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Many Agonists Induce "Quantal" Ca^{2+} Release or Adaptive Behavior in Muscle Ryanodine Receptors

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SUMMARY

Ryanodine receptors have recently been shown to undergo an unusual kind of inactivation process termed adaptation, which bears similarities to the transient calcium releases induced in other systems by successive incremental additions of inositol-1,4,5-trisphosphate. Such releases are sometimes termed "quantal." In this study we report that many agonists induce similar behavior in muscle sarcoplasmic reticulum and that the responses depend not on the calcium pumps therein but rather on the ryanodine receptors. The chemical diversity of these agonists makes it very unlikely that adaptation simply affects the sensitivity of the receptor to agonists at any one binding site. More likely, this result indicates that adaptive behavior of ryanodine receptors results whenever the ryanodine receptor is activated and that this process affects the action of most, if not all, agonists. Evidence is presented suggesting that the releases observed do not represent all-or-none releases from vesicle subpopulations (true quantal behavior) but rather seem to involve partial release from more homogeneously sensitive stores, a process referred to here as adaptation or increment detection.

Many receptors are activated only transiently and undergo a process of desensitization toward agonists that is analogous to the voltage-dependent inactivation processes that turn off many membrane channels. These desensitization and inactivation processes result in inactive receptors or channels until the stimulus that provoked the initial activity is removed. $InsP_3$ and ryanodine receptors have both been reported to undergo a unique, unconventional, inactivation process that results in the continued ability of the receptors to respond to increases in agonist concentration without the necessity of removing the initial stimulus (1–4). Those studies reported that the physiological ligands for the receptors ($InsP_3$ and Ca^{2+}, respectively) were able to induce such continued responsiveness, or adaptive behavior. Although this behavior has sometimes been termed "quantal" (1, 4), we prefer the terms adaptation (2, 3) or increment detection (2) because true all-or-none release from a subpopulation of vesicles has not been definitively established in most systems in which this behavior has been studied. We present here clear evidence that increment detection is observed in response to many ryanodine receptor agonists that must bind at different sites on the ryanodine receptor. A preliminary version of these results has been communicated in abstract form (5).

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Experimental Procedures

Purified sarcoplasmic reticulum subfractions and microsomal membrane fractions from rabbit skeletal muscle were prepared according to the method of Saito *et al.* (6). A "heavy" microsomal membrane fraction with a higher proportion of terminal cisternae was prepared by sedimenting the supernatant from the second homogenate at 13,500 rpm for 60 min in a Beckman JA-14 rotor. A similar procedure was used for microsomal vesicles from lobster skeletal muscle (7). Canine cardiac microsomes were prepared as described previously (8).

Calcium uptake and release measurements were carried out spectrophotometrically (A_{710}–A_{790}) using antipyrylazo III to monitor Ca^{2+} outside the membrane vesicles (9). The medium in the cuvette consisted of 100 mM KCl, 20 mM K-MOPS, 0.25 mM antipyrylazo III, 1 mM Mg-ATP, 5 mM disodium phosphocreatine, and 20 μg/ml creatine phosphokinase, pH 6.95. In the case of cardiac membranes, the medium was additionally supplemented with 5 mM potassium phosphate at the same pH. Measurements with rabbit and canine membranes were carried out at 37° and those with lobster membranes at 17°. Membranes (0.5–1.0 mg of protein) were added to the cuvette and permitted to take up endogenous calcium in the sample. In some cases additional calcium was administered to the sample in small aliquots (2.5 nmol/addition). At the conclusion of this loading procedure, agonists were added at submaximal doses to elicit calcium release. Because ethanol produced multiple Ca^{2+} releases at low concentrations, stock solutions of lipophilic substances were made up in methanol, which required >1% to produce Ca^{2+} release. No more than 1% methanol was applied in these additions.

