Vol. 152, No. 1, 1988
April 15, 1988
BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 308-314

RECONSTITUTION OF PURIFIED CARDIAC MUSCLE CALCIUM RELEASE CHANNEL (RYANODINE RECEPTOR) IN PLANAR BILAYERS

Lin Hymel and Hansgeorg Schindler
Institute for Biophysics, University of Linz, A-4040 Linz, Austria
Makoto Inui and Sidney Fleischer
Department of Molecular Biology, Vanderbilt University, Nashville, TN 37235

Received March 3, 1988

The purified ryanodine receptor of heart sarcoplasmic reticulum (SR) has been reconstituted into planar phospholipid bilayers and found to form Ca^{2+}-specific channels. The channels are strongly activated by Ca^{2+} (10 nM) in the presence of ATP (1 mM) and ryanodine, and inactivated by Mg^{2+} (3 mM) or ruthenium red (30μM). These characteristics are diagnostic of calcium release from heart SR. The cardiac ryanodine receptor, which has previously been identified as the foot structure, is now identified as the calcium release channel. A similar identity of the calcium release channel has recently been reported for skeletal muscle. The characteristics of the calcium release channel from skeletal muscle and heart are similar in that they: 1) consist of an oligomer of a single high molecular weight polypeptide (M_r 360,000 for skeletal muscle and 340,000 for heart); 2) exist morphologically as the foot structure; 3) are activated (ATP, Ca^{2+}, ryanodine) and inhibited (ruthenium red and Mg^{2+}) by a number of the same ligands. Important differences include: 1) Ca^{2+} activation at lower concentration of Ca^{2+} for the heart; 2) more dramatic stabilization by ryanodine of the open state for the skeletal muscle channel; and 3) different relative permeabilities (P_{Ca}/P_K).

In heart as in skeletal muscle, contraction and relaxation are regulated by the myoplasmic free calcium ion concentration which in turn is regulated by membranes (1,2). In skeletal muscle, depolarization of the transverse tubule rapidly induces calcium release from the SR. In this context, this process has been referred to as "depolarization induced calcium release" (3). The energized reuptake of calcium into the SR enables muscle to relax (4,5). An important difference in heart muscle is that a second pool of Ca^{2+} is involved. Extracellular Ca^{2+} must first enter the heart cell via the slow inward Ca^{2+} channel in the sarcolemma/transverse tubules, which in turn induces Ca^{2+} to be released from the SR compartment. The latter process has been referred to as "Ca^{2+} induced Ca^{2+} release" (6).

In order to compare the Ca^{2+} release process in skeletal muscle and heart, it is essential to isolate and characterize the calcium release machinery of SR. Important progress has been made in this regard. For both skeletal muscle and heart: 1) ryanodine has been identified as a specific ligand for the calcium release channels (7,8); 2) the ryanodine receptor has been localized to the terminal cisternae of SR (7,8); 3) the ryanodine receptor has been isolated (9-11) and identified as the feet structures (9,10). The ryanodine receptor has been found to consist of an oligomer of a single high molecular weight component (M_r of about 360,000 for skeletal muscle and 340,000 for heart) (10,11). The purified ryanodine receptor from skeletal muscle SR has been incorporated into the bilayer and found to form calcium activated channels with calcium release characteristics diagnostic of that in isolated terminal cisternae of SR (12,13). This study now reports on the reconstitution of the purified ryanodine receptor from heart into bilayers which display diagnostic calcium channel activity. These results thereby identify the calcium release channel in heart with the 340 KD polypeptide and the feet structures of terminal cisternae of SR. A preliminary report has appeared (14).

Methods

Purification of Ryanodine Receptor. Ryanodine receptor was purified from canine heart muscle as previously described (10,15) and shipped from Nashville, TN to Linz, Austria at liquid nitrogen temperature.

