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Activation of a potassium current by rapid photochemically generated step increases of intracellular calcium in rat sympathetic neurons

(light flash/calcium jump/superior cervical ganglion)

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ABSTRACT **Although Ca^{2+} is a well-established intracellular messenger, there are many questions concerning the kinetics and spatial localization of its effects. Such problems may now be approached with the photosensitive Ca^{2+} chelator nitr-5. The Ca^{2+} affinity of this molecule decreases by a factor of 40 after absorption of near-UV light; Ca^{2+} is liberated with a time constant of ≈300 μs. Nitr-5 or the related compounds nitr-2 and nitr-7, complexed with Ca^{2+}, were introduced into rat sympathetic ganglion cells by dialysis from a patch pipette electrode operating in the whole-cell, voltage-clamp mode. Light flashes released Ca^{2+} and activated a K^+ current. Flash-induced current relaxations followed a simple exponential time course with time constants as brief as 5 ms. Comparison of the kinetics among the chelators, which photolyze at different rates, suggests that release of Ca^{2+} from nitr-5 is too fast to limit the relaxation. Thus we confirm directly that Ca^{2+} can modulate membrane properties within a few milliseconds after entering a cell. A preliminary kinetic description of K^+ current activation by Ca^{2+} in rat sympathetic neurons is presented; Ca^{2+} appears to bind to the channel with a rate constant of at least 2×10^7 $M^{-1} \cdot s^{-1}$.**

Ca^{2+} is an intracellular messenger for such rapid events as muscle contraction, transmitter release, and ion-channel gating. To study these actions, it is desirable to have methods both for measuring (1, 2) and generating transient changes in intracellular [Ca^{2+}]. This paper addresses the creation of controlled, step increases in intracellular [Ca^{2+}]. Suggestions that this might be achieved with photochemical tactics (3, 4) are now being realized with the introduction of a series of improved photosensitive Ca^{2+} chelators (Fig. 1*A*). These molecules incorporate (*i*) high-affinity, rapidly binding, pH-insensitive chelating groups that select strongly for Ca^{2+} over Mg^{2+} (2, 5) and (*ii*) *ortho*-nitrobenzyl substituents that photolyze efficiently at near-UV wavelengths (4, 6). The first member of the series, nitr-2 (Fig. 1*A*), has been employed to study Ca^{2+}-activated currents in *Aplysia* neurons (7), although its usefulness was limited by the low rate of Ca^{2+} liberation (5–7 s^{-1}).

The present investigation involves other members, nitr-5 and nitr-7 (Fig. 1*A*), which photolyze more rapidly (ref. 8; S. Adams, J. Kao, G. Grynkiewicz, A. Minta, and R.Y.T., unpublished results). Nitr-5 is water soluble and binds Ca^{2+} with a dissociation constant of 145 nM at 0.1–0.15 M ionic strength, increasing to 6.3 μM after photolysis. Ca^{2+} release proceeds with a time constant of ≤300 μs. Nitr-7 binds Ca^{2+} roughly three times more tightly than nitr-5 but releases Ca^{2+} with a time constant of ≈1.8 ms. We used the tight-seal voltage–clamp method (9) to introduce these chelators into rat superior cervical ganglion cells under electrophysiological investigation. Light flashes created Ca^{2+} "jumps" inside the cells and activated a K^+ current, which is thought to underlie the slow after hyperpolarization that follows an action potential in these cells. Kinetic analysis of the flash-induced currents suggests a simple model for Ca^{2+} gating of K^+ channels in these sympathetic neurons.