Results and Discussion

Increment detection by isolated, purified, rabbit skeletal muscle terminal cisternae in response to successive cumulative

ABBREVIATIONS: $InsP_3$, inositol-1,4,5-trisphosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

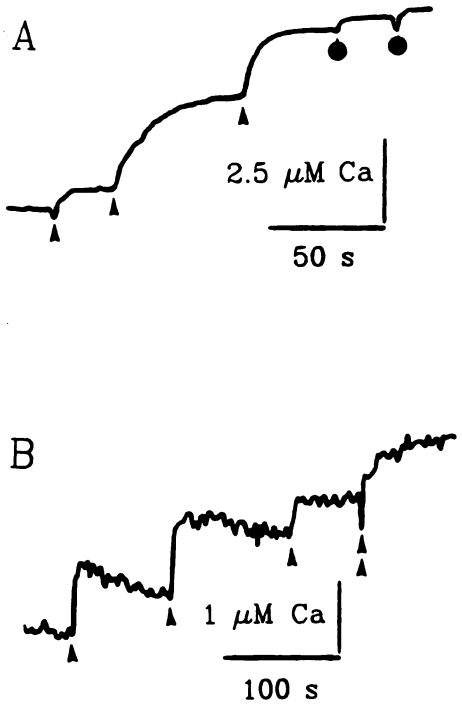

Fig. 1. Multiple Ca^{2+} releases from isolated skeletal and cardiac membranes in response to incremental caffeine additions. Multiple releases were elicited when reuptake was avoided. A, Purified rabbit skeletal muscle terminal cisternae (120 μg) were loaded with endogenous calcium in the sample plus 2.5 nmol (not shown), as described in Experimental Procedures, using antipyrylazo III to monitor Ca^{2+} movements, and were then challenged with three successive 0.2 mM additions of caffeine (*arrowheads*) followed by two successive 1 mM additions (●). B, Canine cardiac microsomes (600 μg of protein) were loaded with eight 2.5-nmol additions of $CaCl_2$ (not shown) before three sequential additions of 0.4 mM caffeine (*arrowheads*) and one addition of 1 mM caffeine (*double arrowhead*). In this figure subsequent caffeine additions were made before the majority of the calcium released could be resequestered by the sample. The calcium concentrations given here and in subsequent figures refer to total calcium added to the cuvette.

additions of caffeine is shown in Fig. 1A. The sample responded to several additions of caffeine before being depleted of Ca^{2+}. Each release of Ca^{2+} was only partial, despite the maintained presence of caffeine. Notwithstanding the apparent desensitization or inactivation, elevation of the caffeine concentration in the cuvette induced further release. Similar responses were obtained with cruder skeletal muscle microsomes (data not shown), as well as with cardiac microsomal membranes (Fig. 1B).

Most studies on incremental calcium release from isolated $InsP_3$-responsive membranes measured release under conditions where the calcium initially released was not permitted to be resequestered before the $InsP_3$ concentration was increased (e.g., see Ref. 2). In the case of both $InsP_3$ and ryanodine receptors, such conditions would tend to increase the likelihood of any Ca^{2+}-dependent inactivation process (10–12) that might contribute to the cessation of each response. The original observations on "quantal" calcium release from $InsP_3$ receptors were carried out instead on cells that resequestered the released calcium (1).

To rule out a possible contribution of the elevated extravesicular (cytoplasmic) Ca^{2+} levels to subsequent responses, we carried out experiments using a larger sample, allowing sufficient time for released Ca^{2+} to be resequestered by the vesicles before the caffeine concentration was raised. Heavy microsomes from rabbit skeletal muscle were exposed to submaximal caffeine concentrations in Fig. 2A. Under these experimental conditions, the caffeine release was only transient, and the calcium released was once again rapidly taken up. Addition of more caffeine to the cuvette was able to reactivate release several times under these conditions. Thus, the combined results of Figs. 1 and 2 suggest that elevated calcium concentrations on the cytoplasmic side of the membrane are not required to be either maintained or removed to restore the ability to respond to agonist.

The partial or transient nature of the releases seen in Figs. 1 and 2 requires some other sort of inactivation or desensitization process as an explanation. A conventional inactivation or desensitization process would dictate that continued exposure to the agonist would prevent any subsequent response to a further increase in agonist concentration. However, in the case of the ryanodine receptors under investigation here, it is clear that elevation of the caffeine concentration in the cuvette from 1.6 to 2.6 mM induced a second release of calcium of almost equal magnitude as the first large release (Fig. 2A). After resequestration of the released calcium, additional releases could be elicited in response to further increases of the caffeine concentration in the cuvette. The amplitude of successive releases diminished, but 10 or more releases could be elicited from certain samples (e.g., Fig. 2A). It is likely that the decreased amplitude of these later releases was due to an increasing proportion of the released calcium being taken up by vesicles lacking ryanodine receptors, because samples less enriched in terminal cisternae tended to respond with multiple releases that became considerably smaller with successive caffeine challenges (data not shown).