0006-291X/88 $1.50

Reconstitution and Bilayer Methods. The ryanodine receptor (0.4 μg protein) was reconstituted with 18 mg acetone-washed soybean phospholipids and 3 mg cholesterol by the "fast dilution" method (17) as described (18). Vesicle-derived solvent-free planar bilayers were formed according to Schindler using a teflon septum with a 100 μm diameter aperture (18,19). The solution for reconstitution and bilayer formation was 0.1 M KCl, 10 mM HEPES-Tris (pH 7.4), 2 mM DTT. Protein-containing vesicles were diluted with phospholipid vesicles to approximate 30 feet structures/100 μm diameter bilayer for macroscopic currents and ~3 feet structures/100 μm diameter bilayer for single channel studies. The calculation of the number of feet structures is based on the ryanodine binding (9,12). After bilayer formation, 50 mM $CaCl_2$ was added to the trans (rear) chamber; additions of channel modulating agents were to the cis (protein-containing) chamber. Judging from effects of activators, sidedness was such that the sarcoplasmic side corresponds to the cis chamber, and the SR lumen to the trans side.

Data Analysis and Presentation. Data storage and analysis were as described (12). Channel openings are shown as downward deflections. Voltage was applied to the front (cis) chamber, while an operational amplifier was used to control the current across a 5 or 10 gigaohm feedback resistor, holding the rear chamber at ground (voltage clamp). Conductance scales assume an ohmic single channel open state current-voltage relationship. The upper end of the scale coincides with the baseline.

General Methods. Biochemical assays (protein determination, ryanodine binding, SDS-PAGE) were as described (9,10). Free Ca^{2+} concentration (cis side) was estimated using the K_d for Ca-EGTA from Owen (20).

Results

The electrophoretic profile of the purified receptor and cardiac terminal cisternae (8) are compared in Figure 1. The purified heart ryanodine receptor consists of an oligomer of a single polypeptide of Mr 340,000 (10).

Incorporation of the receptor into planar bilayers resulted in reproducible Ca^{2+} channel activity. Typical recovery was 20-50%, assuming a conductance of 60 pS/foot structure (see below). Channel activity was approximately proportional to the amount of receptor incorporated in the bilayer. Single

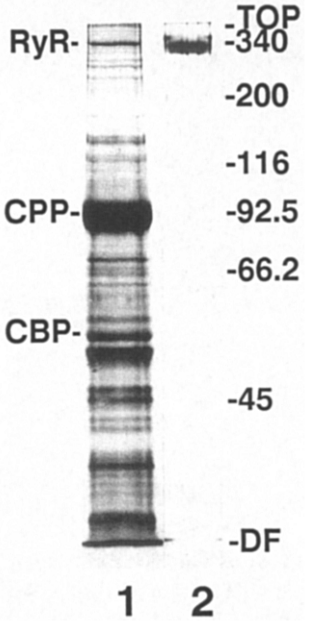

Figure 1. SDS-PAGE of the purified ryanodine receptor from heart. A typical purified receptor preparation used in this study is shown in lane 2, as compared with terminal cisternae of cardiac SR (lane 1). Electrophoresis was carried out in SDS on a 7.5% polyacrylamide gel using the buffer system of Laemmli (28). The gel was double stained with Coomassie blue and silver (29). Molecular weight standards: α2-macroglobulin (non-reduced), M_r 340,000; myosin, 200,000; β-galactosidase, 116,000; phosphorylase b, 92,500; bovine serum albumin, 66,200; ovalbumin, 34,000 were run in parallel gel lanes and indicated on the right side. RyR, ryanodine receptor protein; CPP, Ca^{2+}-pump protein; CBP, Ca^{2+}-binding protein (calsequestrin); TOP, top of gel; DF, dye front.

channel conductance increases with the time after bilayer formation. It is maximal after approximately 30 minutes (data not shown). Channel activity was observed at any voltage applied from -200 mV to well beyond the reversal potential, including zero potential difference. There was no indication for a threshold type of voltage gating. Calcium ion selectivity was tested using bi-ionic gradients. From the reversal potential of +25 ± 1 mV under our measuring conditions (symmetrical 100 mM KCl, trans 50 mM $CaCl_2$), we calculate P_{Ca}/P_K = 3.0 using a constant field equation (21).

