Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 7256–7259, November 1985
Biochemistry

Localization of Ca^{2+} release channels with ryanodine in junctional terminal cisternae of sarcoplasmic reticulum of fast skeletal muscle

(muscle contraction/excitation–contraction coupling/ruthenium red/longitudinal cisternae/gated channels)

SIDNEY FLEISCHER, EUNICE M. OGUNBUNMI, MARK C. DIXON, AND EDUARD A. M. FLEER

Department of Molecular Biology, Vanderbilt University, Nashville, TN 37235

Communicated by William J. Darby, July 8, 1985

ABSTRACT **The mechanism of Ca^{2+} release from sarcoplasmic reticulum, which triggers contraction in skeletal muscle, remains the key unresolved problem in excitation–contraction coupling. Recently, we have described the isolation of purified fractions referable to terminal and longitudinal cisternae of sarcoplasmic reticulum. Junctional terminal cisternae are distinct in that they have a low net energized Ca^{2+} loading, which can be enhanced 5-fold or more by addition of ruthenium red. The loading rate, normalized for calcium pump protein content, then approaches that of longitudinal cisternae of sarcoplasmic reticulum. We now find that the ruthenium red-enhanced Ca^{2+} loading rate can be blocked by the previous addition of ryanodine. The inhibition constant is in the nanomolar range (20–180 nM). Ryanodine and ruthenium red have no effect on the Ca^{2+} loading rate of longitudinal cisternae. Direct binding studies with [^{3}H]ryanodine localized the receptors to the terminal cisternae and not to longitudinal cisternae. Scatchard analysis of the binding data gives a dissociation constant for ryanodine in the range of the drug action on the terminal cisternae (≈100 nM range) with approximately 4 to 20 pmol bound per mg of protein. Ryanodine is known to be toxic in animals, leading to irreversible muscle contractures. These studies provide evidence on the mode of action of ryanodine and its localization to the terminal cisternae. The low concentration at which the drug is effective appears to account for its toxicity. Ryanodine locks the Ca^{2+} release channels in the "open state," so that Ca^{2+} is not reaccumulated and the muscle fiber cannot relax.**

Muscle contraction and relaxation are controlled by the intrafiber free calcium concentration, which, in turn, is regulated by an intricate membrane network referred to as the sarcotubular system (1–4). Energized Ca^{2+} uptake can readily be studied in isolated sarcoplasmic reticulum (SR) (1, 2, 4–7). The Ca^{2+} release process is more difficult to study, *in vitro*, and is consequently less well understood.

The mechanism whereby the action potential at the plasmalemma of the muscle fiber triggers Ca^{2+} release and thereby muscle contraction remains the key unsolved problem in excitation–contraction coupling. There is now much interest in understanding the Ca^{2+} release process (for example, see refs. 3 and 8–12). Our approach to the study of the Ca^{2+} release process (13) has been to isolate and characterize purified membrane fractions referable to defined segments of the sarcotubular system from rabbit skeletal muscle, including plasmalemma (14), triads—i.e., the junctional association of transverse tubule with terminal cisternae (15)—and junctional terminal and longitudinal cisternae of SR (16). The longitudinal cisternae consist mainly of the Ca^{2+} pump membrane, containing the Ca^{2+} pump protein as the major constituent, ≈90% of the protein, whereas the junctional terminal cisternae consist of two types of membranes, the Ca^{2+} pump membrane (80–85%) and the junctional face membrane (15–20%) (16). A unique characteristic of junctional terminal cisternae is that they have a poor Ca^{2+} loading rate, which can be enhanced 5-fold or more by addition of ruthenium red (RR) (ref. 17; unpublished data). This study describes the drug action of ryanodine on the junctional terminal cisternae in blocking the action of RR. It provides evidence that the action of ryanodine is on the Ca^{2+} release mechanism and supports the view that Ca^{2+} release is localized in the terminal cisternae of SR.

MATERIALS AND METHODS

Membrane fractions referable to longitudinal (R_2) and junctional terminal cisternae (R_4) of SR were prepared from rabbit skeletal muscle as described by Saito *et al.* (16). Triads were isolated according to Mitchell *et al.* (15).

