

Time course of the increase in the myocardial slow inward current after a photochemically generated concentration jump of intracellular cAMP

(calcium channel/voltage clamp/*o*-nitrobenzyl ester/cGMP/ β -adrenergic agonist)

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ABSTRACT Voltage-clamped atrial trabeculae from bullfrog hearts were exposed to membrane-permeant photolysable *o*-nitrobenzyl esters of cAMP and cGMP. UV flashes produced intracellular concentration jumps of cAMP or cGMP. With the cAMP derivative, flashes resulted in an increased slow inward current (I_{si}), producing a broadened action potential. The I_{si} reached a maximum 10–30 sec after the flash and decreased over the next 60–300 sec. The first increases were observable within 150 msec; this value is an upper limit imposed by the instrumentation. Responses to flashes lasted longer at higher drug concentrations and in the presence of the phosphodiesterase inhibitor papaverine; effects of flashes developed and decreased faster at higher temperature. Although the amplitude of the I_{si} was increased, its waveform and voltage sensitivity were not affected. Intracellular concentration jumps of cAMP failed to affect the muscarinic K^+ conductance. There were no observable effects of cGMP concentration jumps. The data confirm (i) that cAMP regulates the I_{si} and (ii) that the 5- to 10-sec delay between application of β -agonists and the onset of positive inotropic effects, observed in previous studies, has been correctly ascribed to events prior to the interaction between cAMP and protein kinase.

Cyclic nucleotides are thought to play a pivotal role in the regulation of the strength and frequency of the heartbeat by autonomic neurotransmitters. Electrophysiological investigations show that such inotropic and chronotropic effects arise partly through changes in the slow inward current (I_{si}), which is carried mainly by calcium ions (1–5). The amplitude of the I_{si} is enhanced, and the plateau of the action potential is consequently lengthened, by many treatments that increase intracellular cAMP, such as β -adrenergic stimulation (1, 3, 6), direct intracellular application of cAMP itself (5, 7–11), phosphodiesterase inhibition (12), exposure to membrane-permeant analogues of cAMP (13), and GTPase inhibition (14). On the other hand, this slow inward current is inhibited by muscarinic stimulation (4, 15, 16), which also inhibits adenylate cyclase (17, 18). Presumably the cAMP-mediated effects depend, directly or indirectly, on the phosphorylation of intracellular protein(s). This concept is supported by the recent observation that the I_{si} is increased or decreased by intracellular injection of the catalytic or regulatory subunit, respectively, of cAMP-dependent protein kinase (19).

Which molecular steps limit the rate of onset of the increased I_{si} and other positive inotropic effects? In experiments with rapid application of β -agonists, there is a delay of 5–10 sec before detectable physiological effects (20, 21). This delay approximates the time required for activation of adenylate cyclase

in response to a sudden addition of agonist (22, 23). Therefore, one expects that the positive inotropic effects would appear much more quickly in an experiment in which the intracellular cAMP concentration itself is jumped directly.

We have therefore developed a method for producing concentration jumps of cyclic nucleotides within cells under physiological investigation. Our procedure exploits the properties of *o*-nitrobenzyl cAMP (Fig. 1) and its cGMP analog. These molecules are members of a group of phosphotriesters that enter cells (24–26). The *o*-nitrobenzyl esters can then be photolyzed to produce the cyclic nucleotides themselves, presumably within a few msec (27–30). We find that the I_{si} is indeed increased by photochemically generated concentration jumps of intracellular cAMP and that the latency of this action (<150 msec) is no more than 3% of the latency for β -adrenergic stimulation.

There are additional questions about the detailed kinetics and mechanism of these actions. It is not known whether certain other neurotransmitter effects, such as the muscarinic potassium current, are mediated by changes in intracellular levels of cAMP (11). Furthermore, it is not yet clear whether cGMP affects physiological properties of the heart (31). We find no effect of cAMP concentration jumps on the muscarinic K^+ conductance and, as yet, have not found observable changes in voltage-clamp currents after cGMP concentration jumps.

