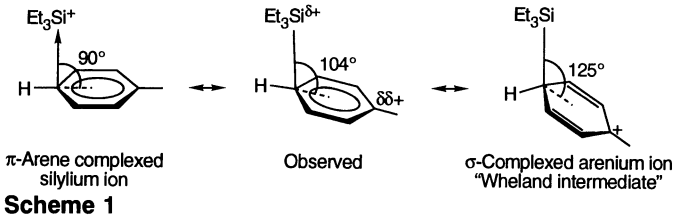
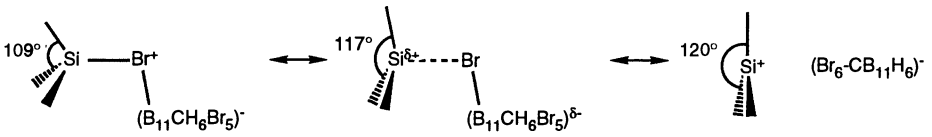


π-Arene complexed silylium ion | Observed | σ-Complexed arenium ion "Wheland intermediate"

Scheme 1



Bromomium ion | Observed "ion-like" | Silylium ion

Scheme 2

observed structure of i-$Pr_3Si(Br_6$-$CB_{11}H_6)$ can be viewed as lying on a continuum between a bromonium ion and a silylium ion (Scheme 2). In describing i-$Pr_3Si(Br_6$-$CB_{11}H_6)$ as a "silylbromonium zwitterion," Olah *et al.* underestimate the contribution of silylium ion character. One can argue about degree, but by the criterion of the C-Si-C angle (117° is observed), the major contributor has silylium rather that bromonium ion character. The silylium ion character is also strongly reflected in the chemical reactivity of i-$Pr_3Si(Br_6$-$CB_{11}H_6)$. It rapidly abstracts chloride from dichlorobenzene and strongly bound fluoride from fluorobenzene.

In summary, while the metaphor "you can't get half pregnant" may apply to carbenium ion chemistry, in silylium ion chemistry the important thing is "being more than halfway through pregnancy." The various criteria currently available for measuring closeness to full-term R_3Si^+ character (^{29}Si NMR, theory, C-Si-C bond angles, reactivity, and so on) give different estimates. But the establishment of substantial, and by most criteria predominant, silylium character in both i-$Pr_3Si(Br_6$-$CB_{11}H_6)$ and $Et_3Si(toluene)^+$ cannot be denied.

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Ca^{2+}-Induced Ca^{2+} Release in Response to Flash Photolysis

S. Györke and M. Fill (*1*) recently used results obtained with DM-nitrophen (caged Ca^{2+}) photolysis to support the hypothesis that Ca release channels from cardiac muscle may "adapt" to a Ca^{2+} stimulus. According to this proposal, a relatively rapid rise in the concentration of Ca^{2+} ($[Ca^{2+}]$) (from about 0.1 μM to 0.2 μM) on the cytoplasmic side of the Ca channels results in the probability that Ca channels of the cell will open (P_O) to increase to a high value and then gradually decline to a lower, steady value (Fig. 1A). Their conclusion was based on the assumption that $[Ca^{2+}]$ rises in a step-like fashion after the photolysis of the DM-nitrophen, and this assumption was apparently supported by measurements with a Ca^{2+}-sensitive electrode. However, the photolysis procedure used by Györke and Fill would have generated an extremely large, transient increase in the $[Ca^{2+}]$ (*2*, *3*) (Fig. 1B), which itself might account for the behavior of the channels without there having to be any Ca channel "adaptation."