Ryanodine (Fig. 1) was obtained from Penick Corp. (Lindhurst, N.J) and ^{3}H label was introduced by bromination of the pyrrole ring with subsequent replacement of the halogen with ^{3}H as described (20). The labeled ryanodine was purified by HPLC chromatography (Waters model 267) using a Whatman ODS-3 column and isocratic elution (75% H_2O/25% acetonitrile). The specific radioactivity obtained was 70 Ci/mmol (1 Ci = 37 GBq). RR (Fig. 1) and antipyrylazo III were obtained from Sigma. The RR concentration was prepared based on the 35% content in the sample. All other chemicals were reagent grade or the best available.

Calcium loading was measured with a Hewlett–Packard UV/visible spectrophotometer model 8450A by using antipyrylazo III as the metallochromic indicator and measuring the difference in absorbancy at 710–790 nm (21). The Ca^{2+} loading assay medium in 1 ml contained 100 mM potassium phosphate buffer (pH 7.0), 1 mM $MgCl_2$, 0.2 mM antipyrylazo III, and ≈50 μg of protein per ml, similar to that described (22). Na_2ATP (1 mM) was then added and the reaction at 25°C was initiated by addition of 50 μM $CaCl_2$. When ryanodine was added, it was preincubated in the assay medium for 2 min prior to addition of ATP and Ca^{2+}, unless otherwise indicated, and RR was added ≈100 sec thereafter (see Fig. 2). At the end of the assay an additional aliquot of 50 μM $CaCl_2$ was added for recalibration (22).

Ca^{2+}-stimulated ATPase was measured spectrophotometrically by using a coupled enzyme assay, containing pyruvate kinase and lactate dehydrogenase, monitoring NADH oxidation as described (unpublished data).

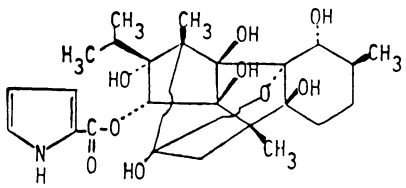
Ryanodine Binding Assay. [^{3}H]Ryanodine was diluted with carrier to a specific radioactivity of about 14,500 cpm/pmol. The binding assay in 1 ml contained binding buffer (0.15 M KCl/10 mM sodium Hepes, pH 7.4), 0.2 mg of protein of the subcellular fraction, and varying concentrations of ryanodine. The concentration of ryanodine was usually varied from

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: RR, ruthenium red; SR, sarcoplasmic reticulum.

$[(NH_3)_5\text{-Ru-O-Ru}(NH_3)_4\text{-O-Ru}(NH_3)_5]Cl_6$

RR



Ryanodine

FIG. 1. Structural formulae of RR and ryanodine (19).

28 to 224 nM. For each concentration, in duplicate, nonspecific binding controls were run containing the same contents but also with a 100-fold excess of unlabeled ryanodine. The samples were incubated at room temperature for 30 min and then filtered through GSWP 0.22-μm Millipore filters, presoaked for several minutes in binding buffer. The filtered samples were washed sequentially with 4 ml of binding buffer and then washed twice with 4 ml each of 10% ethanol in water. The filters were placed in 12 ml of ACS scintillation fluid (Amersham), and radioactivity was measured to 1 σ% in a Searle Analytic 81 scintillation counter using quench correction. The binding was calculated by Scatchard analysis using least-squares linear regression analysis. Nonspecific binding accounted for less than 10% of the counts.

Protein was estimated according to Lowry *et al.* (23) with bovine serum albumin as standard.

RESULTS

Junctional terminal cisternae of SR have a poor Ca^{2+} loading rate, which can be enhanced about 5-fold or more by the addition of RR; the stimulation is rapid, occurring within seconds (Fig. 2) (ref. 17; unpublished data). The addition of ryanodine prior to the addition of RR was found to block stimulation of Ca^{2+} loading while not appreciably affecting the basal rate (the rate without RR). The extent of such inhibition was found to be dependent on the concentration of ryanodine. A double reciprocal plot of the RR-stimulated rate vs. ryanodine concentration gives a straight line from which