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METHODS

Superior cervical ganglion cells, dissociated from neonatal rats, were maintained in cell culture and used for experiments within 3 days after plating. Their electrophysiological properties have been described elsewhere (10). Membrane currents were recorded, at 20–22°C, using the tight-seal voltage–clamp technique, in the whole-cell mode (9), with a Dagan (Minneapolis, MN) model 8900 amplifier. Unless otherwise noted, the external solution contained (mM): NaCl, 140; $CaCl_2$, 1; $MgCl_2$, 1.5; glucose, 5; Hepes, 10, pH 7.3. K^+ currents were isolated by blocking voltage-dependent Na^+ and Ca^{2+} currents with, respectively, tetrodotoxin (1 μM) and $CdCl_2$ (100 μM) included in the solution. Pipettes were filled with solutions containing (mM): KCl, 140; Hepes, 10, pH 7.3. Resistances were 1–4 MΩ. Series resistance was <8 MΩ, of which 60–90% could be compensated. The chelators (2–10 mM), partially complexed with Ca^{2+}, were included in the pipette solutions. It is assumed that the pipette contents equilibrate with the cell interior and that the photosensitive molecule is the dominant intracellular Ca^{2+} buffer. Photolysis of chelator in the pipette was prevented by coating the pipette to within 50–100 μm of the tip with an opaque paint (M-coat D, Measurements Group, Raleigh, NC), which also reduced the pipette capacitance. The recording chamber was mounted on the stage of an inverted microscope (Leitz Dialux); the illuminator was mounted separately and during an experiment was replaced with a flashlamp focused on the cell (11). Light flashes were filtered to remove wavelengths <305 nm. Flash intensity was varied by charging the capacitor bank to different voltages; we assume that the photon flux in the near-UV region, where the chelators absorb, is proportional to the discharge energy.

For photochemical calibrations, a 2-μl drop of chelator solution (2 mM) was placed in a circular well (0.75-mm deep, 2-mm diameter). Evaporation was prevented with a drop of mineral oil, and the well was positioned in the usual location of the cell. Transmission of 350-nm light from the microscope illuminator was measured with a UV-enhanced photodiode (United Detector Technology, Santa Monica, CA), before and after flashes. Maximal flashes (1 J, total output energy) photolyzed 20% per flash of free nitr-2 or nitr-5 and 34% per flash when complexed with Ca^{2+}. Corrections for screening by the molecule itself yield photochemical efficiencies of 39% and 66% photolyzed per flash, for uncomplexed and Ca^{2+}-