The activation properties of reconstituted calcium channel activity was studied using macroscopic currents (~30 feet structures/bilayer). As expected by analogy to Ca^{2+} release studies with SR vesicles, the channel proved to be highly dependent on Ca^{2+} concentration (Fig. 2A). While 3 nM Ca^{2+} was insufficient to induce stationary Ca^{2+} currents, an increase to merely 10 nM caused a dramatic and rapid (within the 30 seconds mixing time) increase in Ca^{2+} current. This dramatic effect was often followed by spontaneous inactivation in the next few minutes (Fig. 2C, left side). If the Ca^{2+} activation experiment was performed in the absence of adenine nucleotide, the effect of Ca^{2+} was much less dramatic. Figure 2B shows only limited channel activity in 100 nM Ca^{2+} in the absence of ATP, which

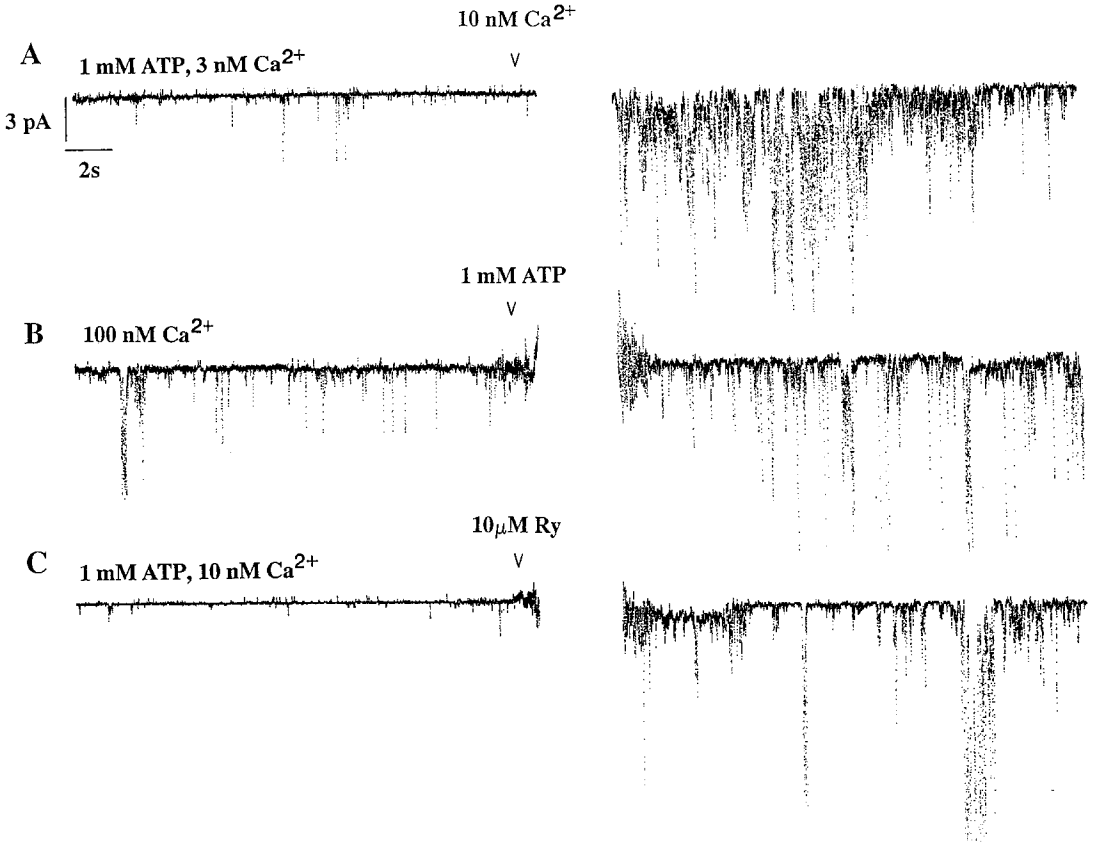

Figure 2. Activation of Ca^{2+} channel activity by Ca^{2+}, ATP and ryanodine. A-C. Macroscopic conductance traces in symmetrical 100 mM KCl, 10 mM HEPES-Tris, pH 7.4 plus 50 mM $CaCl_2$, trans. Activators were added to the cis side at the end of each trace on the left, followed by a 30 s stirring interval (not shown). Traces on the right are in each case for the same membrane as on the left, shown just after stirring was turned off. A. Activation by Ca^{2+} in the presence of 1 mM ATP. On the left, the free Ca^{2+} concentration was 3 nM (0.54 mM $CaCl_2$, 10 mM EGTA), and on the right the [Ca^{2+}] is 10 nM (1.59 mM $CaCl_2$, 10 mM EGTA). After several minutes this activation is often followed by spontaneous inactivation (see Fig. 1C). B. Activation by 1 mM ATP in the presence of 100 nM Ca^{2+} (6.55 mM $CaCl_2$, 10 mM EGTA). C. Activation by 10 μM ryanodine in the presence of 1 mM ATP and 10 nM free Ca^{2+}. The holding potential is at -50 mV (A) and -100 mV (B,C). The data in figures 2 to 4 were filtered at 30 Hz.