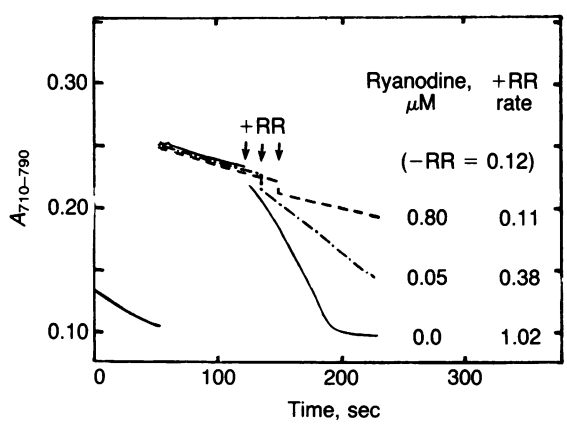


FIG. 2. Ryanodine inhibits RR stimulation of Ca^{2+} loading in junctional terminal cisternae. The Ca^{2+} loading assay medium was admixed with terminal cisternae and ryanodine (when added) and incubated for 2 min. At zero time, Na_2ATP was added. The decline in absorbancy indicates uptake of calcium from the medium. Calcium ions (50 μM) were added at 60 sec, resulting in an increase in absorbancy and a slow "basal" Ca^{2+} loading rate (0.12 μmol/min·mg of protein) was observed. RR (7 μM) stimulated this rate 8.5-fold. Preincubation with ryanodine decreased the enhancement of Ca^{2+} loading with RR.

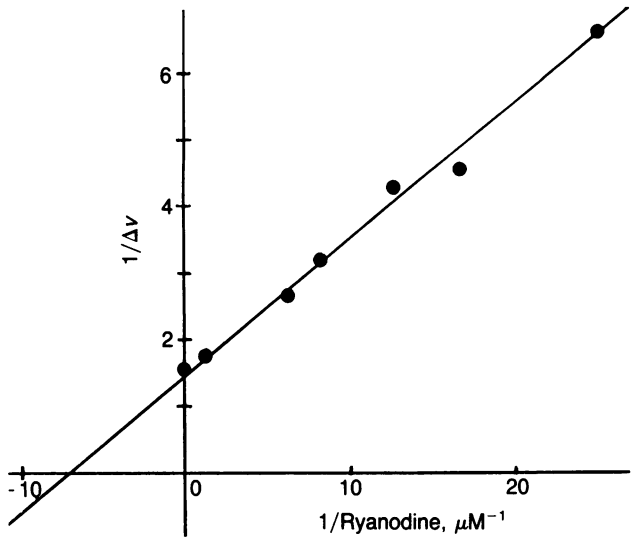


FIG. 3. Double reciprocal plot of ryanodine inhibition of RR-stimulated loading of junctional terminal cisternae. The basal rate was subtracted from the RR-stimulated rate to give the maximal RR-stimulated rate (see Fig. 2, 1.02 − 0.12 = 0.90 μmol/min·mg of protein). This gives the point on the y axis. The RR-stimulated rate in the presence of ryanodine was subtracted from the maximal RR-stimulated rate to give Δv. The apparent inhibition constant (K_i) for ryanodine was obtained from the double reciprocal plot of Δv vs. ryanodine concentration. A straight line was obtained with an apparent K_i for this sample of 138 nM ($-1/x$ intercept). For the junctional terminal cisternae fraction in Fig. 2, the K_i was 21 nM.

an apparent inhibition constant (K_i) can be obtained (Fig. 3). Apparent K_i values for ryanodine for a number of different preparations are summarized in Table 1. They ranged between 20 and 180 nM for most preparations. The K_i for ryanodine was influenced only slightly by the RR concentration (see legend to Table 1).

Table 1. Ryanodine inhibition of RR-stimulated Ca^{2+} loading in SR fractions

SR fraction			Ca^{2+} loading rate			
No.	Assay date	RR,* μM	Without RR	With RR	Ratio†	K_i for ryanodine, nM
			Terminal cisternae			
310	06/14/84	7.0	0.104	0.655	6.3	42; 54
488	10/24/84	7.0	0.132	1.018	7.7	21
517	11/29/84	1.4	0.140	0.971	6.9	120
454	01/03/85	7.0	0.122	0.657	5.4	167
606	03/11/85	7.0	0.233	1.01	4.3	98
			Triads			
426	07/17/84	1.4	0.745	1.43	1.92	56
			Longitudinal cisternae			
488	10/29/84	7.0	2.32	2.49	1.07	−‡
310	06/20/84	7.0	1.54	1.54	1.0	−‡
415	06/11/84	2.0	1.85	1.99	1.07	−‡

Determination of the Ca^{2+} loading rates of each SR fraction and measurement of the K_i values of ryanodine were carried out as described in the legends to Figs. 2 and 3. The linear regression coefficients varied from 0.91 to 0.997.