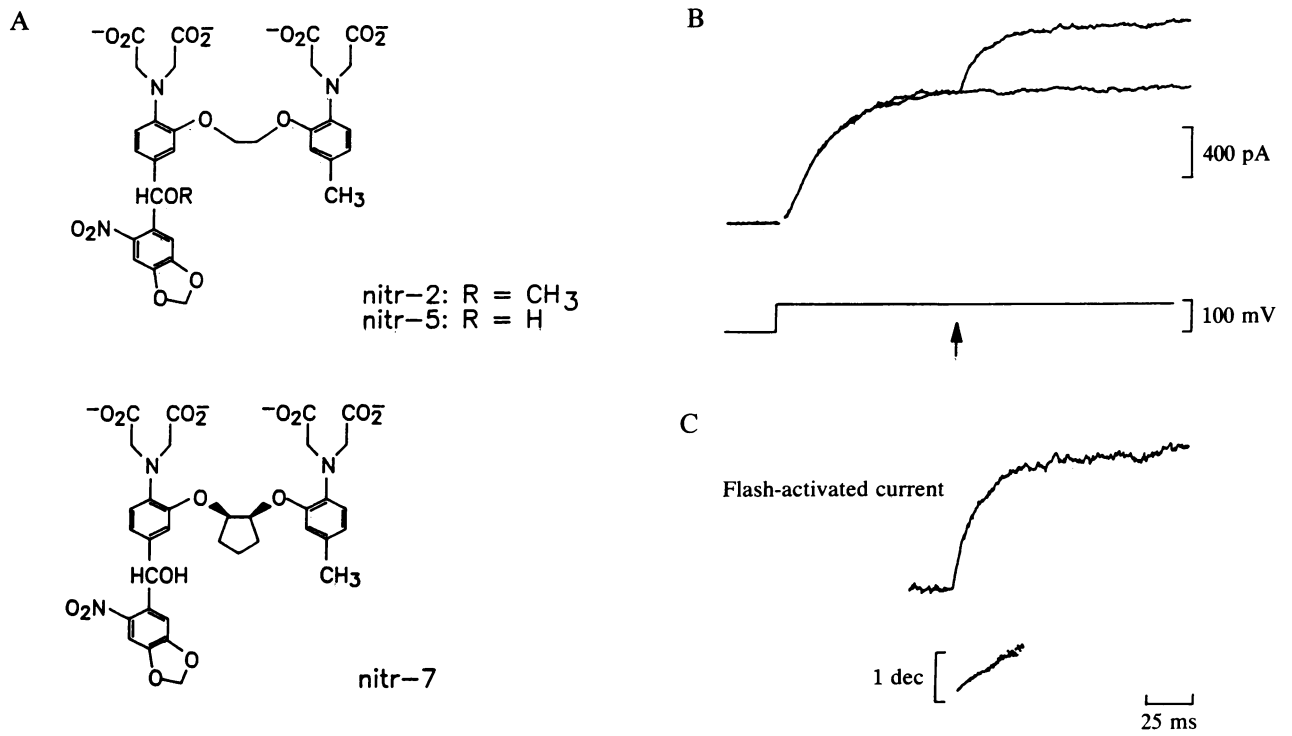

FIG. 1. (*A*) Structures of nitr-2, nitr-5, and nitr-7. (*B*) Voltage-clamp currents from a rat superior cervical ganglion cell are shown in the *Upper* traces, and the voltage protocol is shown in the *Lower* trace. The starting conditions were 2 mM nitr-5 with 1.5 mM added Ca^{2+}. The membrane potential was jumped from the holding potential of −50 mV to a test potential of 40 mV. Episodes occurred at intervals of 10 s. During the second episode, a flash was delivered at the time indicated by the arrow, jumping $[Ca^{2+}]$ from 430 nM to 5.5 μM. As a result, the outward current increased to a new steady state. The current observed during a third episode had only partially returned to the preflash level. (*C*) (*Upper*) Light-flash relaxation, evoked during episode 2, on an expanded scale. The approach to equilibrium is plotted (*Lower*) on semilogarithmic coordinates. The time constant in this example was 16 ms.

complexed chelators, respectively, for a sample with negligible thickness—this is the case for the cultured cells used in these experiments. The total nitr-5 photolyzed by a flash was estimated by separately calculating the concentrations of free and Ca^{2+}-bound chelator before and after the flash. From the concentrations of total nitr-5(N) after a flash, total photolyzed [nitr-5](P) [calculated for each flash energy according to the methods of Sheridan and Lester (12)] and total $[Ca^{2+}](C_t)$, the free $[Ca^{2+}](C)$ after the flash, was calculated by solving the following equation for the equilibrium established by two competing buffers:

$$C^3 + (K_N + K_P - C_t + N + P)C^2 +$$
$$[K_NP + K_PN - C_t(K_N + K_P) + K_NK_P]C - K_NK_PC_t = 0,$$

where K_N and K_P are the dissociation constants for Ca^{2+} binding to nitr-5 and to the photoproduct, respectively. These values were determined as described (7). The amplitude of the $[Ca^{2+}]$ jump is the difference between the free $[Ca^{2+}]$ before and after the flash.

The speed of Ca release was measured by converting the Ca release to proton release in the presence of *N*-(hydroxyethyl)ethylene diaminetriacetic acid (10 mM). The pH jump in turn was measured by monitoring the absorption of bromothymol blue.

Nitr-5 and nitr-7 were synthesized by Stephen Adams as described elsewhere (S. Adams, J. Kao, G. Grynkiewicz, A. Minta, R.Y.T., unpublished results). Nitr-5 is available commercially from Calbiochem.