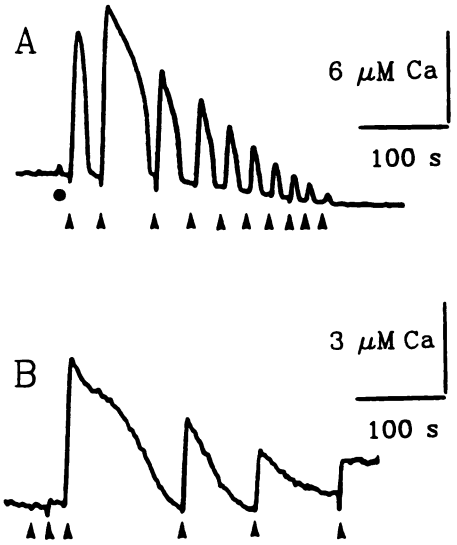

Fig. 2. Multiple Ca^{2+} releases in response to caffeine and other agonists, with all releases starting from a similar resting Ca^{2+} concentration. A, Rabbit skeletal muscle heavy microsomes (600 μg) were loaded with ten 1.25-nmol additions of $CaCl_2$. After completion of the loading procedure (not shown), an 0.6 mM caffeine addition (●) produced only a tiny release, but subsequent 1 mM caffeine additions made consecutively (*arrowheads*) resulted in multiple transient releases of calcium from the sample. B, In this equivalent experiment, 600 μg of rabbit skeletal muscle heavy microsomes were loaded with five 2.5-nmol additions of $CaCl_2$ before sequential 5 μM additions of W-7 (*arrowheads*) to induce multiple releases.

TABLE 1
Agonists that produce incremental calcium releases from sarcoplasmic reticulum

Source of membranes	Substance	Concentration[a]	
Rabbit skeletal muscle	Caffeine	0.2–11 mM	R, X[b]
	Doxorubicin	10–70 μM	R
	Ethanol	0.1–1.0%	R
	pCMB[c]	1.5–10 μM	R
	Sphingosyl phosphorylcholine	10–20 μM	X
	Sulmazole	350–550 μM	R
	Thymol	20–200 μM	R
	W-7	1–15 μM	R
Lobster skeletal muscle	Butanedione monoxime	3–10 mM	R
	Caffeine	5–10 mM	R
	Doxorubicin	10–80 μM	R
	Ethanol	0.1–5.0%	R
	Sphingosyl phosphorylcholine	10–90 μM	R
Canine cardiac muscle	Caffeine	0.4–3.0 mM	X
	Doxorubicin	2–16 μM	X
	Sphingosyl phosphorylcholine	10–40 μM	X
	Sulmazole	50–400 μM	X
	W-7	1–30 μM	X

[a] Concentrations represent the minimum concentrations tested that elicited release and the final cumulative concentrations tested that still elicited further release.
[b] R, released calcium reaccumulated before agonist concentration increased; X, released calcium mostly not reaccumulated before agonist concentration increased.
[c] pCMB, *p*-chloromercuribenzoate.

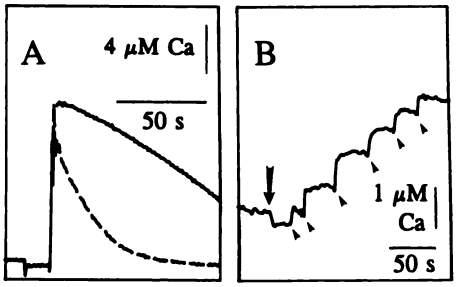

Fig. 3. Multiple Ca^{2+} releases in the presence of the pump blocker thapsigargin. A, Rabbit skeletal muscle heavy microsomes (200 μg) were administered 12.5 nmol of $CaCl_2$ under control conditions (*dashed trace*) and in the presence of 2 μM thapsigargin (*solid trace*), both in the presence of 1.25 mM phosphate. Inhibition of uptake was calculated to be >70%. B, The same microsomes were loaded with seven 3.75 nmol additions of $CaCl_2$ (not shown), exposed to 2 μM thapsigargin (*downward arrow*), and then challenged with six successive additions of 0.2 mM caffeine (*arrowheads*), resulting in multiple releases.

