the problems associated with these compounds (see Results). Insufficient loading with DM-nitrophen will allow Mg^{2+} to displace all the bound Ca^{2+}, whereas nitr-5 may buffer resting Ca^{2+} to levels that activate the current being studied. The latter problem was noted also in previous studies using nitr-5 to activate a voltage-independent K^+ current (Gurney *et al.* 1987). Problems associated with illumination, such as tissue absorbance or lamp focusing, can be ruled out because, under the same conditions, consistent success was obtained in diazo-4 photolysis, despite a quantum efficiency comparable to nitr-5 and DM-nitrophen.

The slow AHP is sensitive to a number of transmitters, the best characterized of which is the action of noradrenaline acting at β-receptors (Madison & Nicoll, 1986). Since flash responses, like the postspike AHPs, were sensitive to the β-receptor agonist isoprenaline, this confirms the identity of the flash response as AHP current. Thus, following Ca^{2+} release from a photolabile buffer the AHP current is capable of much more rapid activation than following Ca^{2+} entry through voltage-dependent channels.

Calcium released from DM-nitrophen or nitr-5 did not cause activation of the large conductance Ca^{2+}-activated K^+ channel. Under the conditions of our experiments (membrane potential -60 to -70 mV), small conductance Ca^{2+}-activated K^+ channels are more sensitive to Ca^{2+} than the large conductance channels (Lancaster, Nicoll & Perkel, 1991), which would require Ca^{2+} concentrations in excess of 10 μM for significant activation (Franciolini, 1988; Barrett, Magleby & Pallotta, 1982). Of the two calcium buffers we used, nitr-5 is incapable of raising Ca^{2+} to these levels. Partly loaded DM-nitrophen can raise Ca^{2+} to between 10 and 100 μM, but only for periods of a few milliseconds (Zucker, 1993), which would be coincident with the flash artifact and not resolved in our experiments.

Production of calcium buffer within the cell by diazo-4 photolysis caused irreversible reduction of the spike-evoked AHP amplitude. This result is in keeping with the block of the slow AHP by EGTA- or BAPTA-containing electrodes (Schwartzkroin & Stafstrom, 1980; Lancaster & Nicoll, 1987). The important question for the purpose of this paper is the rapidity with which photolysed diazo-4 acts to block an evoked AHP. The diazo-4 flash response was a sudden step decrease in the AHP current that was complete within 10 ms; a time limit imposed mainly by the

settling time of the single electrode voltage clamp. These results agree with previous experiments in muscle fibres using the sibling compound diazo-2 (see Table 1), where rapid relaxation was produced and Ca^{2+} estimated to fall within 3 ms (Mulligan & Ashley, 1989; Palmer, Mulligan, Nunn & Ashley, 1990). The protons produced by diazo-4 photolysis are unlikely to result in significant changes in pH (Malenka, Lancaster & Zucker, 1992) and would be buffered by bicarbonate and organic anions and thus unlikely to produce irreversible block of the AHP following a single flash. The immediate response to diazo-4 photolysis was observed despite Ca^{2+} entry having ceased 400–500 ms earlier. Since it is assumed that the only functional consequence of diazo-4 photolysis is to buffer and decrease free Ca^{2+}, the very rapid change following a flash is evidence that a raised, free intracellular Ca^{2+} level is required for the complete time course of the AHP.

Two other aspects of the diazo-4 results deserve further consideration. Following weak flashes, which caused a reduction in the slow AHP, there was a concurrent increase in the time to peak compared with prephotolysis values in the same cell. Although this result is compatible with modelling studies in which an increase in fast calcium buffer caused prolongation of Ca^{2+} redistribution within the cell (Sala & Hernandez-Cruz, 1990), this is characteristic of an immobile buffer, which would not be expected for the diazo-4 photolysis product. In fact, a presumably mobile buffer like diazo-4 should speed the diffusion of free Ca^{2+} (Irving, Maylie, Sizto & Chandler, 1990). We have no satisfactory explanation to account for the prolonged time to peak of the AHP following diazo-4 photolysis. A second interesting observation was that a flash could produce an immediate step decrease in an evoked AHP, but not necessarily 100 % block even though the ability to block subsequent AHPs was complete. Thus Ca^{2+} at the channels was lowered only enough to reduce an AHP in progress, but the same amount of buffer blocked AHP generation. This finding is consistent with the possibility that Ca^{2+} must diffuse from the site of entry at voltage-dependent calcium channels to the site of action at the AHP channels.