RESULTS

Flash-Activated Currents. Ca^{2+}-dependent K^+ currents have been described in rat superior cervical ganglion cells (10, 13). These currents are thought to underlie the prolonged (>100 ms) hyperpolarization that follows an action potential in these cells. For voltage steps from a holding potential of −50 mV to a test potential in the range 40–80 mV, outward currents were decreased 40–60% by extracellular application of 100 μM $CdCl_2$, by 50 μM apamin (the latter effect was only partially reversible), or by both agents together (A.M.G. and J. M. Nerbonne, unpublished data). Thus these cells display a Ca^{2+}-activated K^+ current similar to the current component termed I_{AHP} in bullfrog sympathetic ganglion cells (14, 15). Further experiments were performed with Cd^{2+} in the bathing solution to prevent Ca^{2+} influx and with nitr-5 in the patch pipette (Fig. 1). In the absence of light, the voltage-dependent K^+ currents presumably included both a component that was Ca independent and another that was activated by the basal intracellular $[Ca^{2+}]$ (≈430 nM in the experiment of Fig. 1*B*).

Light flashes presented during the plateau phase of these currents induced an additional outward current (Fig. 1 *B* and *C*). Flash-activated currents were resolved only at membrane potentials more positive than −40 mV. At more negative potentials the currents were probably too small to be distinguished against the background noise. Flash effects were specifically associated with the photochemistry of nitr-5, because they were absent either (*i*) when EGTA was substituted for nitr-5 in the pipette or (*ii*) when flashes were filtered to remove wavelengths absorbed by nitr-5.

After a flash, currents relaxed with a simple exponential time course to a new maintained level within a few tens of milliseconds (Fig. 1*C*). Little inactivation was apparent during 500-ms voltage steps. The currents did eventually return to the preflash level, at least in part because fresh, unphotolyzed nitr-5 diffused from the pipette into the cell. The current relaxation could, therefore, be reproduced by

successive flashes, provided they were presented at intervals ≥30 s.

To determine the events that limit the kinetics of the flash-induced current, we compared the effects of flashes using different photolabile chelators (Fig. 2), under similar conditions (i.e., 2 mM chelator, 0.2 mM added Ca^{2+}). When the pipette contained nitr-2 (7; S. Adams, J. Kao, G. Grynkiewicz, A. Minta, and R.Y.T., unpublished results), which has a similar Ca^{2+} affinity to nitr-5 but photolyzes with a time constant of 140–200 ms, flashes (0.8–1 J) induced current relaxations with a time constant of 169 ± 14 ms (SEM, $n = 6$). With nitr-7, which binds Ca more tightly than nitr-5 but releases it with a time constant of ≈1.8 ms, less-intense flashes (0.25 J) activated currents that relaxed with a time constant of 24 ms (mean of two cells), compared to 28 ± 5 ms (SEM, $n = 4$) with nitr-5. The amplitude of the $[Ca^{2+}]$ jump was comparable in the nitr-5 and nitr-7 experiments. We, therefore, suggest (*i*) that the kinetics of the nitr-2 relaxations reflect predominantly the rate of photorelease of Ca^{2+}, but (*ii*) that nitr-5 and nitr-7 release Ca^{2+} so quickly that the kinetics are dominated by a subsequent event, presumably Ca^{2+} binding to the K^+ channel or opening of the channel itself.

$[Ca^{2+}]$ Dependence of Flash-Activated Currents. Both the amplitudes and kinetics of the flash-activated currents varied with the magnitude of the $[Ca^{2+}]$ jump. Current amplitude increased with increasing $[Ca^{2+}]$ (Fig. 3*A*). In the cell of Fig. 3*A*, which was studied over a wide concentration range from a basal free $[Ca^{2+}]$ of 430 nM (i.e., 2 mM nitr-5, 75% Ca^{2+} bound), a maximal response was approached with jumps of ≈5 μM, and the response was half maximal with a jump of ≈350 nM. Half-maximal activation appeared to be in the range 350–1000 nM for all cells studied. For the same cell as in Fig. 3*A*, the Hill coefficient was 0.90 (Fig. 3*B*). For all experiments under similar conditions, the slope of a double log plot of current amplitude versus the $[Ca^{2+}]$ jump was 0.80 ± 0.04 (SEM, $n = 5$). As this value was likely to be distorted by the high preflash $[Ca^{2+}]$, we repeated the experiments beginning with a free $[Ca^{2+}]$ estimated at 16 nM (2 mM nitr-5, 10% Ca^{2+} bound). The slope of a similar plot was 0.96 (mean of two cells), indicating an approximately linear dependence on $[Ca^{2+}]$ and confirming the findings at higher $[Ca^{2+}]$. There may, however, be errors in our estimates of the free $[Ca^{2+}]$ particularly with low levels of added Ca^{2+}, because in a few cells where 2 mM nitr-5 was included without added Ca^{2+}, flashes induced measurable currents.