The occurrence of a Ca^{2+} "spike" in response to photolysis of DM-nitrophen can be explained as follows. Györke and Fill added 3 mM total nitrophen and 2 mM $CaCl_2$ (*1*), so initially there would have been 2 mM of Ca-nitrophen and 1 mM of free nitrophen in the solution, and thus 0.5 mM of the total nitrophen must have been photolysed in order to raise the steady-state $[Ca^{2+}]$ by a factor of 2. As the quantum efficiency of Ca-nitrophen is 2.5 times that of free nitrophen (*3*), the laser flash would have liberated about 0.42 mM of Ca^{2+} from Ca-nitrophen. Because this liberation would have been faster (half-time <180 μs) (*4*) than the association of Ca^{2+} with the remaining free nitrophen (initial half-time about 1 ms) (*3*), the free $[Ca^{2+}]$ would have increased briefly to about three orders of magnitude greater than the final steady-state concentration (Fig. 1). In order to raise the steady state $[Ca^{2+}]$ by a factor of another 2.5, the second laser flash must have photolysed about 0.27 mM Ca-nitrophen and hence generated another large, though slightly smaller, Ca^{2+} spike. (Even if the free nitrophen were only 0.1 mM, owing to a possible error in the calculated total amount of added nitrophen, the Ca^{2+} spikes would still be two orders of magnitude greater than the change in steady $[Ca^{2+}]$.) Such Ca^{2+} spikes would not have been detected by the Ca^{2+}-sensitive electrode used by Györke and Fill, which only responded with a time constant of 30 to 50 ms (dotted line in Fig. 1B). Nevertheless, the Ca release channels should have responded acutely to such a fast transient $[Ca^{2+}]$ change (the half-activation time was stated as 1.2 ms) (*1*), with the probability of being open rising markedly during the Ca^{2+} spike before settling to a lower value after the $[Ca^{2+}]$ reached steady-state.

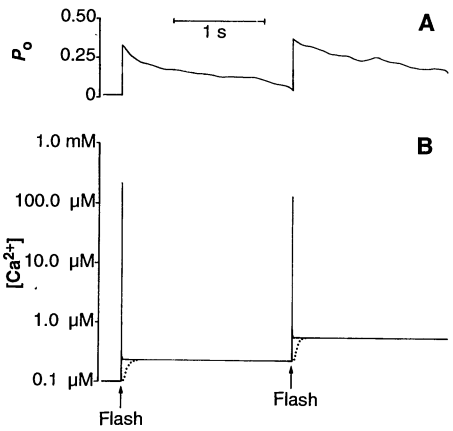

Fig. 1. Channel activity in response to changes in $[Ca^{2+}]$ [modeled on data in figure 3 of (*1*)]. (**A**) Change in the probability (P_o) of a cardiac Ca^{2+} release channel being open. (**B**) Time course of $[Ca^{2+}]$ resulting from flash photolysis of DM-nitrophen, calculated (solid line) and as measured by a Ca^{2+}-sensitive electrode (dotted line).

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18 June 1993; accepted 13 October 1993

Response: The legitimate concern expressed by Lamb *et al.* does not change the interpretation or significance of our result and is only one of many possible concerns that could not be addressed in a short report.

There are two misinterpretations in the comment of Lamb *et al.* First, we did not assume that the Ca^{2+} electrode could accurately track the Ca^{2+} waveform immediately after the flash [note 20 in (*1*)]. Second, the experimental conditions Lamb *et al.* describe are impossible. The apparent Ca^{2+} binding affinity of DM-nitrophen is 5×10^{-9} M (*2*, *3*). Thus, the 3 mM total nitrophen buffering "[Ca^{2+}] at 100 nM" [(*1*), p. 807, paragraph 4] was essentially saturated with Ca^{2+}. We carefully titrated the free [Ca^{2+}] to 10^{-7} M using a macroscopic Ca^{2+} electrode, so the concentration of free nitrophen ([nitrophen]) was not 1 mM, as Lamb *et al.* state. Thus, if the putative fast [Ca^{2+}] spikes had occurred, they would have been significantly smaller than those calculated by Lamb *et al.* Our response addresses two questions.

1) Do fast [Ca^{2+}] spikes occur?