We have observed multiple release responses in the presence of incremental additions of many different agonist substances. As seen in Fig. 2B, the sample also responded to application of W-7 with multiple transient releases. Table 1 lists the agonists we have tested, their concentration ranges, and the kinds of membrane preparations that responded to them. In addition, we document in Table 1 that ryanodine receptors from several different muscle sources all display the capability of responding repetitively to successive submaximal incremental agonist additions. Thus, the incremental release phenomenon appears to be a common property of muscle ryanodine receptors. The muscles from which these membranes were prepared display different forms of excitation-contraction coupling (13, 14), indicating that incremental release is most likely a property of the ryanodine receptor itself, rather than being reflective of the manner in which the ryanodine receptor interacts with the dihydropyridine receptor in excitation-contraction coupling.

In some cases in Table 1 the responses were transient, and calcium was permitted to be resequestered (R); in other cases (X), second and subsequent agonist additions were made before the majority of the calcium released could be reaccumulated by the sample. This raises the possibility that calcium might have inactivated each release in Fig. 2 but that the ensuing calcium reuptake permitted some recovery from inactivation and thus restoration of the ability to respond to agonist.

The results presented thus far were all obtained under conditions where the sarcoplasmic reticulum calcium pump was capable of being activated by the released calcium. The termination of net release thus could be due to either diminished efflux through the ryanodine receptor or greatly enhanced influx mediated via the pump. Although pump activation might have explained termination of the first releases in Fig. 1, the pump should have remained maximally activated with elevated extravesicular calcium concentrations and thus would not have been able to terminate subsequent releases by being further activated.

Additionally, pump inhibitors did not alter the basic character of such releases. As shown in Fig. 3, it is clear that release slows precipitously even in the presence of concentrations of thapsigargin that decrease the uptake rate considerably. If pump activation were the cause of release termination, such strong inhibition of the pump should have reduced its ability to terminate release and resulted in continued efflux, albeit at a reduced rate. Further additions of caffeine continue to support multiple releases, even with calcium pumping being partially compromised by thapsigargin. We conclude that the mechanism of "quantal" calcium release from muscle microsomes is inherent to the ryanodine receptor and does not involve the sarcoplasmic reticulum calcium pump. The results detailed here document the ability of ryanodine receptors from a variety of different sources to respond to successive incremental increases in agonist concentration by releasing only a portion of their calcium.

Very recently it was reported that caffeine-sensitive stores undergo incremental Ca^{2+} release that was termed "quantal" (4), even though all-or-none release from individual stores was not actually demonstrated. Before this, there had been some indications in the literature of sequential responses to agonists such as caffeine (15–17) or bromo-eudistomin D (17) in the continued presence of the agonist. No inferences regarding

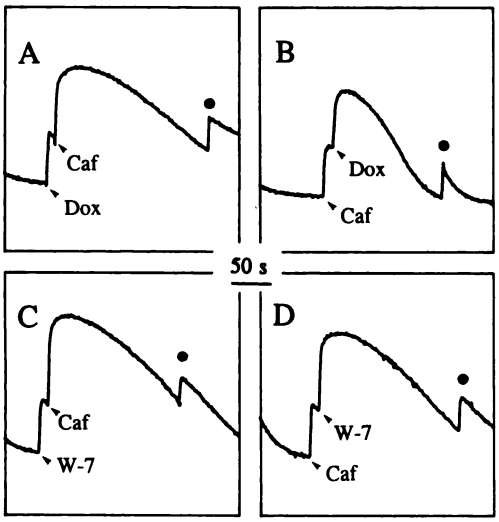

Fig. 4. Evidence that a submaximal dose of one agonist does not deplete a subpopulation of vesicles that are maximally responsive to another agonist. Rabbit skeletal muscle heavy microsomes (200 μg) were loaded with five 5-nmol additions of $CaCl_2$ in the presence of 1.25 mM phosphate (not shown). A, The microsomes were subsequently challenged with 4 μM doxorubicin (*Dox*). After the doxorubicin-induced release had stopped, 3 mM caffeine (*Caf*) was added. B, The microsomes were instead challenged with 3 mM caffeine, followed by 4 μM doxorubicin. C, The microsomes were instead challenged with 15 μM W-7. After the W-7-induced release had stopped, 2 mM caffeine was added. D, The microsomes were instead challenged with 2 mM caffeine, followed by 150 μM W-7. ●, Calibrating additions of 2.5 nmol of $CaCl_2$ (equivalent to 2.5 μM total calcium).

