Activation of Ryanodine Receptors by Flash Photolysis of Caged Ca^{2+}

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ABSTRACT Flash photolysis of DM-nitrophen generates an extremely large [Ca^{2+}] transient ("Ca^{2+} spike") at the start of each Ca^{2+} "step." The Ca^{2+} spike greatly increases the speed of activation of the ryanodine receptor channel ("supercharging") and could be responsible for apparent channel adaptation.

INTRODUCTION

We wish to point out that the recent paper by Györke et al. (1994), which used flash photolysis of DM-nitrophen to activate ryanodine receptor (RyR)/Ca^{2+} release channels, does not accurately convey the true rate of channel activation by 1 μM Ca^{2+} and should not be taken as evidence for a role of Ca^{2+}-induced Ca^{2+} release (CICR) in skeletal muscle under physiological conditions. Furthermore, neither it nor the preceding papers (Györke and Fill, 1993, 1994) offer clear evidence that either skeletal or cardiac RyRs "adapt" to a steady [Ca^{2+}].

Rather than producing a simple "step" change in [Ca^{2+}], the flash photolysis procedure of Györke et al. (1994) actually elicits a very large transient peak in [Ca^{2+}] ("Ca^{2+} spike") before the [Ca^{2+}] reaches its low steady-state level (Lamb et al., 1994). This Ca^{2+} spike has been observed directly by other workers (McCray et al., 1992; Zucker, 1993). Ca^{2+} spikes occur because the flash has to destroy a large amount of nitrophen to achieve the final steady-state [Ca^{2+}], and most of this nitrophen has Ca^{2+} bound, which is rapidly liberated by the photolysis and takes a finite time to rebind to some of the remaining free nitrophen (Fig. 1).

DETERMINANTS OF SPIKE SIZE

The key to understanding the size and time course of the Ca^{2+} spike involves consideration of the concentration of free nitrophen, [N_f], at each instant. Consider the case in question where the total amount of nitrophen is initially 3 mM and the steady-state [Ca^{2+}] is increased by photolysis from 0.1 to 1 μM (i.e., pCa 7 to pCa 6) (Györke et al., 1994). Assuming that the dissociation constant of Ca^{2+} from nitrophen is 5 nM (although it may actually be slightly higher at the ionic strength used), initially there must be ~143 μM N_f and 2.86 mM Ca-nitrophen (N_{Ca}). Thus, if the steady-state [Ca^{2+}] is to be raised 10-fold to 1 μM, the flash must destroy enough of the total nitrophen so that N_f drops eventually by 10-fold, i.e., 0.9 × 143 μM = 129 μM total nitrophen must be destroyed. Because 1) 95% of the total nitrophen is in the N_{Ca} form, and 2) N_{Ca} is photolysed 2.5 times more easily than N_f (Zucker, 1993), virtually all of the nitrophen destroyed (98%) will be N_{Ca}, and consequently ~126 μM of Ca^{2+} must be transiently liberated. To reach the final steady-state [Ca^{2+}], all but 0.9 μM of this liberated Ca^{2+} must be rebound by remaining N_f, which will thus drop from about 140 μM (as 3 μM is photolysed) to 14 μM. The rate of photolysis of nitrophen, and hence liberation of Ca^{2+}, is extremely fast (time constant $\leq$30 μs; Vergara and Escobar, 1993). If the association rate constant of Ca^{2+} to N_f (k_{on}) is 1.5×10^6 M^{-1} s^{-1} (Zucker, 1993), the corresponding time constant for Ca^{2+} association would be 5 ms initially (i.e., $(k_{on} \times [N_f])^{-1}$), increasing to 50 ms as the [N_f] dropped to its equilibrium value. Even if k_{on} were 100 times higher (i.e., 1.5×10^8 M^{-1} s^{-1}), approaching the theoretical diffusion-limited maximum, the time constant of Ca^{2+} association would be 50 μs initially but would increase to 500 μs before steady state was reached. Thus, the rebinding of Ca^{2+} by N_f is not fast enough to prevent a large transient increase in [Ca^{2+}] upon photolysis (Fig. 2, *A* and *B*).

Györke et al. (1994) argue that such Ca^{2+} spikes do not activate the RyR channel, citing earlier work in which the spike was supposedly "maximized" and had no effect on channel activity (Györke and Fill, 1994). However, the Ca^{2+} spike in question was actually *not* maximized, and in fact was smaller, and most importantly considerably briefer, than the spikes that activated the channel. This can be seen by considering the [N_f] changes involved: a single flash raising [Ca^{2+}] from 0.1 to 0.2 μM must lower N_f from ~140 to 70 μM, whereas the three equal-intensity flashes raising [Ca^{2+}] from 0.05 to 0.2 μM (Fig. 1, Györke and Fill, 1994) must lower [N_f] ~273 μM → 205 μM → 137 μM → 68 μM. Thus, the first of the three flashes liberates slightly less Ca^{2+} (68 μM) than the single flash (70 μM), and the reassociation of Ca^{2+} with unphotolysed nitrophen will occur initially about two times faster (i.e., $(k_{on} \times 273\ \mu M)^{-1}$ vs. $(k_{on} \times 140\ \mu M)^{-1}$), and then later three times faster ($(k_{on} \times 205\ \mu M)^{-1}$ vs. $(k_{on} \times 70\ \mu M)^{-1}$) than in the single-flash case. Thus, it is not surprising that the first of the three flashes did not elicit any channel activity, because 1) the Ca^{2+} spike would have been smaller (much smaller, if the Ca^{2+} association rate is close to the photolysis rate) and much briefer than the Ca^{2+} spike for the single flash, and 2) the initial level of occupancy