The possibility that fast [Ca^{2+}] spikes occur is not universally accepted. Recently, Vergara and Escobar (*4*) showed that there was no experimental evidence of [Ca^{2+}] spikes with nitrophen buffering Ca^{2+} at 10^{-7} M. They used a fast (μs) fluorescent Ca^{2+} indicator (Ca^{2+}-green 5N; Molecular Probes, Eugene, Oregon) that had an appropriate K_D (μM range). Their conclusion is consistent with the precarious theoretical basis of the fast [Ca^{2+}] spike. The proposal that [Ca^{2+}] spikes occur is based on the relatively small (≈ fivefold) difference between the Ca^{2+} liberation and association rates of nitrophen (*3*). A small error in the estimate of either rate could eliminate the theoretical basis for the fast [Ca^{2+}] spike. Thus, it is far from certain that fast [Ca^{2+}] spikes actually occur.

2) If [Ca^{2+}] spikes occur, then how would they affect data interpretation?

Lamb *et al.* imply that the measured slow decay in channel activity after a fast [Ca^{2+}] spike is driven by the rate at which Ca^{2+} falls off its binding site. However, the putative [Ca^{2+}] spike (≈1 ms) is 1300 times faster than the measured spontaneous decay in single channel activity. Using conservative estimates for the rate constant and K_D of Ca^{2+} binding, one can estimate the rate at which Ca^{2+} comes off the channel to be approximately 10 ms (100 times faster than the measured decay in channel activity). Furthermore, the P_O in the single channel records decreases gradually over seconds (*1*). If the decay were simply a result of decreased Ca^{2+} activation site occupancy, then one would expect an abrupt decrease in P_O after the [Ca^{2+}] spike.

In the initial stages of our project, control experiments were designed to determine the contribution (if any) of the putative fast [Ca^{2+}] spikes (*2*, *3*). Experimental conditions were set to maximize the amplitude of the fast [Ca^{2+}] spikes (Fig. 1). The resting free [Ca^{2+}] was adjusted to 50 nM so that the free [nitrophen] available for re-equilibration with Ca^{2+} was relatively high. Sets of three low-intensity laser flashes were applied to elevate [Ca^{2+}] to approximately 200 nM in a stepwise fashion. According to the interpretation of Lamb *et al.*, each flash produces a fast

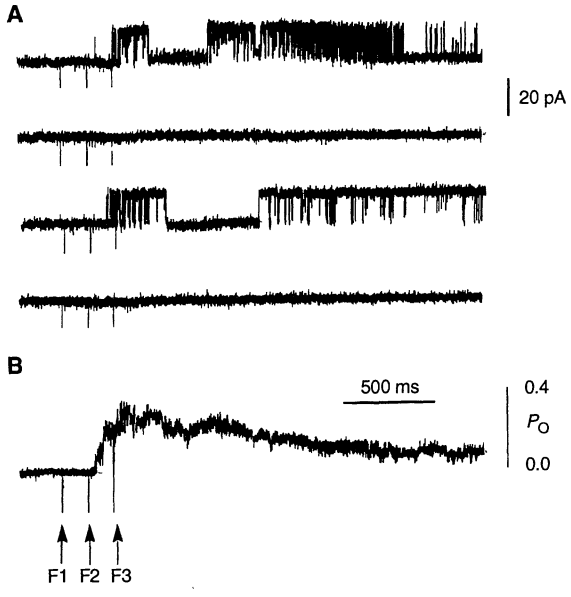

Fig. 1. The effect of maximizing the putative [Ca^{2+}] spike. Resting free [Ca^{2+}] (≈50 nM) was adjusted so that ample free nitrophen was present. Total [nitrophen] was 3 mM. A train of three low-intensity ultraviolet (UV) flashes was repetitively applied to the channel. Resting conditions were reestablished between flash train applications. Theoretically, the first flash (F1) induced a large [Ca^{2+}] spike (≈100 μM) and little change in baseline [Ca^{2+}]. Subsequent flashes (F2 and F3) should induce somewhat smaller spikes but larger changes in baseline [Ca^{2+}]. Final baseline [Ca^{2+}] after the third flash was ≈200 nM. (**A**) Sample single channel data sweeps. Three flashes applied during each sweep. The downward deflections are flash artifacts. (**B**) Ensemble current generated by summing 23 single channel sweeps.