increases considerably upon addition of 1 mM ATP. Figure 2C demonstrates, further, that spontaneous inactivation after Ca^{2+} addition (10 μM, left side of trace) can rapidly be reversed by 10 μM ryanodine (right side).

Ruthenium red effectively blocked cardiac ryanodine receptor channel activity at micromolar concentrations (Fig. 3A). This effect was strongly dependent on the state of activation of the channel. Higher Ca^{2+} concentrations (100 nM) or ryanodine (10 μM) completely eliminated the ruthenium red effect (not shown). Similarly, 3-5 mM $MgCl_2$, but not 1 mM, quickly blocked the channel (Fig. 3B), but the block was usually transient, some activity returning spontaneously or when a higher driving force (applied potential) was applied. Ryanodine did not prohibit the Mg^{2+} block.

At the single channel level, the cardiac Ca^{2+} release channel reconstituted into the bilayer yielded a variety of conductance states, complicating analysis of channel properties. Ryanodine (10 μM) stabilized the conductance states of the channel somewhat, and so was applied to the study of single channel activity (Fig. 4). Nonetheless, the long open times (order of seconds) was not observed in the heart receptor as it was for the skeletal muscle (12). The smallest level observed was ~4 pS, also seen as a sublevel in ~8 pS bursts (trace a). More common events of up to 30 pS also revealed even multiples

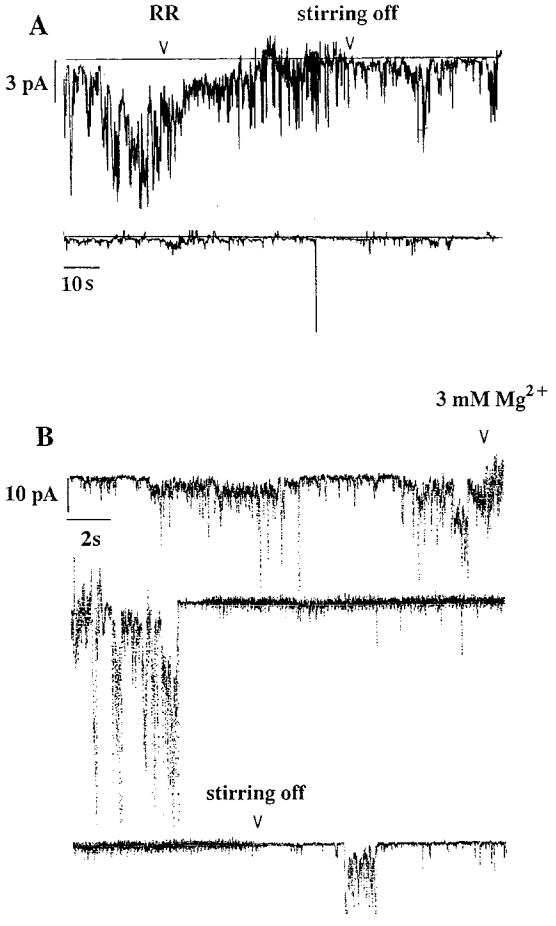

Figure 3. Blockage of cardiac calcium release channel by ruthenium red and Mg^{2+}. Solutions were the same as in Figure 2. A. Ruthenium red (30 μM) blocks Ca^{2+} current in the presence of 1 mM ATP and 10 nM Ca^{2+}. B. Cis 1 mM $MgCl_2$ (beginning of trace) is insufficient to block the channel in the presence of 1 mM ATP, 30 nM free Ca^{2+}, and 10 μM ryanodine. Increasing to 3 mM $MgCl_2$ (cis) blocks the channel effectively, but some residual activity remains. The three traces are continuous in time. The holding potential is clamped at -50 mV (A) and -120 mV (B).