To determine the origin of the flash-induced currents in the absence of added Ca^{2+}, a series of cells were studied with the pipette solution alternately containing 2 mM or 10 mM nitr-5, but no added Ca^{2+}. In this series, no flash-induced currents were observed in four cells with 10 mM nitr-5, whereas flashes induced small but measurable currents in two out of five cells at the lower nitr-5 concentration. This result suggests that the currents were not an effect of a photolysis byproduct, although there is no way of testing this directly.

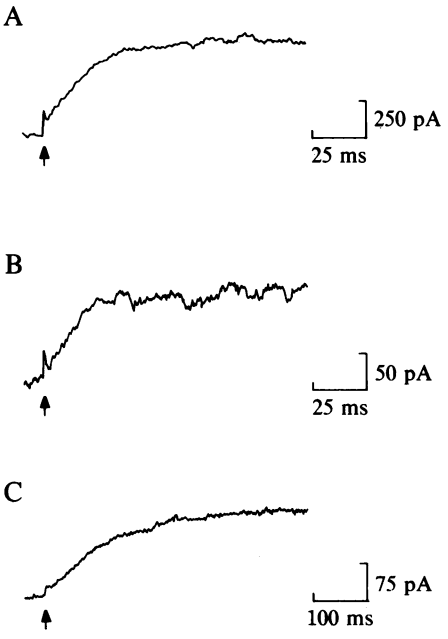

FIG. 2. Flash-activated outward currents at 40 mV, with 2 mM chelator and 0.2 mM added Ca^{2+} in the patch pipette. (*A*) Nitr-5. (*B*) Nitr-7. (*C*) Nitr-2. The total output energy of the flash, delivered at the arrow, was 0.25 J in *A* and *B* and 0.84 J in *C*. The different relaxation amplitudes in *A* and *B* typify the range of observations with different cells tested under identical conditions.