MATERIALS AND METHODS

The electrophysiological and optical apparatus has been described in detail (32). In brief, atrial trabeculae from bullfrogs (*Rana catesbeiana*) were investigated with the double sucrose-gap voltage clamp (33, 34) while exposed to light from a xenon flash tube (30). The current under investigation in the present study, I_{si} , was large enough to obviate the need to correct for the nonspecific light-flash relaxations noted previously (32). The fast sodium current was eliminated with tetrodotoxin, allowing for good records of the I_{si} . Signals were led to an oscilloscope and a pen recorder and, in some experiments, to analog-to-digital converters for numerical analysis. Temperature was 23°C except where noted.

The flash tube discharged a capacitor bank (1,800 μ F) charged to 400 V. The flash lasted about 2 msec. Wavelengths <300 nm were excluded with a filter (WG295, Schott Optical Glass, Duryea, PA).

The *o*-nitrobenzyl esters of cAMP and cGMP were synthesized and purified as described (24–26). The *o*-nitrobenzyl esters were first dissolved in dimethyl sulfoxide and then diluted

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Abbreviation: I_{si} , slow inward current.

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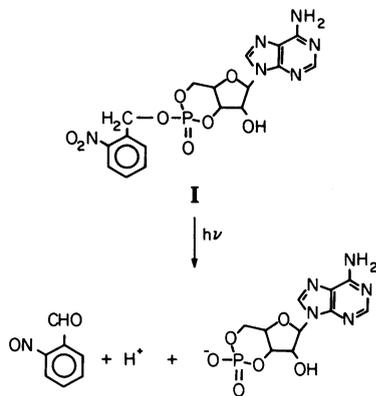


FIG. 1. Photochemical strategy used in the experiments. A fiber containing *o*-nitrobenzyl cAMP (I) is exposed to UV flashes. The photolysis yields *o*-nitrosobenzaldehyde, a proton, and cAMP itself.

into Ringer solution so that the final dimethyl sulfoxide concentration was <1%, a concentration that by itself did not affect voltage-clamp currents.

Thermal or light-induced hydrolysis of the triesters was measured by HPLC on a reversed-phase column. We confirmed that thermal hydrolysis proceeds at a rate of <5%/day at 22°C (24). A 25- μ l drop of Ringer solution containing 60 μ M *o*-nitrobenzyl ester was placed at the usual position of the cell. The drops were exposed to flashes and then analyzed by HPLC. Each flash hydrolyzed 2.5% of the *o*-nitrobenzyl cAMP or 1.2% of the *o*-nitrobenzyl cGMP to produce the corresponding cyclic nucleotide; photolysis proceeded with the same efficiency for the axial and equatorial isomers.

RESULTS

When *o*-nitrobenzyl cAMP (0–40 μ M) was perfused through the experimental chamber for 5–10 min, there was no change in the waveform of an unclamped action potential and there was no effect or a small increase (<5%) in the peak amplitude of the I_{si} . A single flash then produced a broadened action potential (Fig. 2A) and an increase in I_{si} over the next 30–40 sec (Fig. 2B). This increase was most pronounced in preparations treated with acetylcholine or propranolol to decrease the initial size of I_{si} ; in such cases, the increase reached a factor of 3. Over the next few min, I_{si} then decreased to the value before the flash (Fig. 3). In 10–20% of the preparations tested, there was a small “irreversible” effect so that the amplitude of I_{si} never returned to its value before the flash. Similar lack of reversibility is also often observed with β -adrenergic stimulation.

Several observations support the conclusion that the flash-induced changes in the I_{si} result from a photochemically generated jump of intracellular cAMP. (i) The effect had an action spectrum appropriate to *o*-nitrobenzyl photolysis (29): it was abolished by filters that eliminated UV radiation and was still present, although smaller, when a filter (Schott, UG11) that had a transmission peak at 320 nm was used. (ii) There were no flash effects in the absence of added drug or after a 15- to 30-min washout period, except for the small “nonspecific” relaxations previously described (32). (iii) I_{si} was not affected by flashes in the presence of 100 μ M *o*-nitrobenzyl acetate or of several related compounds that produce the same intracellular pH jump and aldehyde photoproducts as *o*-nitrobenzyl cAMP but not the cyclic nucleotide itself (30). (iv) Neither resting potential, resting conductance, nor I_{si} were affected by flashes in the presence of *o*-nitrobenzyl cGMP. Because the photolysis by-product of the reaction, *o*-nitrosobenzaldehyde, is identical for the