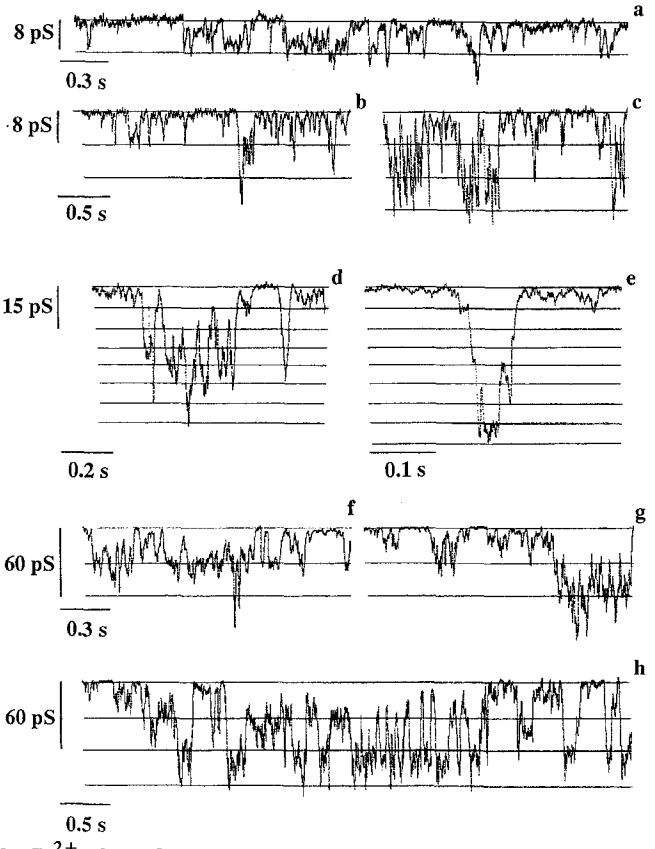

Figure 4. Single Ca^{2+} channel activity of the reconstituted cardiac ryanodine receptor. Solutions were same as Figure 2. In addition at the cis side the following activators were added: 1 mM ATP, 30 nM free Ca^{2+}, and 10 μM ryanodine. Approximately 3 foot structures were incorporated per bilayer. The voltage was clamped at different holding potentials: -50 mV (e), -75 mV (d), -100 mV (a), -140 mV (b,c,f-h). The reversal potential was +25 ± 1 mV.

of 4 pS (traces b,c). Conductances in the range 30-60 pS were also frequently encountered (traces d-h). Trace 3 presents most of the even multiples of 4 pS up to ~60 pS and tentatively is assumed to represent a single foot structure. Finally, for larger conductances, 15 and 30 pS events and conductance jumps were especially noticeable (traces f-h).

Discussion

This study identifies the cardiac ryanodine receptor as the SR calcium release channel. The recent finding that the isolated receptor from fast twitch skeletal muscle is equivalent to the junctional feet structures (9) which join the SR junctional face membrane to the transverse tubule, has confirmed its central role in signal transduction at the triad junction. A similar identity was made for the heart receptor (10). The properties described here for the ryanodine receptor/Ca^{2+} channel are similar to the properties of the Ca^{2+} release channel exhibited in vesicle transport studies (8,22-24): activation by submicromolar Ca^{2+}, millimolar ATP, and block by micromolar ruthenium red, millimolar Mg^{2+} and ryanodine. The channel can be activated by ryanodine in the nanomolar range (7,16), although higher concentrations of ryanodine (10 μM) were used for activation to speed up the response time (Fig. 3). An interesting property of the reconstituted channel is its tendency to show time correlated subconductance states which are integral multiples of the smallest observed stable conductance levels, 4 and 8 pS. This is strong evidence for an oligomeric channel structure (oligochannel) and is consistent with the observed size and four-fold symmetry of the foot structure, estimated to consist of 12-16 copies

of the 340,000 polypeptide (9,10). This is further supported by the increasing conductances of single events with increasing time, indicating aggregation of channel proteins with concommitant channel synchronization.