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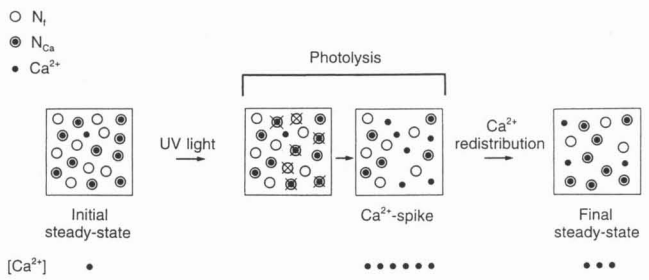

FIGURE 1 Schematic illustration of the basis of the Ca^{2+} spike, showing the large increase in free $[Ca^{2+}]$ caused by the extremely rapid destruction of some of the nitrophen (predominantly Ca-nitrophen (N_{Ca})) by the UV laser flash, and the subsequent, slower rebinding of most of this newly liberated Ca^{2+} by the remaining free nitrophen (N_f).

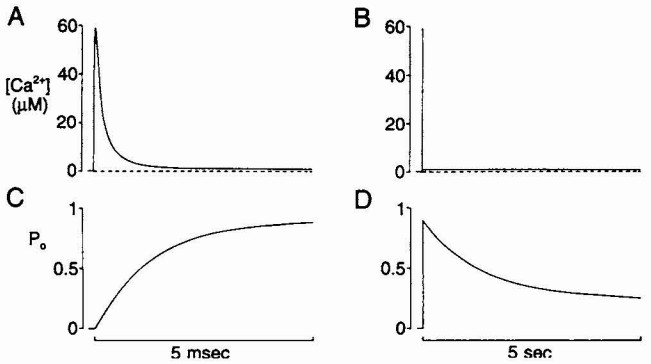

FIGURE 2 Comparison of the time courses of the Ca^{2+} spike and the opening and closing of the RyR channel upon flash photolysis of DM-nitrophen. Calculated minimum size of the Ca^{2+} spike accompanying a $[Ca^{2+}]$ "step" from 0.1 μM to 1 μM, displayed on a fast (*A*) and a slow (*B*) time scale; data modeled using the upper limit of the rate of Ca^{2+} reassociation with nitrophen (1.5×10^8 M^{-1} s^{-1}; see text), 5 nM dissociation constant, 3 mM total nitrophen, and a photolysis time constant of 30 μs. (*C* and *D*) Corresponding time course of the average open P_o of a single cardiac channel, based on data of Györke and Fill (1993) (activation time constant, 1.3 ms; maximum P_o, 0.9; adaptation time constant, 1.3 s; final P_o, 0.25). The rate of channel activation (*C*) is greatly enhanced by the high $[Ca^{2+}]$ during the Ca^{2+} spike, and the subsequent decrease in channel P_o over several seconds, i.e., adaptation (*D*), may result simply from dissociation of Ca^{2+} from activation sites on the channel, as they equilibrate with the final low steady-state $[Ca^{2+}]$ (see text).

of the Ca^{2+} activation site(s) on the channel must have been lower at a $[Ca^{2+}]$ of 0.05 μM than at 0.1 μM. And as the third of the three flashes should have elicited a Ca^{2+} spike nearly identical to that of the single-flash case (free $[N_f]$: 137 μM → 68 μM vs. 140 μM → 70 μM), it is not surprising that they caused nearly identical channel activation (Györke and Fill, 1993, 1994).