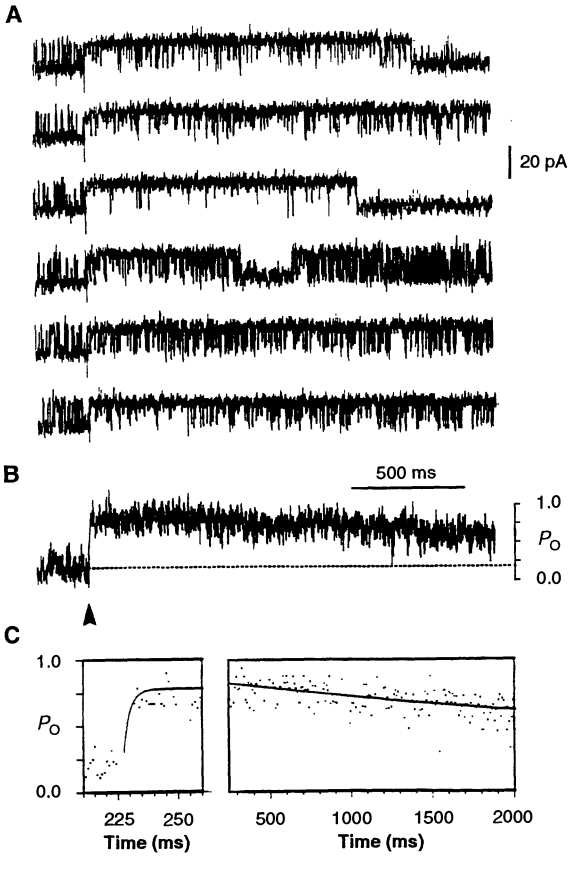

Fig. 2. The effect of minimizing the putative [Ca^{2+}] spikes. Resting free [Ca^{2+}] (1 μM) was adjusted so that little free nitrophen was present. Total [nitrophen] was 3 mM. A single high-intensity UV flash was repetitively applied to the channel. Resting conditions were reestablished between flashes. Theoretically, the flash (arrow) induced a free [Ca^{2+}] step (1 → 10 μM) with essentially no free [Ca^{2+}] spike. (**A**) Sample single channel data sweeps. One flash was applied during each sweep. The downward deflection is the flash artifact. Pre-flash single channel activity (P_o ≈ 0.25 at 1 μM free [Ca^{2+}]) was consistent with the steady-state Ca^{2+} dependence of the channel. (**B**) Ensemble current generated by summing 42 single channel sweeps. (**C**) Expansion and fit of ensemble current. Channel activation (left panel) was fit by a single exponential with a time constant of 2.89 ms. The spontaneous decay (right panel) was fit with a single exponential with a time constant of 2277 ms.

$[Ca^{2+}]$ spike that is larger than those which occurred in our experiments (*1*). Yet, the first and second flashes that theoretically produced large $[Ca^{2+}]$ spikes did not trigger channel activity.

Experimental conditions were then set to minimize the amplitude of the $[Ca^{2+}]$ spike (Fig. 2). The resting free $[Ca^{2+}]$ was adjusted to 1 μM so that the free [nitrophen] was extremely low. The pre-flash P_O was about 0.25, as one would predict from the steady-state Ca^{2+} dependence of the channel (*1*). One high-intensity laser flash was applied to elevate the free $[Ca^{2+}]$ to 10 μM. According to the interpretation of Lamb *et al.*, the fast $[Ca^{2+}]$ spike would be essentially absent under these conditions. Yet, in the absence of $[Ca^{2+}]$ spikes, photolysis induced fast activation followed by a slow decay in channel activity.