*The value of K_i is somewhat dependent on RR concentration. In a study with one terminal cisternae preparation, ryanodine titrations were carried out at four RR concentrations, 0.7, 1.75, 7.0, and 17.5 μM; the K_i values increased from 120 to 132, 147, and 211 nM, respectively.

†Ratio of Ca^{2+} loading rates (+RR/−RR).

‡RR does not significantly stimulate Ca^{2+} loading in longitudinal cisternae and ryanodine even at 10 μM does not affect Ca^{2+} loading in this fraction.

The action of ryanodine in blocking RR stimulation of Ca^{2+} loading is time-dependent. Preincubation of junctional terminal cisternae with ryanodine is required prior to the addition of RR. When ryanodine was added after the RR even at much higher concentrations (20 μM), it was without effect. As the interval between ryanodine and RR addition was shortened (in this study ryanodine was added after the basal rate was obtained), ryanodine became less effective. A plot of the logarithm of the RR-stimulated rate as a function of the time interval gives a straight line, thereby conforming to first-order kinetics, with a half-time of 36 sec (±2 sec for two studies).

Ryanodine even in relatively high concentrations (10 μM), in the presence or absence of the Ca^{2+} ionophore A23187, does not affect Ca^{2+}-stimulated ATPase activity of either terminal or longitudinal cisternae (not shown).

The Ca^{2+} loading rate of triads is stimulated by RR by only a factor of 2 (ref. 24; unpublished data). Ryanodine also blocked the stimulated rate in triads with a K_i in a similar concentration range. Longitudinal cisternae that already had a high Ca^{2+} loading rate (Table 1) (ref. 17; unpublished data) were not appreciably stimulated by RR. Preincubation of this fraction with ryanodine did not significantly affect the Ca^{2+} loading rate.

The effect of ryanodine at low concentrations in blocking the stimulation of Ca^{2+} loading by RR indicates a selective and highly specific drug action of ryanodine on junctional terminal cisternae. This assay can be used to measure the drug potency of ryanodine *in vitro*. In this way, ryanodine after labeling with ^3H was found to be equally effective at comparable concentrations of the original ryanodine.

We next measured the binding of ryanodine to junctional terminal and longitudinal cisternae fractions. The binding of ryanodine is shown in the Scatchard plot (Fig. 4). Single component binding is indicated with an apparent dissociation constant (K_d) in the range of 100 nM, with ≈4 pmol/mg of protein for junctional terminal cisternae. Ca^{2+} binding was essentially eliminated when EGTA (1 mM) was added to the binding assay. The addition of RR eliminated the binding of ryanodine (not shown). The binding of ryanodine to longitudinal cisternae was found to be negligible (Fig. 4 *Inset*). The K_d of ryanodine by junctional terminal cisternae is in the concentration range (K_i) in which ryanodine is effective in blocking the stimulation of Ca^{2+} loading by RR (see Table 1).

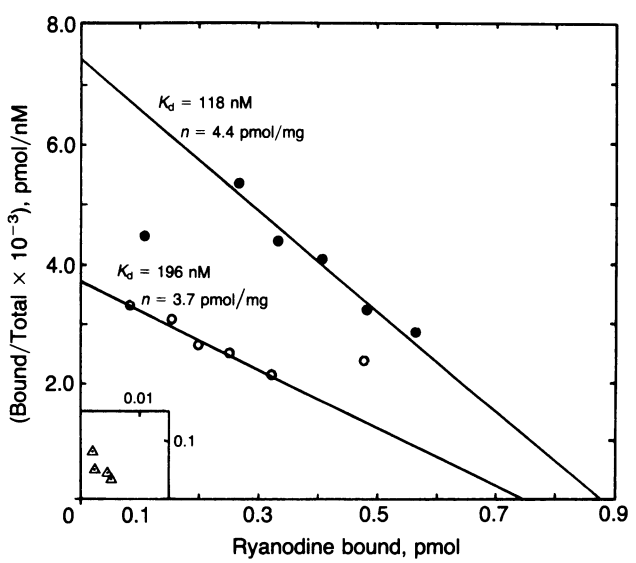


FIG. 4. Scatchard analysis of ryanodine binding to junctional terminal cisternae and longitudinal cisternae of SR. The binding assay was carried out on 0.20 mg of protein per tube. Thus, the x-intercept multiplied by 5 gives the ryanodine bound (pmol/mg of protein) for the two preparations (●, ○). The dissociation constants (K_d) are also given. The binding of ryanodine to longitudinal cisternae of SR was miniscule (△) (see *Inset*; note that the scale is 10× greater). The prior addition of RR (7 μM) to junctional terminal cisternae practically eliminated ryanodine binding (not shown). The value of n varied from approximately 4 to 20 pmol/mg.