One mechanism postulated to account for the slow onset and decay of a Ca^{2+}-activated K^+ current in cortical neurones is a calcium-dependent enzymatic cascade (Schwindt *et al.* 1992). The results with nitr-5, DM-nitrophen and diazo-4 show that the slow AHP in hippocampal neurones is instantaneously responsive to changes in calcium buffering during the whole time course; thus any enzyme system would have to share this rapid and persistent dependence upon Ca^{2+}. In addition it should be noted that the time course of spike-evoked AHPs is compatible with the decay of intracellular Ca^{2+} following voltage-gated entry (Mayer, MacDermott, Westbrook, Smith & Barker, 1987; Müller & Connor, 1991) More problematically, enzymatic cascades are normally considered as mechanisms for amplification of a response, which is not compatible with the graded activation of AHP current seen with stimulus protocols that increase Ca^{2+} entry (Lancaster & Adams, 1986). Finally, any delay due to enzyme kinetics should be present regardless of the method of raising cytoplasmic Ca^{2+}, yet Ca^{2+} release from DM-nitrophen and nitr-5 can activate the AHP current almost an order of magnitude more rapidly than Ca^{2+} entry through the membrane.

If there were release of Ca^{2+} from internal stores, could this explain the time course of the slow AHP? Although evidence exists for such a mechanism in vagal motoneurones (Sah & McLachlan, 1991), there are a number of impediments to this interpretation in hippocampal neurones. Most obviously, if Ca^{2+}-induced Ca^{2+} release was involved, photolytically induced rises in Ca^{2+} might be expected to produce an AHP with a slow time course, in contradiction of our experimental results. Secondly, ryanodine binding sites are sparse in CA1 compared to area CA3 and the dentate gyrus (Padua, Wan, Nagy & Geiger, 1991). Furthermore, when release of Ca^{2+} from stores does activate outward current, as in smooth muscle, step voltage commands evoke transient outward currents (Zholos, Baidan & Shuba, 1991) consistent with the pulsatile nature of Ca^{2+} release (Friel & Tsien, 1992). In hippocampal neurones, however, AHP current evoked by step voltage commands is always graded such that larger amplitude or duration voltage jumps give rise to larger AHP current (Lancaster & Adams, 1986). Recently, Hocherman, Werman & Yarom (1992) have noted the graded activation of slow AHPs in vagal motoneurones and also have questioned whether this is compatible with the mechanism of Ca^{2+} release.

Calcium-dependent outward currents have a high temperature sensitivity (Barish & Thompson, 1983) and this is true also for the AHPs of hippocampal and vagal motoneurones (Lancaster & Adams, 1986; Sah & McLachlan, 1991). This temperature sensitivity was used to exclude free diffusion of Ca^{2+} as an explanation of AHP time course (Sah & McLachlan, 1991), but clearly it does not exclude buffered diffusion of Ca^{2+}, which is a more realistic assumption for cytoplasm in any case. The high temperature coefficient over 10 °C (Q_{10}) might represent the temperature dependence of the calcium buffers and pumps upon which Ca^{2+} diffusion depends (Barish & Thompson, 1983), or it may reflect a property of the AHP channels.

Experiments were also performed to manipulate Ca^{2+} influx to see if this alone could lead to changes in peak latency. The methods used (glutamate-induced dendritic depolarization or current-evoked calcium spikes) were designed to increase the relative amount of Ca^{2+} entering dendritic membrane. Under either of these conditions, Ca^{2+} influx was more likely to generate an AHP with an earlier peak latency than following sodium spikes. This observation is consistent with measurements of aminophosphonovalerate (APV)-insensitive Ca^{2+} influx in these cells (Regehr & Tank, 1992), which showed that somatic Ca^{2+} levels took longer to reach peak levels

following influx than dendritic Ca^{2+}. Thus as the ratio of dendritic/somatic Ca^{2+} influx increases, one would predict an earlier peak to a Ca^{2+}-dependent event such as the slow AHP if the latter arises from channels with a majority distribution on dendritic membrane. This result suggests that some AHP channels are located on pyramidal cell dendrites.