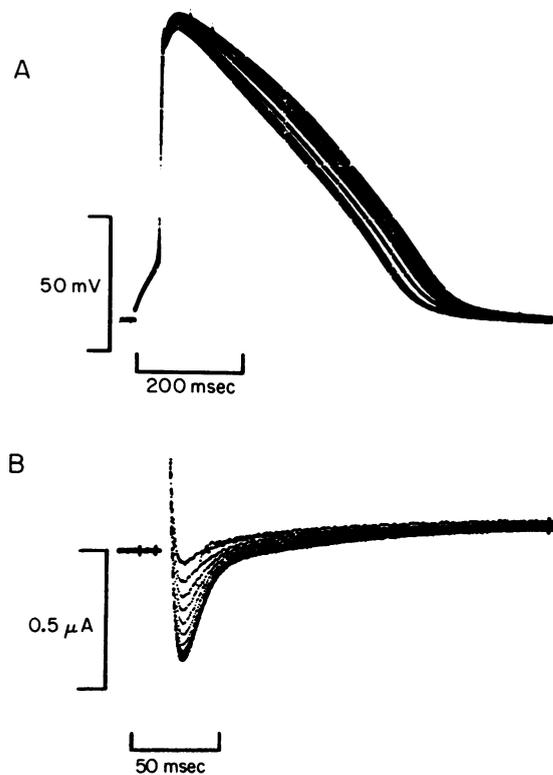


FIG. 2. (A) Action potentials stimulated by externally applied current pulses, at intervals of 4 sec, in an atrial trabeculum in the sucrose-gap apparatus. The Ringer solution contained 30 μ M *o*-nitrobenzyl cAMP. The stimulation was started 1 min before the episodes shown, so that there were no further changes due to facilitation. The action potential is briefest in the first trace; a flash was delivered to the fiber just before the second trace, which shows a longer action potential. The broadening continues for the next six traces. Similar results were obtained with three other preparations. (B) Waveforms of I_{si} in voltage-clamped bullfrog atrium. The Ringer solution contained 40 μ M *o*-nitrobenzyl cAMP, 1 μ M tetrodotoxin to suppress fast Na^+ currents, 20 mM Cs^+ to maintain the inward rectifier in a linear high-resistance state, and 1 μ M carbachol/0.1 μ M propranolol to decrease the initial amplitude of I_{si} . The fiber was held hyperpolarized by 10 mV with respect to the normal resting potential and then stepped to a depolarization of 72 mV at intervals of 4 sec. As in A, this stimulation was started 1 min before the episodes shown, so that the preparation was in a steady state of facilitation. Passive currents have not been subtracted. In the first two episodes of the trial, the currents are smallest, and they superimpose until a flash, delivered just after the peak of current, produces a small voltage-clamp artifact. The next episode, 4 sec later, reveals a larger I_{si} , and this trend continues for seven episodes until the amplitude levels off. These records are typical of results obtained with 30 preparations.

two nucleotides, this result also rules out any effects of the nitroso compound (28). (v) There was no effect on I_{si} when previously photolyzed solutions of *o*-nitrobenzyl cAMP were perfused through the experimental chamber.

Duration and Amplitude of the Flash Effects. The effects of flashes on I_{si} may be summarized by (i) time to maximum increase, (ii) percent maximum increase, and (iii) half-decay time. As the concentration of *o*-nitrobenzyl cAMP was increased (within the range 4–40 μ M), each of these parameters increased: the flash-induced increases were larger and lasted longer (Fig. 3), possibly because phosphodiesterase or phosphatase activity was saturated at higher cAMP concentrations. In three preparations treated with 10 μ M papaverine to block phosphodiesterase, the time to maximum effect was increased by an average of 43% and the half-time of decay was increased by an average of 36%, although the papaverine treatment itself increased the I_{si} . The effects developed and decreased more rapidly at higher tem-