adaptive behavior were drawn in those studies, however. It has now been reported that caffeine and 9-methyl-7-bromoeudistomin D bind at the same binding site (18). Drawing upon analogies of shifts in agonist sensitivity proposed to underlie adaptive behavior of the $InsP_3$ receptor (19–21), this might mean that only a shift in sensitivity at the caffeine binding site accompanies adaptation. However, the results detailed here demonstrate that many different agonists are capable of eliciting such responses. Given the likelihood that so many chemically diverse structures interact with the ryanodine receptor at different binding sites (22), these results indicate that a shift in agonist sensitivity at any one binding site is unlikely to account for the observed adaptive behavior. Rather, these results tend to suggest that adaptation is triggered by activation of the channel at any site and that adaptive behavior probably influences subsequent activation of the channel regardless of which site is used for the activation. This is likely to represent an effect exerted at some step after agonist binding, perhaps mediated by an associated "memory" molecule (23). Adaptive behavior at more than one binding site on the $InsP_3$ receptor could account for sequential transient Ca^{2+} releases from brain microsomes seen in response to superfusion with $InsP_3$ followed by superfusion with the same $InsP_3$ concentration at higher coagonist Ca^{2+} concentrations (20).

One school of thought regarding "quantal" calcium release from $InsP_3$-sensitive stores suggests that alternative splicing or post-translational modification of $InsP_3$ receptors might result in subpopulations of $InsP_3$-sensitive stores with differing sensitivity to $InsP_3$. If we were to interpret our multiple-release results from ryanodine receptors in terms of the "quantal" release model described above, we would need to take into account the fact that many different agonists display equivalent behavior. If "quantal" release behavior is not due to any one ligand binding to its site but instead is due to some common step in an activation sequence, then it is likely that those receptors that are most sensitive to one ligand would be most sensitive to the other ligands, whether the ligands interacted at the same site or not. If the receptors that are most sensitive to caffeine are also those that are most sensitive to all other ligands, then it should be possible to devise tests to selectively deplete the most sensitive stores.

Accordingly, we selected concentrations of caffeine (2–3 mM) and doxorubicin (4 μM) or W-7 (15 μM) that generated similarly sized, submaximal, calcium releases from muscle microsomes. Then, after application of one agonist, we applied the second agonist. As seen from the results of Fig. 4, regardless of the order of addition, the second agonist always produced a similarly sized (or even larger) release than when it was added first. According to the expectations of true quantal release behavior, if the stores most sensitive to caffeine were also most sensitive to doxorubicin or W-7, then total release of calcium from those most sensitive stores should have emptied them and rendered them incapable of responding to a low concentration of the second agonist. As seen, caffeine clearly did not deplete the most doxorubicin-sensitive or W-7-sensitive stores, nor did doxorubicin or W-7 deplete the most caffeine-sensitive stores.

The results of Fig. 4 are incompatible with an explanation involving true quantal calcium release from a subpopulation of store vesicles that are most sensitive to all agonists. The results of Fig. 4 could be compatible with a scheme in which vesicles with the highest fixed sensitivity to caffeine were not those most sensitive to doxorubicin. Alternatively, caffeine, doxorubicin, W-7, and other agonists may induce a more dynamic alteration of ryanodine receptor sensitivity, akin to the adaptation recently described at the single-channel level (3) or the increment detection described for $InsP_3$ receptors (2).

Therefore, an additional test was devised to discriminate between true quantal release and adaptation/increment detection exerted at the level of each ryanodine receptor. Muscle microsomes were loaded with calcium and exposed to a dose of caffeine that produced a submaximal release of calcium. Before the calcium could be resequestered, the caffeine concentration was reduced by one half by diluting the sample with an equal volume of assay medium. Finally, the caffeine concentration was restored to its original 2 mM level. If caffeine had selectively released all of the calcium from only a subpopulation of most sensitive vesicles, then in the absence of reuptake of the released calcium the most sensitive vesicles should have been empty and a second identical caffeine challenge should have produced no release (Fig. 5A). At variance with this expectation, we observed a clear release of calcium in response to the second caffeine challenge (Fig. 5C). This second release was far greater than the amount of initially released calcium that could possibly have been reaccumulated by the vesicles prior to the second challenge. This result argues that caffeine does not release all of the calcium from a selective subpopulation of highly sensitive vesicles (Fig. 5A) but rather releases some of the calcium from most of the vesicles, as diagrammed in Fig. 5B. A very similar experimental result and interpretation have just been reported for cerebellar $InsP_3$ receptors (24).