DISCUSSION

Ryanodine is a neutral alkaloid that is toxic in low doses (≈20–300 mg/kg) in both vertebrates and invertebrates, which leads to irreversible skeletal muscle contractures resulting in death (25). Previous studies on isolated subcellular muscle membrane fractions with ryanodine made use of concentrations far in excess of pharmacological significance (0.1 mM) (26–28). This study provides direct evidence that ryanodine at concentrations in the range of 100 nM reacts specifically with the terminal cisternae of SR, apparently with the Ca^{2+} release channels. The following observations are relevant: (*i*) junctional terminal cisternae in contrast with longitudinal cisternae have a low Ca^{2+} loading rate, which is stimulated 5-fold or more with RR so that, normalized for calcium pump protein concentration, the stimulated loading rate approaches that of longitudinal cisternae; (*ii*) RR is without effect on the rate of Ca^{2+} loading in longitudinal cisternae, which is already maximal; (*iii*) ryanodine, at nanomolar concentrations (20–200 nM), blocks the action of RR in stimulating Ca^{2+} loading in junctional terminal cisternae, although ryanodine has no effect by itself on Ca^{2+} loading in longitudinal cisternae; (*iv*) for both junctional terminal cisternae and longitudinal cisternae, ryanodine does not affect Ca^{2+}-stimulated ATPase activity assayed in the presence or absence of the Ca^{2+} ionophore A23187; (*v*) ryanodine binding is found to be localized to the terminal cisternae, whereas it does not bind in significant amount to longitudinal cisternae (Fig. 4). The binding of ryanodine is blocked by RR.

Points *i–v* taken together lead to the conclusion that the action of RR and ryanodine is not on the calcium pump protein but on the Ca^{2+} release channels localized in the junctional terminal cisternae. The drug action of ryanodine on junctional terminal cisternae in blocking RR-enhanced Ca^{2+} loading can be explained by its action on "the calcium release channels being locked in the open state." The apparent binding affinity of ryanodine on junctional terminal cisternae is in the same low concentration range as its drug action as well as the pharmacological levels that are lethal to animals. Hence, the toxic action of ryanodine can be explained by the inability of the SR to store Ca^{2+} so that the muscle cannot relax.

Relaxation of muscle requires the uptake and storage of Ca^{2+} in SR by the Ca^{2+} pump membrane, consisting mainly of the calcium pump protein (≈90% of the protein). The calcium pump protein in SR is present at a concentration range of 7–9 nmol/mg of protein (29). The Ca^{2+} pump membrane is the major membrane (80–85%) of terminal cisternae and essentially the only membrane of longitudinal cisternae. Hence, Ca^{2+} uptake can take place in both longitudinal and terminal cisternae of SR, although Ca^{2+} is stored in the terminal cisternae since the calcium binding protein [also referred to as calsequestrin (30)] is localized there (16). Thus, in skeletal muscle, Ca^{2+} is stored within the terminal cisternae until it is released consequent to an action potential, which triggers muscle contraction. From this perspective, our finding that the junctional terminal cisternae are leaky to calcium suggests that, *in situ*, the "resting potential" on the transverse tubules, which are junctionally associated with terminal cisternae, maintains the Ca^{2+} release channels in the "closed state." This is also consistent with the observation that isolated triads are less leaky to Ca^{2+} (Table 1) (24) and

the rapid kinetic studies by Ikemoto *et al.*, which suggest that transverse tubules are required to modulate Ca^{2+} release as studied *in vitro* (12).

The Ca^{2+} release process is a rapid event occurring in milliseconds. It is reasonable that Ca^{2+} release occurs via gated channels. The drug action of ryanodine on terminal cisternae described here indicates that ryanodine exerts its effect by direct binding to such Ca^{2+} release channels apparently locking them in an "open state." The concentration of such channels estimated here for junctional terminal cisternae, and in "heavy SR fragments" (18), is three orders of magnitude lower than the concentration of calcium pump protein in SR. The studies described here localize the ryanodine drug action and binding to the terminal cisternae of SR. Both actions occur at pharmacologically significant concentrations.