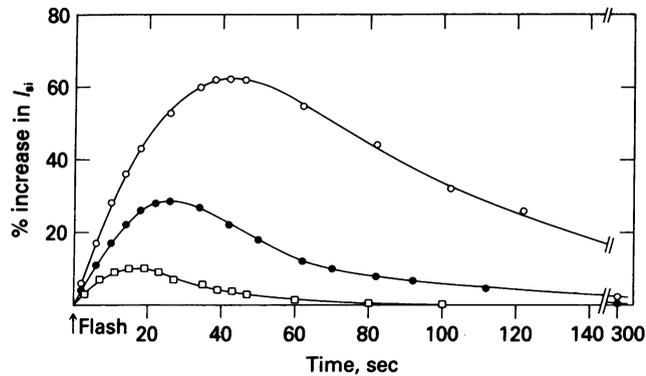


FIG. 3. Time course of increase in the I_{si} in a fiber exposed to 30 μM (\circ), 10 μM (\bullet), and 4 μM (\square) *o*-nitrobenzyl cAMP. The depolarizing pulse was 80 mV, and the flash occurred at time zero. Similar results were obtained with four other preparations.

peratures: in three preparations warmed from 25°C to 35°C, the time to peak and the half-decay time were decreased by factors of 1.8 and 1.7, respectively; on cooling from 25°C to 16°C, these parameters were increased by factors of 2.3 and 3.3.

Latency of the Response to cAMP Jumps. In five experiments in which the flow through the experimental chamber was switched to a solution containing a β -adrenergic agonist, the I_{si} began to increase after a delay of ≈ 6 sec (Fig. 4A). On the other hand, in 10 experiments in which flashes were delivered to a preparation exposed to *o*-nitrobenzyl cAMP, the increase extrapolated back to the time of the flash, with an uncertainty of ≈ 150 msec (Fig. 4B). In three exceptionally stable preparations, increases of 2% to 3% were directly observed within 150 msec after the flash (Fig. 5). The first electrophysiological effects of cAMP concentration jumps might occur much earlier than 150 msec; the value given is limited by interference from the "nonspecific light-flash relaxations" described earlier (32).

Waveform and Voltage Sensitivity of the I_{si} . The experiment of Fig. 2B suggests that cAMP concentration jumps, like β -adrenergic stimulation, produce no marked change in the time course of the I_{si} while increasing its amplitude (3). This impression was confirmed in five other preparations with step depolarizations at 8- or 10-mV increments between the resting potential and a depolarization of +120 mV (Fig. 6). When scaled to the same peak amplitude, the waveforms of I_{si} superimposed well before and after the flash. Furthermore, there was only a small change in the voltage sensitivity of I_{si} : after three or four flashes, the amplitude was increased by an average factor of 2.24 but the peak of the current-voltage curve was shifted by an average of only 5.8 mV to more negative potentials. This small shift could arise because increased intracellular Ca^{2+} accumulation enhances inactivation of I_{si} (35-37); it might also arise because of systematic errors in the sucrose-gap voltage-clamp circuit.

Other Observations with *o*-Nitrobenzyl cAMP. In fibers exposed to Ca^{2+} -, Mg^{2+} -free Ringer solution containing 1 mM EGTA, the I_{si} is increased severalfold in amplitude and duration, probably because the channels become more permeable to Na^+ (38). When such fibers were exposed to *o*-nitrobenzyl cAMP, flashes produced a further augmentation of I_{si} . Likewise, the flash-induced increase in I_{si} still occurred when Li^+ was substituted for Na^+ in the Ringer solution. Thus, the cAMP concentration jumps seem to be affecting the I_{si} channels themselves, regardless of the specific permeant ion.