The rapid activation of TEA-sensitive Ca^{2+}-dependent K^+ current involved in repolarizing the action potential leads to the suggestion of a close proximity of the two channel types (Lancaster & Nicoll, 1987). Such co-localization has been demonstrated clearly in saccular hair cells, where the data implied Ca^{2+} and K^+ channels lying within a 300 nm diameter area (Roberts, Jacobs & Hudspeth, 1990). Our experiments in hippocampus indicate that, in contrast to the TEA-sensitive K^+ channel, co-localization cannot be the situation for the majority of calcium and AHP-K^+ channels. If buffered diffusion of Ca^{2+} is the limiting factor in the rise of the AHP it is worthwhile considering some estimates of the distance involved. That is, how far can buffered diffusion carry calcium ions within the 400 ms available? This distance s can be estimated from the equation $s = \sqrt{(2Dt)}$, where t is time and D the diffusion coefficient for calcium ions. In aqueous solution this value is 6×10^{-6} cm^2 s^{-1}, but a 50- to 100-fold lower value is necessary to account for the slower diffusion in cytoplasm containing immobile buffers (Smith & Zucker, 1980; Neher & Augustine, 1992; Allbritton, Meyer & Stryer, 1992). Thus assuming an effective D of $0{\cdot}12 \times 10^{-6}$ cm^2 s^{-1}, in 400 ms calcium ions in cytoplasm diffuse about 3 μm. It is clear that, even given variable assumptions concerning buffering, the distance involved is small.

The simplest scheme consistent with our observations is that localized Ca^{2+} entry acts on a uniform distribution of AHP channels. Thus one possibility for the delay of the slow AHP peak is redistribution of Ca^{2+} from areas of the cell (soma and proximal dendrites; Jaffe *et al.* 1992) where entry occurs. However, the small distance available for this redistribution ($< 5\ \mu$m) would require a sharp cut-off between the areas of Ca^{2+} entry and the rest of the cell. Alternatively, within the area of membrane containing the active calcium channels, there may be clustering of these channels (Hirano & Hagiwara, 1989; O'Dell & Alger, 1991), which would give rise to punctate Ca^{2+} entry. In this case, buffered diffusion into areas between Ca^{2+} domains would account for the rising phase of the slow AHP.

REFERENCES

Adams, S. R., Kao, J. P. Y., Grynkiewicz, G., Minta, A. & Tsien, R. Y. (1988). Biologically useful chelators that release Ca^{2+} upon illumination. *Journal of the American Chemical Society* **110**, 3212–3220.

Adams, S. R., Kao, J. P. Y. & Tsien, R .Y. (1989). Biologically useful chelators that take up Ca^{2+} upon illumination. *Journal of the American Chemical Society* **111**, 7957–7968.

Allbritton, N. L., Meyer, T. & Stryer, L. (1992). Range of messenger action of calcium ion and inositol 1,4,5-trisphosphate. *Science* **258**, 1812–1815.

Barish, M. E. & Thompson, S. H. (1983). Calcium buffering and slow recovery kinetics of calcium-dependent outward current in molluscan neurones. *Journal of Physiology* **337**, 201–219.

Barrett, J. N., Magleby, K. L. & Pallotta, B. S. (1982). Properties of single calcium-activated potassium channels in cultured rat muscle. *Journal of Physiology* **331**, 211–230.

Delaney, K. R. & Zucker, R. S. (1990). Calcium released by photolysis of DM-nitrophen stimulates transmitter release at squid giant synapse. *Journal of Physiology* **426**, 473–498.

Franciolini, F. (1988). Calcium and voltage dependence of single Ca^{2+}-activated K^+ channels from cultured hippocampal neurons of rat. *Biochimica et Biophysica Acta* **943**, 419–427.

Friel, D. D. & Tsien, R. W. (1992). Phase-dependent contributions from Ca^{2+} entry and Ca^{2+} release to caffeine-induced $[Ca^{2+}]_i$ oscillations in bullfrog sympathetic neurons. *Neuron* **8**, 1109–1125.

Fryer, M. W. & Zucker, R. S. (1993). Ca^{2+}-dependent inactivation of Ca^{2+} current in *Aplysia* neurons: kinetic studies using photolabile Ca^{2+} chelators. *Journal of Physiology* **464**, 501–528.