Thus, the true mechanism of "quantal" release might involve a more dynamic adaptation/increment detection process taking

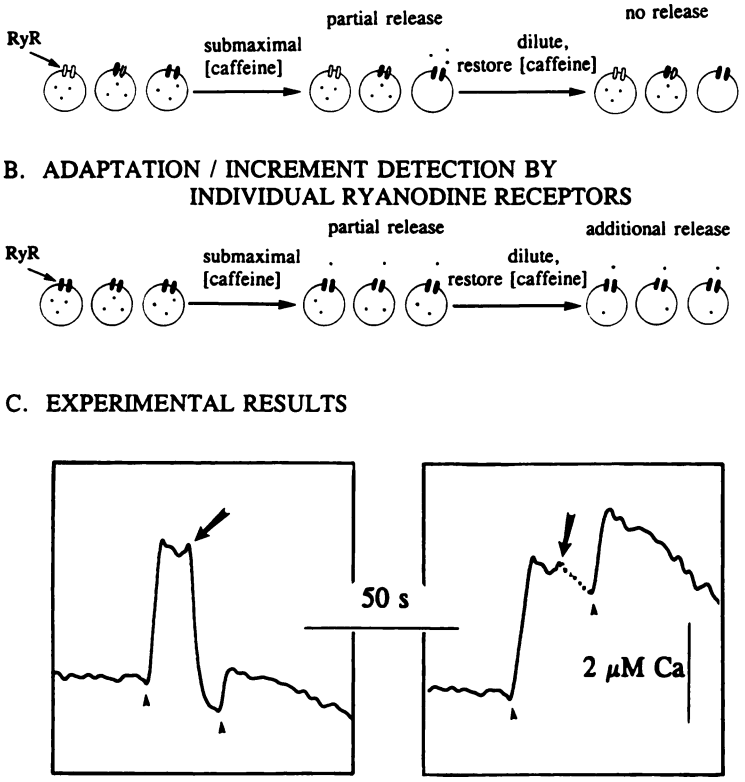

Fig. 5. Evidence that a submaximal concentration of caffeine releases some of the calcium from most of the vesicles rather than all of the calcium from a maximally responsive subpopulation of vesicles. A, Predictions of a true quantal release of calcium from a heterogeneously sensitive population of vesicles. The doubly *shaded* ryanodine receptors (*RyR*) are assumed to be the most responsive. B, Predictions of the release behavior of a homogeneous population of ryanodine receptors individually responding by adaptation or increment detection. C, *Left*, rabbit skeletal muscle heavy microsomes (300 μg) were loaded with seven 5-nmol additions of $CaCl_2$ (not shown). They were subsequently challenged with an addition of 2 mM caffeine (arrowhead). After the caffeine-induced calcium release had attained its maximal amplitude, 1 ml of assay medium was added to the cuvette (downward arrow), diluting its contents by one half and resulting in a large downward shift in the trace as indicated. The caffeine concentration was then restored to 2 mM (arrowhead), resulting in another release of calcium. This trace is not calibrated for calcium because the calibration would have changed after the dilution, *right*. Note that, because of the dilution, the second release of calcium at *left* could have been expected to be at most one half the size of the first one, right. The results at *left* were replotted to remove the dilution artifact in the base line, with allowance being made for estimated reuptake (*dotted portion of trace*) before the second caffeine addition, and were scaled to compensate for the effect of the dilution on the size of the second calcium release. The results correspond to the predictions of adaptive behavior (B) rather than true quantal release (A).

place at the level of each ryanodine receptor. This process would dynamically slow release after each activation of the receptor but still allow reactivation of the same receptor by an increased concentration of agonist. This appears to be the case *in situ*, at least with respect to the ryanodine receptors of crayfish skeletal and rat cardiac muscle (12, 25).

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