At *o*-nitrobenzyl cAMP concentrations $\geq 100 \mu\text{M}$, there were large (2-fold) increases in I_{si} even without flashes, as described

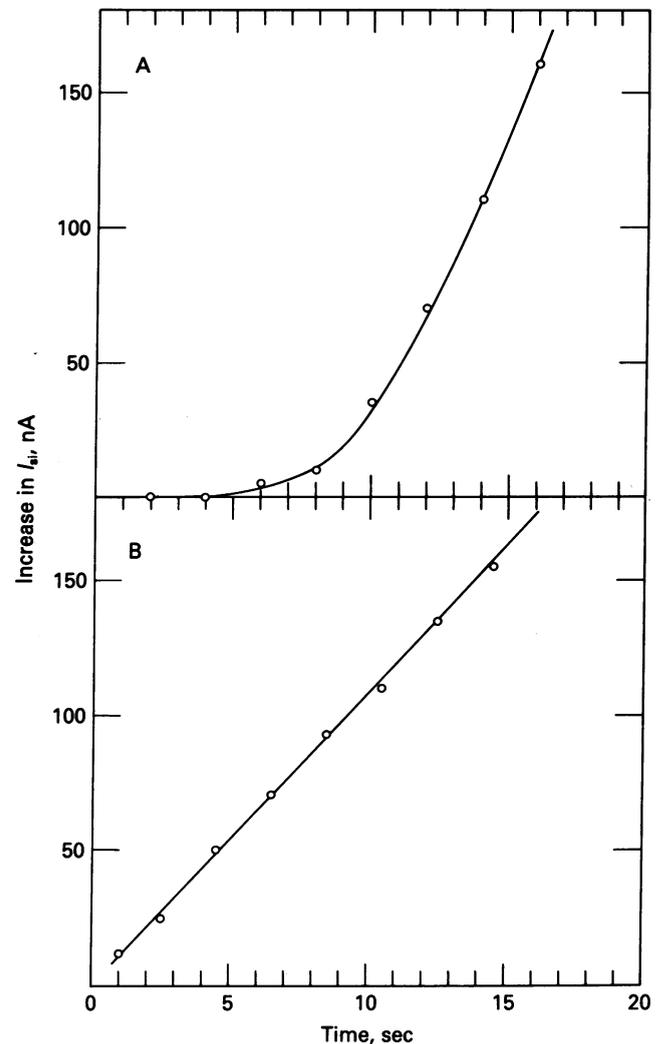


FIG. 4. Comparison of initial increase in the I_{si} after a concentration jump of extracellular isoprenaline (A) or intracellular cAMP (B). (A) At zero time, the solution flowing through the chamber was suddenly switched to one containing isoprenaline (3 μM). Data have been corrected for the dead time of the perfusion apparatus (measured with depolarizations due to high-KCl Ringer solution; resolution, ± 300 msec). (B) Another fiber from the same heart. The preparation was equilibrated for 5 min with *o*-nitrobenzyl cAMP (30 μM) and a flash was delivered at zero time. Depolarizing pulses were 60-mV in A and 75 mV in B.

previously (26), and flashes produced little or no further increases. In three preparations in which I_{si} was maximally increased with isoprenaline (10 μM), there was no further effect of *o*-nitrobenzyl cAMP, with or without flashes. There were small increases in delayed rectifier currents (I_x) as a result of the cAMP concentration jumps but we have not studied this effect systematically. The flash-induced increases in I_{si} were accompanied by increases in twitch tension. There was no effect of *o*-nitrobenzyl cAMP, either before or after flashes, on the increased potassium conductance induced by carbachol.

Experiments with *o*-Nitrobenzyl cGMP. The experiments of Figs. 1-6 were also conducted with *o*-nitrobenzyl cGMP at concentrations up to 500 μM . The compound was also tested in the presence of *o*-nitrobenzyl cAMP at various concentrations and in the presence of 1 μM isoprenaline. Action potentials, I_{si} , and the background conductance were measured. The cGMP derivative itself had no effects in any of these experiments, either before or after flashes were delivered to the preparation.