ACTIVATION RATE AND "SUPERCHARGING"

The occurrence of large Ca^{2+} spikes greatly affects the interpretation of the rate of channel activation, because the channels activate on a comparable time scale to the spike (Fig. 2). Thus, the apparent channel activation time constant of about 1.3 ms for a $[Ca^{2+}]$ "step" to 1 μM must be a considerable underestimate of the true time constant for Ca^{2+} binding and channel activation, because the binding was being driven by a much higher $[Ca^{2+}]$ than 1 μM for an appreciable part of the time. This is confirmed by the finding that the cardiac RyR channels activated about 2.4 times *more slowly* for a "step" to 10 μM Ca^{2+} (time constant of activation: 2.9 ms, Györke and Fill, 1994) than they did when supposedly driven by a 10 times lower $[Ca^{2+}]$ in the case of a "step" to 1 μM (1.1 ms, Györke and Fill, 1993; 1.4 ms, Györke et al., 1994). Thus, for the "step" to 10 μM Ca^{2+}, where the Ca^{2+} spike is only 1.45 times the final steady $[Ca^{2+}]$, the apparent rate constant of association of Ca^{2+} with the channel is 3×10^7 M^{-1} s^{-1}, rather than the extremely high value of 8×10^8 M^{-1} s^{-1}, which would be calculated for the 1 μM "step." The increase in the apparent association rate constant caused by a Ca^{2+} spike is highly analogous to the speeding of a voltage-clamp step in the supercharging procedure (Armstrong and Chow, 1987), except that in the case of channel activation there is no feedback to ensure that the percentage occupancy of the Ca^{2+} activation site(s) on the channel does not temporarily exceed the level occurring at the final steady-state $[Ca^{2+}]$.

ADAPTATION

Consequently, RyR "adaptation" (Györke and Fill, 1993; Györke et al., 1994) may simply reflect the closing of the channels after the $[Ca^{2+}]$ has dropped to its equilibrium level and Ca^{2+} comes back off the activation site(s) (Fig. 2). Of course, the channels might not close immediately upon Ca^{2+} vacating the activation site, and closure may also involve some cooperative function of the four subunits composing each RyR. This could be tested directly using flash photolysis of Diazo-2, which Györke et al. (1994) have already shown is capable of rapidly lowering the $[Ca^{2+}]$ (their Fig. 2 *C*): does this cause channel closure with the same or a much faster time constant than seen in adaptation? An alternative test of the existence of adaptation would be to try to activate the channels by rapid replacement of a solution weakly buffered (e.g., 50 μM EGTA) at pCa 7.0 with a solution strongly buffered (e.g., 10 mM EGTA) at pCa 6.7; this would provide a rapid increase in $[Ca^{2+}]$, which does not overshoot the final equilibrium level (Moisescu, 1976) and one could find out whether the channels indeed activated and then adapted to such a small $[Ca^{2+}]$ change as would be expected from the data of Györke and Fill (1993). The decrease in channel open probability (P_o) after a $[Ca^{2+}]$ "step" from 1 to 10 μM (Fig. 2 in Györke and Fill, 1994), where the Ca^{2+} spike was supposedly minimized, is not proof of adaptation, because 1) there still was a considerable Ca^{2+} spike, which peaked at 14.5 μM and dropped back comparatively slowly (10 times more slowly than the spike for a $[Ca^{2+}]$ "step" from 0.1 to 1 μM: initial $[N_f]$ 14 μM vs. 140 μM); and 2) the $[Ca^{2+}]$ may not really have been constant for several seconds at 10 μM, perhaps because of diffusion, given that Fig. 2 *B* in Györke et al. (1994) shows an example where the $[Ca^{2+}]$ decreased 50% (approximately pCa 6.0 to pCa 6.3) in about 2.5 s.

ACTIVATION AND CICR

Finally, the occurrence of Ca^{2+} spikes readily explains why flash photolysis can fully activate the skeletal RyRs ($P_o \approx 1$), even though the maximum P_o for any steady-state [Ca^{2+}] is only about 0.21 (Györke et al., 1994). The bell-shaped curve of P_o vs. [Ca^{2+}] is apparently the result of Ca^{2+} binding to an activation site ($K_d \approx 2$–5 μM) and also to an inactivation site ($K_d \approx 200$ μM) (Ma et al., 1988; Fill et al., 1990; Chu et al., 1993). If the rate of association of Ca^{2+} is faster at the activation site than at the inactivation site, as is plausible considering the relative K_ds, a Ca^{2+} spike could cause maximal binding to the activation site with little or no binding to the inactivation site. This disparity could be expected to cause a greater level of channel activation than any steady-state [Ca^{2+}], as was observed. And as would be predicted, in cardiac RyRs, which are inactivated at only very high [Ca^{2+}] (Sitsapesan et al., 1991; Chu et al., 1993), the maximum steady-state P_o is higher (0.63) and not as markedly different from the maximum found upon photolysis of nitrophen (Györke et al., 1994).

Importantly, even if skeletal RyRs in situ did experience Ca^{2+} spike stimuli, they would not show the large increase in P_o observed with the photolysis procedure of Györke et al. (1994), because the inactivation site has almost the same affinity for Mg^{2+} as it does for Ca^{2+} (Meissner et al., 1986) and consequently the channel will be already almost fully inhibited at the physiological [Mg^{2+}] of 1 mM (Lamb, 1993). Thus, Ca^{2+} will activate the skeletal RyR in situ far less rapidly and potently than indicated by Györke et al. (1994), and therefore their data should not be taken as evidence for a major role of CICR in skeletal muscle.

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