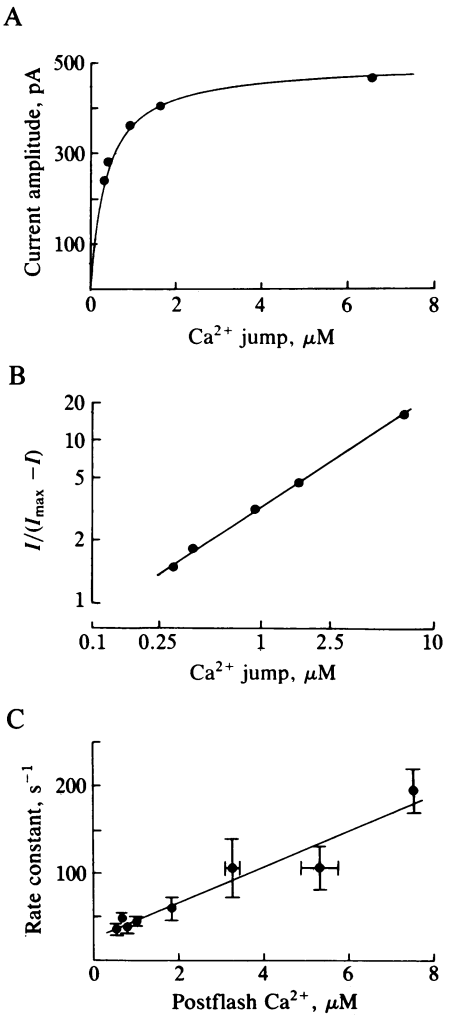

FIG. 3. Concentration dependence of flash activated currents induced at 40 mV. Note that the ordinate is the $[Ca^{2+}]$ jump in *A* and *B*, but the total $[Ca^{2+}]$ in *C*. (*A*) Relaxation amplitude versus the amplitude of the $[Ca^{2+}]$ jump on linear coordinates. The pipette contained 2 mM nitr-5 with 1.5 mM added Ca^{2+}. The continuous line represents a fit to the data with the relation $I = I_{max}/(1 + K/[Ca^{2+}])$, where I_{max} (the maximum current amplitude) = 490 pA and K (the $[Ca^{2+}]$ jump at which there is half-maximal activation) = 350 nM. (*B*) Same data are plotted on "Hill" coordinates. The line represents the least-squares fit to the data and has a slope of 0.90. (*C*) The relationship between the relaxation rate constant and the $[Ca^{2+}]$ at the end of the jump. Points represent the mean values from 5 to 22 cells. Error bars show the SEM. The best fitting line has a slope of $2.05 \times 10^7\ M^{-1}s^{-1}$ and zero-concentration intercept of $25\ s^{-1}$.

It seems more likely that they were activated by released Ca^{2+}. This Ca^{2+} is unlikely to have been contributed by contamination of the KCl pipette solutions: with 5 μM contamination, flashes would increase $[Ca^{2+}]$ from a basal level of 0.4 nM to a value only 50% higher. Other workers have noted that $[Ca^{2+}]$ control is improved by chelator concentrations of $\geq$10 mM (16); it, therefore, seems more likely that the Ca^{2+} originated from within the cell.

The rate constants of the flash-induced current relaxations also increased with the $[Ca^{2+}]$ throughout the range measured (up to 7 μM; Fig. 3*C*). A linear fit to this relation provides a slope of 2.05×10^7 $M^{-1} \cdot s^{-1}$ and an intercept of 25 s^{-1}, when the preflash free $[Ca^{2+}]$ was 430 nM (2 or 10 mM nitr-5, 75% complexed with Ca^{2+}).

Voltage Sensitivity. The amplitudes of the flash-induced current relaxations increased at more positive voltages (Fig. 4). Because currents could not usually be resolved at membrane potentials more negative than −40 mV, there are insufficient data to rule out a sigmoidal start to the current versus voltage relationship. In the voltage range studied, however, the flash-activated currents varied in a roughly linear fashion with membrane potential (Fig. 4), suggesting that the flash-activated conductance was not strongly voltage dependent. There was also little or no apparent voltage sensitivity in kinetics of current activation. The time constants of the flash-induced current relaxations were compared at various membrane potentials for the larger $[Ca^{2+}]$ jumps, which yielded reliable kinetic data. Time constants varied measurably with voltage in <45% of the cells examined; where present, voltage dependence was rather shallow, with an *e*-fold change for 102 ± 11 mV (SEM; n = 9).

A rather voltage-insensitive flash-activated conductance is further suggested by the behavior of the tail currents, observed upon membrane repolarization after a flash (Fig. 5*A*). Voltage steps, applied during the flash-activated current, caused a change in current amplitude. There was usually no clear relaxation associated with this change in amplitude. It should be noted, however, that the tail current resulting from intracellular Ca^{2+} release was measured from the difference between two current records collected at 10-s intervals, and accurate resolution relied on the exact superposition of the preflash currents in both records. We cannot rule out the possibility of a very slow relaxation. The amplitude of the tail current varied linearly with membrane potential (Fig. 5*B*). The current reversed close to the K^+ equilibrium potential, confirming that the flash-activated current was carried by K^+ ions and ruling out a significant contribution from Ca^{2+}-dependent, nonselective cation (17, 18) or Cl^- (19–22) channels.

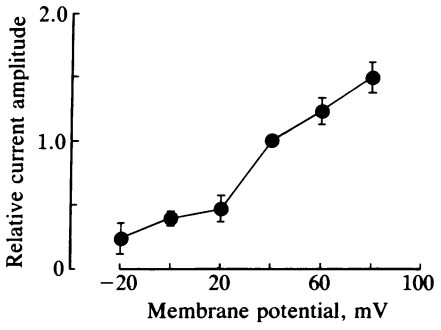

FIG. 4. The amplitude of the flash-induced current plotted against membrane potential. For each cell the amplitude was normalized against the amplitude of the current induced at 40 mV. The pipette contained either 2 or 10 mM nitr-5, 75% complexed with Ca^{2+}. Data obtained using various flash intensities have been combined. Points represent the mean from 3 to 16 cells. Error bars represent the SEM.