We are pleased to acknowledge the helpful comments of Drs. Alice Chu, Makoto Inui, and Brian Costello. We thank Dr. Murray Smigel for advice on the binding assay. This investigation was supported in part by grants from the National Institutes of Health (AM14632) and the Muscular Dystrophy Association.

1. Tada, M., Yamamoto, T. & Tonomura, Y. (1978) *Physiol. Rev.* **58,** 1–79.
2. Ikemoto, N. (1982) *Annu. Rev. Physiol.* **44,** 297–317.
3. Endo, M. (1977) *Physiol. Rev.* **57,** 71–108.
4. Martonosi, A. (1984) *Physiol. Rev.* **64,** 1240–1320.
5. deMeis, L. & Inesi, G. (1982) in *Membrane Transport of Calcium*, ed. Carafoli, E. (Academic, London), pp. 141–180.
6. Tanford, C. (1983) *Annu. Rev. Biochem.* **52,** 379–409.
7. Pickert, C. M. & Jencks, W. (1984) *J. Biol. Chem.* **259,** 1629–1643.
8. Winegrad, S. (1982) *Ann. Rev. Physiol.* **44,** 451–462.
9. Meissner, G. (1984) *J. Biol. Chem.* **259,** 2365–2374.
10. Fabiato, A. (1985) *J. Gen. Physiol.* **85,** 291–320.
11. Nagasaki, K. & Kasai, M. (1983) *J. Biochem. (Tokyo)* **90,** 749–755.
12. Ikemoto, N., Antonius, B. & Kim, D. H. (1984) *J. Biol. Chem.* **259,** 13151–13158.
13. Fleischer, S. (1985) in *Structure and Function of Sarcoplasmic Reticulum*, eds. Fleischer, S. & Tonomura, Y. (Academic, New York), pp. 119–145.
14. Seiler, S. & Fleischer, S. (1982) *J. Biol. Chem.* **257,** 13862–13871.
15. Mitchell, R., Palade, P. & Fleischer, S. (1983) *J. Cell Biol.* **96,** 1008–1016.
16. Saito, A., Seiler, S., Chu, A. & Fleischer, S. (1984) *J. Cell Biol.* **99,** 875–885.
17. Chu, A., Volpe, P., Costello, B. R. & Fleischer, S. (1984) *Biophys. J.* **45,** 317 (abstr.).
18. Pessah, I. N., Waterhouse, A. L. & Casida, J. E. (1985) *Biochem. Biophys. Res. Commun.* **128,** 449–456.
19. Weisner, K., Valenta, Z. & Findlay, J. A. (1967) *Tetrahedron Lett.* **3,** 221–225.
20. Fairhurst, A. S. (1971) *J. Labelled Compd. Radiopharm.* **7,** 133–136.
21. Scarpa, A. (1979) *Methods Enzymol.* **56,** 301–338.
22. Palade, P., Mitchell, R. D. & Fleischer, S. (1983) *J. Biol. Chem.* **258,** 8098–8107.
23. Lowry, O. H., Rosebrough, W. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
24. Mitchell, R. D., Volpe, P., Palade, P. & Fleischer, S. (1983) *J. Biol. Chem.* **258,** 9867–9877.
25. Jendon, D. J. & Fairhurst, A. S. (1969) *Pharmacol. Rev.* **21,** 1–25.
26. Jones, L. R., Besch, H. R., Sutko, J. L. & Wilkerson, J. T. (1979) *J. Pharmacol. Exp. Ther.* **209,** 48–55.
27. Chamberlain, B. K., Volpe, P. & Fleischer, S. (1984) *J. Biol. Chem.* **259,** 7547–7553.
28. Feher, J. J. & Lipford, G. B. (1985) *Biochim. Biophys. Acta* **813,** 77–86.
29. Meissner, G., Conner, G. E. & Fleischer, S. (1973) *Biochim. Biophys. Acta* **298,** 246–269.
30. MacLennan, D. H. & Wong, P. T. S. (1971) *Proc. Natl. Acad. Sci. USA* **68,** 1231–1235.