Finally, our interpretation of the data led to the hypothesis that single channels adapt to a Ca^{2+} stimulus (*1*). This complex single channel behavior correlates well with phenomena reported in other studies. For example, the self-adjusting mechanism described for mechanosensitive channels (*5*) is phenomenologically similar to adaptation. The "incremental" or "quantal" activation described in populations of IP_3 receptor channels (*6*) is also consistent with the existence of single channel adaptation.

Thus, in the absence of experimental data to the contrary, we have confidence in the interpretation of our results and in the existence of single channel adaptation.

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Fertilization and Ion Channels

We would like to point out flaws in the experimental design of a recent study by Kupitz and Atlas (*1*). The first part of the report describes a Na^+ channel in the immature oocyte that is activated by external concentrations of 0.2 to 2.0 mM of adenosine 5′-triphosphate (ATP) (*2*). Kupitz and Atlas then suggest that this ATP-activated Na^+ channel might be involved in fertilization of the mature egg. There are major differences, however, between an immature oocyte and a mature egg. Many changes in the plasma membrane occur during maturation, greatly decreasing the ion channel density and membrane permeability (*3*). Therefore, before suggesting that the sperm might activate a Na^+ channel in the mature egg, Kupitz and Atlas should have first determined if mature eggs also exhibited ATP-sensitive channels. In addition, they implied in their opening paragraph that an increase in Na^+ permeability normally occurs at fertilization, but this does not occur in the frog egg, where the sperm activates a Ca^{2+}-gated Cl^- efflux that depolarizes the mature egg and provides an electrical block to polyspermy (*4*).

The second flaw in their report is the proposal that the high amounts of ATP inside the sperm could activate the putative ATP-sensitive channel on the exterior surface of the egg's plasma membrane. This would require that the sperm secrete millimolar amounts of ATP during a period in which it is in need of this energy source to swim through the 0.5-mm-thick jelly layer surrounding the egg. While implausible, this idea could have been investigated directly, but was not. Instead, Kupitz and Atlas designed experiments in which sperm, jellied eggs, and various compounds were added together in the same dish and egg cleavage was used as an indirect indicator of fertilization. Many compounds will not easily diffuse through the thick egg jelly layer, so these conditions do not guarantee that the applied concentration of each compound was present at the membrane surface. Moreover, a more likely target for these compounds is the sperm. If any of the applied compounds interfered with sperm activation or motility, a similar absence of normal development could result. Yet Kupitz and Atlas do not describe control experiments that would test for the effects of the various compounds on the ability of the sperm to fertilize unexposed eggs.

These flaws in experimental design make it impossible to judge the reliability of this report.

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2. This current-voltage response is similar to that reported by I. Lotan *et al.* [*Nature* **298**, 572 (1982)] for ATP-induced currents in the immature *Xenopus* oocyte, in which it was concluded that Cl^- was the main current carrier.
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Response: In our report, we suggested that the involvement of ATP-activated channel in fertilization is suggested on the basis of analogy with oocytes.

1) It is clear that membranes of eggs and oocytes are absolutely different from each other; however, as different as they are, we found a strong correlation between inhibition of fertilization and inhibition of ATP-induced current in oocytes by guanosine 5′-triphosphate (GTP), guanosine .5′-[β,γ-imido]triphosphate (GppNHp), and amiloride.

2) ATP-activated current can be upstream to the Ca^{2+}-gated Cl-efflux.

3) High ATP concentration inside the sperm was mentioned in our report as a possible source for the ATP that acts at the membrane. We agree that the actual mechanism should be worked out.

4) Neither GTP, GppNHp, or amiloride had an effect on the mobility of the sperm in our study. More rigorous tests should be applied, as suggested in the comment.

5) Applying the antagonists to the jellied eggs at concentrations mentioned in our report would not reflect their actual concentration at the membrane. Nevertheless, the three compounds, at those concentrations, were effective at inhibiting sperm-induced fertilization.

A direct electrophysiological recording from mature eggs, compared with our oocytes recording, would help to confirm the indirect approach we took in comparing inhibitory action of various ligands at oocytes and mature eggs.

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