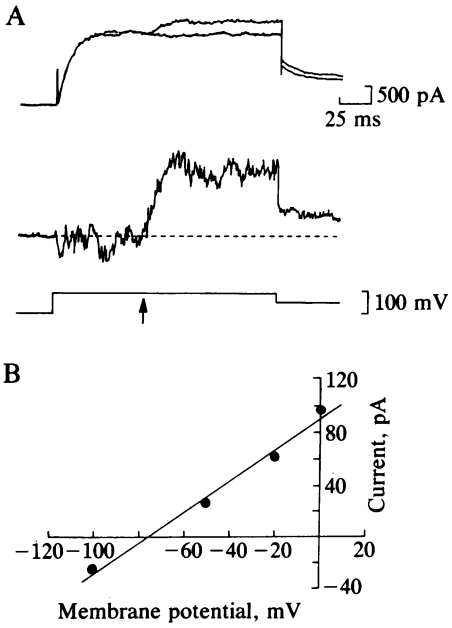

FIG. 5. (*A*) (*Top*) Current records during voltage steps from a holding potential of −50 mV to 40 mV and during repolarization to 0 mV. The voltage–clamp protocol is shown (*Bottom*). Two episodes evoked at 10-s intervals are superimposed. A flash was presented during the second episode at the arrow. The tail current during repolarization was larger following the flash. (*Middle*) The difference current, constructed by subtracting the preflash record from the one during which the flash was presented on a 5-fold expanded current scale, represents the flash-activated component of the currents. The zero-current level is indicated by the broken line. (*B*) The amplitude of the flash-activated tail current, from the same cell as in *A*, is plotted as a function of the membrane potential during the tail. The current reversed at −76 mV. The intracellular and extracellular $[K^+]$ were, respectively, 140 mM and 5 mM. The preflash conditions were 2 mM nitr-5 with 1 mM Ca^{2+} added. A maximal flash was employed, but in this experiment it was delivered through the fluorescence port of the microscope and has not been calibrated.

DISCUSSION

These experiments establish the usefulness of flash-induced Ca^{2+} concentration jumps as a means to study kinetic and equilibrium aspects of ion-channel gating. Some uncertainties remain about how completely the $[Ca^{2+}]$ is controlled by nitr-5 within the cell and, therefore, about the accuracy of the quantitative estimates of Ca^{2+} potency near the membrane. Hence, responses to small $[Ca^{2+}]$ jumps, which might yield the most interesting data on functional stoichiometry, are not well understood. Nonetheless it is already clear that K^+ channels open within a few milliseconds after a submicromolar concentration of Ca^{2+} appears at the cytoplasmic face of the membrane. This supports suggestions that a cell's firing rate can be modulated on a millisecond time scale by Ca^{2+}, entering through voltage-sensitive Ca^{2+} channels and increasing K^+ conductance (23, 24).

At present it is thought that two classes of Ca^{2+}-activated K^+ conductances exist in vertebrate tissues. These are usually distinguished by sensitivity either to charybdotoxin and tetraethylammonium ion (TEA) (25) or to apamin (26). Rat sympathetic ganglion cells are apparently capable of displaying both types of conductance (14, 32); however, in the present experiments, the current was sensitive to apamin but not to low concentrations of charybdotoxin (A.M.G., unpublished data). The present data, therefore, pertain to the apamin-sensitive conductance. Relatively little is known about gating or permeation in the apamin-sensitive channel, mainly because the current is not easily isolated from other

membrane currents and the single-channel conductance is small (27, 28). Ca^{2+} jumps provide a means of selectively activating Ca^{2+}-dependent conductances, facilitating their study. Although our results are still preliminary, they do permit a simple kinetic description for Ca^{2+} activation of K^+ channels in rat superior cervical ganglion neurons.