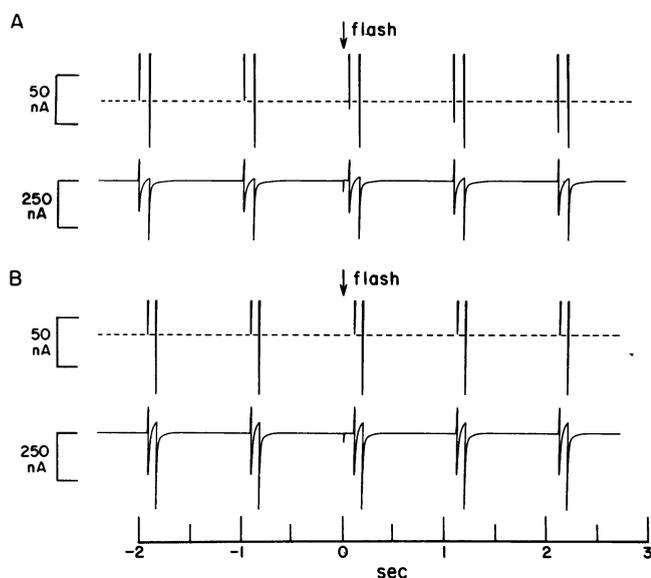


FIG. 5. (A) Rapid response to flashes in an especially stable preparation exposed to 30 μM *o*-nitrobenzyl cAMP. The lower trace shows the voltage-clamp currents associated with a depolarizing step of 80 mV every 2 sec. The upper trace, displayed with an offset and at higher gain, is off scale except during the peak of I_{si} (as well as the larger peak of the tail current on repolarization). The flash occurred at zero time and produces a small artifact on the low-gain trace. ----, Amplitude of the I_{si} before the flash. I_{si} increases after the flash; the increase is noticeable even for the step that occurs 150 msec after the flash. (B) Control experiment on another fiber in the absence of the photolabile drug.

DISCUSSION

The speed of the observed effects, along with the previous observations summarized in the Introduction (1–19), leave little doubt that cAMP can serve as an intracellular second messenger for modulation of the I_{si} . In eukaryotes, all known cAMP actions are mediated through cAMP-dependent protein kinase. Our results confirm earlier conclusions that the “lag phase” after the introduction of a β -adrenergic agonist arises from a step(s) preceding the interaction between cAMP and protein kinase. Such a step might be the activation of adenylate cyclase (22, 23) or the diffusion of cAMP to a special compartment (39). The evolution of I_{si} after the flash agrees well with the time course of the positive inotropic effect and of protein phosphorylation

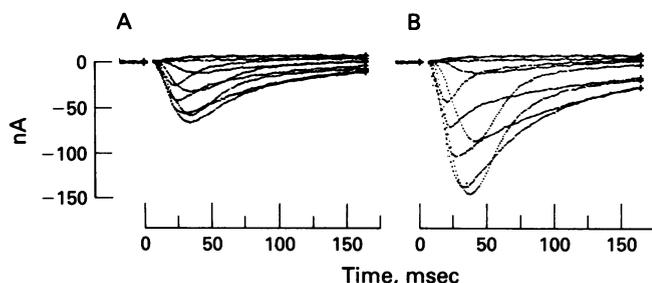


FIG. 6. Records of I_{si} associated with step depolarizations at intervals of 1 sec. The holding potential was 5 mV more negative than the resting potential and the depolarizing step was incremented by 8 mV in each episode; traces from alternate episodes are shown. Trials were taken just before (A) and 30 sec after (B) a flash in the presence of 40 μM *o*-nitrobenzyl cAMP; the Ringer solution also contained 20 mM Cs^+ , 0.1 μM carbachol, and 0.5 μM propranolol as in the experiment of Fig. 2B. Passive currents have been eliminated by subtraction of appropriately scaled responses to hyperpolarizing voltage jumps. The first 5 msec after the jump have been deleted because the capacitive transient currents saturated the recording apparatus.

after the lag phase in previous studies: all these effects develop over a period of 10–30 sec (20, 40–42). Rather low levels of phosphorylation evidently produce detectable increases in I_{si} : phosphorylation would proceed to the extent of at most a few percent within 150 msec after the flash (40–42), when the first increases in I_{si} were detected (Fig. 5).

In our experiments, the linear increase in I_{si} extrapolates to the time of the flash with a delay of <150 msec. This suggests that a single rate-limiting step is involved in this increase. This step might be the binding of cAMP to the kinase, the dissociation of the regulatory and catalytic subunits, possible translocation of the catalytic subunit to its site of action (39), the phosphorylation, or a subsequent event. Our experiments do not allow for a decision among these possibilities.