Gurney, A. M., Tsien, R. Y. & Lester, H. A. (1987). Activation of a potassium current by rapid photochemically generated step increases of intracellular calcium in rat sympathetic neurons. *Proceedings of the National Academy of Sciences of the USA* **84**, 3496–3500.

Hirano, T. & Hagiwara, S. (1989). Kinetics and distribution of voltage-gated Ca, Na and K channels on the somata of rat cerebellar Purkinje cells. *Pflügers Archiv* **413**, 463–469.

Hocherman, S. D., Werman, R. & Yarom, Y. (1992). An analysis of the long-lasting after-hyperpolarization of guinea-pig vagal motoneurones. *Journal of Physiology* **456**, 325–349.

Imredy, J. P. & Yue, D. T. (1992). Submicroscopic Ca^{2+} diffusion mediates inhibitory coupling between individual Ca^{2+} channels. *Neuron* **9**, 197–207.

Irving, M., Maylie, J., Sizto, N. L. & Chandler, W. K. (1990). Intracellular diffusion in the presence of mobile buffers. Application to proton movement in muscle. *Biophysical Journal* **57**, 717–721.

Jaffe, D. B., Johnston, D., Lasser-Ross, N., Lisman, J. E., Miyakawa, H. & Ross, W. N. (1992). The spread of Na^+ spikes determines the pattern of dendritic Ca^{2+} entry into hippocampal neurons. *Nature* **357**, 244–246.

Kaplan, J. H. & Ellis-Davies, G. C. R. (1988). Photolabile chelators for the rapid photorelease of divalent cations. *Proceedings of the National Academy of Sciences of the USA* **85**, 6571–6575.

Kimitsuki, T. & Ohmori, H. (1992). The effect of caged calcium release on the adaptation of the transduction current in chick hair cells. *Journal of Physiology* **458**, 27–40.

Lancaster, B. & Adams, P. R. (1986). Calcium-dependent current generating the afterhyperpolarization of hippocampal neurons. *Journal of Neurophysiology* **55**, 1268–1282.

Lancaster, B. & Nicoll, R. A. (1987). Properties of two calcium-activated hyperpolarizations in rat hippocampal neurones. *Journal of Physiology* **389**, 187–203.

Lancaster, B., Nicoll, R. A. & Perkel, D. J. (1991). Calcium activates two types of potassium channels in rat hippocampal neurons in culture. *Journal of Neuroscience* **11**, 23–30.

Lancaster, B. & Zucker, R. S. (1991). Photolytic manipulation of $[Ca^{2+}]_i$ controls hyperpolarizations in hippocampal pyramidal cells. *Society for Neuroscience Abstracts* **17**, 1114.

Landò, L. & Zucker, R. S. (1989). "Caged calcium" in *Aplysia* pacemaker neurons: characterization of calcium-activated potassium and nonspecific cation currents. *Journal of General Physiology* **93**, 1017–1060.

LEA, T. J., FENTON, M. J., POTTER, J. D. & ASHLEY, C. C. (1990). Rapid activation by photolysis of nitr-5 in skinned fibres of the striated adductor muscle from the scallop. *Biochimica et Biophysica Acta* **1034**, 186–194.

LLINÁS, R., SUGIMORI, M. & SILVER, B. (1992). Microdomains of high calcium concentration in a presynaptic terminal. *Science* **256**, 677–679.

MCCRAY, J. A., FIDLER-LIM, N., ELLIS-DAVIES, G. C. R. & KAPLAN, J. H. (1992). Rate of release of Ca^{2+} following laser photolysis of the DM-nitrophen–Ca^{2+} complex. *Biochemistry* **31**, 8856–8861.

MADISON, D. V. & NICOLL, R. A. (1986). Cyclic adenosine 3′,5′-monophosphate mediates β-receptor actions of noradrenaline in rat hippocampal pyramidal cells. *Journal of Physiology* **372**, 245–259.

MALENKA, R. C., LANCASTER, B. & ZUCKER, R. S. (1992). Temporal limits on the rise in postsynaptic calcium required for the induction of long-term potentiation. *Neuron* **9**, 121–128.

MAYER, M. L., MACDERMOTT, A. B., WESTBROOK, G. L., SMITH, S. J. & BARKER, J. L. (1987). Agonist-and voltage-gated calcium entry in cultured mouse spinal cord neurons under voltage clamp measured using arsenazo III. *Journal of Neuroscience* **7**, 3230–3244.