That the flash-induced currents were maintained for several seconds suggests little distortion by the cell's endogenous Ca buffers, in contrast to the situation with microinjected cells (7). Furthermore, the presence of only one kinetic component in the flash-induced current relaxations suggests that one step, either Ca^{2+} binding or channel opening, is rate limiting. In addition, the linear relationship between current amplitude and the magnitude of the $[Ca^{2+}]$ jump does not require us to propose, at present, that more than one Ca^{2+} ion is needed to open the channel. As the currents did not display voltage-dependent inactivation, we, therefore, consider the following simple scheme for channel gating:

$$Ca^{2+} + R \rightleftarrows Ca^{2+}\cdot R \rightleftarrows Ca^{2+}\cdot R^*,$$

where R and R* represent the closed and open states of the channel, respectively. Interpretation of the model depends upon the rate-limiting step. If the Ca^{2+}-binding step is slow relative to channel opening and is rate limiting, then the forward rate constant for binding is simply the slope of the line relating the relaxation rate constant to $[Ca^{2+}]$ (Fig. 3*C*). If, however, the binding step is fast, binding is governed by a rate constant greater than the slope. This simple model thus suggests that the forward binding rate is at least 2×10^7 $M^{-1}\cdot s^{-1}$ at 20°C. By a similar argument, the rate constants governing both channel closure and dissociation of Ca^{2+} are at least equal to the zero-concentration intercept of this plot ($25\ s^{-1}$).

Regardless of the exact molecular interpretation, the model predicts half-maximal activation when the overall rate constant for channel opening equals that for closing, i.e., when the relaxation rate constant is double the zero-concentration intercept. According to Fig. 3*C*, this occurs at a $[Ca^{2+}]$ of about 1.2 μM, resulting from a jump of 800 nM. This value is in good agreement with the observed half-maximal activation for jumps of 300–1000 nM under similar conditions (Fig. 3*A*). Although our model may be oversimplified, it does appear to account for the major features of the data presented here and in single-channel experiments (27).

The rather small voltage dependence to the amplitude and kinetics of the flash-induced current suggests that it is similar to the apamin-sensitive, Ca^{2+}-activated currents in other tissues (15, 27, 28). However, our voltage-sensitivity experiments were performed at high $[Ca^{2+}]$. Some ligand-activated conductances display voltage sensitivity at low levels of activation but not at high concentrations that produce maximal activation (29, 30). Our results are also consistent with the idea that the slow time course of I_{AHP} in bullfrog sympathetic neurons reflects the rate of Ca^{2+} removal from the cell rather than the kinetics of channel closure (15).

Flash-activated molecules have proven useful for a variety of physiological studies (4, 31). The present photolabile Ca^{2+} chelators have obvious applications in determining the role of Ca^{2+} as an intracellular messenger in many systems. Neither nitr-5 nor its photoproduct had any apparent toxic effects on the cells studied. Some of the uncertainties about Ca^{2+} sensitivity, and the adequacy of $[Ca^{2+}]$ control in intact cells, might be resolved with studies on the $[Ca^{2+}]$ dependence of single-channel currents in membrane patches. It will also be useful to combine the technique with methods of measuring intracellular $[Ca^{2+}]$.

We thank Dr. S. Adams for synthesizing nitr-5 and nitr-7, Dr. J. M. Nerbonne for advice and helpful discussions, for participating in early experiments, and for providing facilities for some of the later experiments. We also thank Dr. C. Miller for a gift of charybdotoxin and for much discussion, Drs. P. R. Adams, R. Brett, S. Jones, J. Nargeot, and R. Zucker for discussion; and B. Tanamachi, D. Martin, and J. Doyle for preparing the cells. This research was supported by the American Heart Association Postdoctoral Fellowship to A.M.G., by Grant 83-K-111 from the Searle Scholars Program, by Grants GM-29836, GM-31004, and EY04372 from the National Institutes of Health, and by the Medical Research Council and British Heart Foundation.

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