It has been suggested that cAMP increases the I_{si} by stimulating direct phosphorylation of a Ca^{2+} channel (3, 43, 44). It is also possible that I_{si} is modulated by local changes in cytoplasmic ion concentration via a less direct mechanism. Phosphorylation of the sarcoplasmic reticulum, for example, can lead to increased Ca^{2+} pumping activity (45, 46) and this might account for the cAMP-induced modulation of I_{si} (34–36). One might predict that these mechanisms would result in different response latencies between the concentration jump of cAMP and the change in I_{si} . However, in both cases, these delays are expected to be less than the 150-msec limit of our present technique; therefore, we cannot resolve this issue in our experiments. Improved time resolution and other techniques will be required to describe the complete chain of events between activation of protein kinase and increased I_{si} .

Although muscarinic agonists decrease both cAMP in intact cells and adenylate cyclase activity in homogenates from cardiac muscle (17, 18), it has been reported that the acetylcholine-induced K^+ conductance is not affected by manipulations of the intracellular cAMP concentration (11). In the present experiments, cAMP concentration jumps also failed to affect the muscarinic K^+ conductance. This observation argues against an involvement of cAMP in the muscarinic response.

One uncertainty associated with the light-flash technique is the actual amplitude of the intracellular concentration jumps. At an *o*-nitrobenzyl cAMP concentration of 10 μM , each flash produces a jump of 250 nM cAMP in the external solution. This value roughly equals the intracellular concentration estimated to produce half-maximal activation of protein kinase (47). We do not know, however, to what extent *o*-nitrobenzyl cAMP accumulates within the cell; because the *o*-nitrobenzyl esters have high lipid solubility, the accumulation could be substantial and locally variable and therefore play a role in determining the kinetics of the response.

It is not yet known why *o*-nitrobenzyl cGMP is photolyzed to produce cGMP with only half the efficiency of the corresponding cAMP derivative; preliminary studies suggest that there is a competing intramolecular reaction for this derivative that does not lead to cGMP release. Despite the uncertainties, in some of our experiments, the cGMP concentration jump amounted to 4 μM in the external solution—i.e., 10–100 times the K_m for activation of cGMP-activated protein kinase in several tissues (48) and 40 times the minimum cAMP concentration that produced observable effects. Our negative results with *o*-nitrobenzyl cGMP confirm previous observations with this compound (26) and suggest that cGMP does not play a role in modulating I_{si} or the background conductance in frog atrium (see also ref. 31).

The present technique may be generally useful for studies on the regulation of cellular properties by intracellular cyclic nucleotides. Flash effects are observable at *o*-nitrobenzyl cAMP concentrations as low as 4 μM ; the unphotolyzed drug itself

produces cAMP-like effects at about 100 μM , like many other cyclic nucleotide analogues (49). Thus, one has an available concentration range of 25-fold. With simple improvements in the optical apparatus,[¶] the procedure could probably be applied to any nonpigmented cell in a nearly transparent preparation. However, the technique is likely to be marginally effective for opaque tissues. For instance, to produce afterdischarges in bag cells of isolated intact *Aplysia* abdominal ganglia exposed to *o*-nitrobenzyl cAMP, 3–10 times more UV irradiation is required than in the present experiments (unpublished data). Perhaps the most important improvement would be development of *o*-nitrobenzyl cyclic nucleotide derivatives that undergo photolysis at longer wavelengths, allowing for large concentration jumps of cyclic nucleotides without danger of damage to cells. Preliminary investigations have been reported with such derivatives (29, 30, 50, 51).

[¶]With the apparatus used in the present experiments, 2.5% of the *o*-nitrobenzyl cAMP molecules were photolyzed per flash. This could be improved by a factor of 1.5 if a WG280 filter, passing wavelengths down to 280 nm, was substituted for the WG295 filter. An additional factor of 2 could be gained with a 50-mm secondary focusing lens rather than the 75-mm lens used at present (microelectrode impalements would be cumbersome). It would be technically possible to produce higher conversions by using (i) larger storage capacitors to produce brighter flashes or (ii) a laser (28).

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