MORAD, M., DAVIES, N. W., KAPLAN, J. H. & LUX, H. D. (1988). Inactivation and block of calcium channels by photo-released Ca^{2+} in dorsal root ganglion neurons. *Science* **241**, 842–844.

MÜLLER, W. & CONNOR, J. A. (1991). Cholinergic input uncouples Ca^{2+} changes from K^+ conductance activation and amplifies intradendritic Ca^{2+} changes in hippocampal neurons. *Neuron* **6**, 901–905.

MULLIGAN, I. P. & ASHLEY, C. C. (1989). Rapid relaxation of single frog skeletal muscle fibres following laser flash photolysis of the caged calcium chelator diazo-2. *FEBS Letters* **255**, 196–200.

NEHER, E. & AUGUSTINE, G. J. (1992). Calcium gradients and buffers in bovine chromaffin cells. *Journal of Physiology* **450**, 273–301.

NEHER, E. & ZUCKER, R. S. (1993). Multiple calcium-dependent processes related to secretion in bovine chromaffin cells. *Neuron* **10**, 21–30.

O'DELL, T. J. & ALGER, B. E. (1991). Single calcium channels in rat and guinea-pig hippocampal neurons. *Journal of Physiology* **436**, 739–767.

PADUA, R. A., WAN, W., NAGY, J. I. & GEIGER, J. D. (1991). [^{3}H]Ryanodine binding sites in rat brain demonstrated by membrane binding and autoradiography. *Brain Research* **542**, 135–140.

PALMER, R. E., MULLIGAN, I. P., NUNN, C. & ASHLEY, C. C. (1990). Striated scallop muscle relaxation: fast force transients produced by photolysis of diazo-2. *Biochemical and Biophysical Research Communications* **168**, 295–300.

REGEHR, W. G. & TANK, D. W. (1992). Calcium concentration dynamics produced by synaptic activation of CA1 hippocampal pyramidal cells. *Journal of Neuroscience* **12**, 4202–4223.

ROBERTS, W. M., JACOBS, R. A. & HUDSPETH, A. J. (1990). Colocalization of ion channels involved in frequency selectivity and synaptic transmission at presynaptic active zones of hair cells. *Journal of Neuroscience* **10**, 3664–3684.

SAH, P. & MCLACHLAN, E. M. (1991). Ca^{2+}-activated K^+ currents underlying the afterhyperpolarization in guinea-pig vagal neurons: a role for Ca^{2+}-activated Ca^{2+} release. *Neuron* **7**, 257–264.

SALA, F. & HERNANDEZ-CRUZ, A. (1990). Calcium diffusion modelling in a spherical neuron. Relevance of buffering properties. *Biophysical Journal* **57**, 313–324.

SCHWARTZKROIN, P. A. & STAFSTROM, C. E. (1980). Effects of EGTA on the Ca^{2+}-activated afterhyperpolarization in hippocampal CA3 pyramidal cells. *Science* **210**, 1125–1126.

SCHWINDT, P. C., SPAIN, W. J. & CRILL, W. E. (1992). Calcium-dependent potassium currents in neurons from cat sensorimotor cortex. *Journal of Neurophysiology* **67**, 216–226.

SMITH, S. J. & ZUCKER, R. S. (1980). Aequorin response facilitation and intracellular calcium accumulation in molluscan neurones. *Journal of Physiology* **300**, 167–196.

STORM, J. F. (1987). Action potential repolarization and a fast after-hyperpolarization in rat hippocampal pyramidal cells. *Journal of Physiology* **385**, 733–759.

WESTENBROEK, R. E., AHLIJANIAN, M. K. & CATTERALL, W. A. (1990). Clustering of L-type Ca^{2+} channels at the base of major dendrites in hippocampal pyramidal neurons. *Nature* **347**, 281–284.

ZHOLOS, A. V., BAIDAN, L. V. & SHUBA, M. F. (1991). Properties of the late transient outward current in isolated intestinal smooth muscle cells of the guinea-pig. *Journal of Physiology* **443**, 555–574.

ZUCKER, R. S. (1993). The calcium concentration clamp: spikes and reversible pulses using the photolabile chelator DM-nitrophen. *Cell Calcium* **14**, 87–100.

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