In a previous study (23) the cardiac SR calcium channel was shown to form 75 pS channels in planar bilayers (53 mM trans Ca^{2+}). For the purified, reconstituted Ca^{2+} channels in this study, we find a range of conductance states from 4 to 60 pS and more (50 mM trans Ca^{2+}) with time correlated sublevels in multiples of 4 or 8 pS, which we interpret as evidence for allosteric interactions (positive cooperativity) in an oligomeric channel complex (oligochannel). Assuming that the smallest observed conductance (~4 pS) is referable to a single 340,000 Dalton polypeptide, and considering that a single foot structure is estimated to contain 12-16 such polypeptides (10), we tentatively assign the 60 pS conductance level to a single foot structure (cf. Fig. 4e). It is interesting to note that similarly-sized channel associates have been indicated in skeletal muscle for both the ryanodine receptor (12) and dihydropyridine receptor (25) calcium channels, where evidence for interaction between the two channels has been presented (26).

In a related study, we have characterized the purified Ca^{2+} release channel/ryanodine receptor from skeletal muscle in planar bilayers (12). A secondary aim of the current study is to begin to define differences and similarities between the Ca^{2+} release channels in cardiac and skeletal muscle. The similarities predominate: i) monochannel conductance for both channels is about 4 pS in 50 mM $CaCl_2$; ii) both channels have similar associative properties, forming cooperatively interacting oligochannels as witnessed by integral multiple subconductance states showing substates with multiples of four, with predominant conductance states of 15, 30 and 60 pS; iii) both channels are highly dependent on submicromolar Ca^{2+} for activity under our conditions, and show similar modulation characteristics by ATP, ryanodine, ruthenium red and Mg^{2+}. Important differences include: a) Ca^{2+} activation at lower concentrations of Ca^{2+} for cardiac ryanodine receptor (10 nM) compared with 50-100 nM for similar activation with skeletal muscle receptor); b) more dramatic stabilization of open state for skeletal ryanodine receptor (cf. 12); and c) a six times lower permeability ratio (P_{Ca}/P_K) for heart compared to skeletal muscle (12) under comparable conditions.

The greater sensitivity to Ca^{2+} by heart Ca^{2+} release channels is of interest with respect to the presumed Ca^{2+}-induced Ca^{2+} release mechanism in cardiac muscle (6). Contraction of heart muscle is clearly dependent on external Ca^{2+}, in contrast to skeletal muscle. Further studies are required to relate the Ca^{2+} concentration for opening of the calcium release channels from heart and skeletal muscle SR under physiological conditions, i.e., [Mg^{2+}], adenine nucleotides, pH, etc.

Acknowledgements. This investigation was supported in part by a grant from the NIH (HL32711) to SF and project grant S-45/3 from the Osterreichischer Forschungsforderungsfonds to HS. MI is Investigator of the American Heart Assoc., TN Affil.

References

1. Tada, M., Yamamoto, T., and Tonomura, Y. (1978) Physiol. Rev. 58, 1-79.
2. Fleischer, S. (1985) In Structure and Function of Sarcoplasmic Reticulum (S. Fleischer and Y. Tonomura, Eds.) pp. 119-145. Academic Press, Orlando, FL.
3. Endo, M. (1977) Physiol. Rev. 57, 71-108.
4. Martonosi, A. (1984) Physiol. Rev. 64, 1240-1320.
5. Inesi, G. (1985) Annu. Rev. Physiol. 47, 573-601.
6. Fabiato, A. (1983) Am. J. Physiol. 245, C1-C14.
7. Fleischer, S., Ogunbunmi, E.M., Dixon, M.C., and Fleer, E.A.M. (1985) Proc. Natl. Acad. Sci. USA 82, 7256-7259.
8. Wang, S., Inui, M., Ogunbunmi, E., Saito, A., and Fleischer, S. (1987) Biophys. J. 51, 354a.
9. Inui, M., Saito, A., and Fleischer, S. (1987) J. Biol. Chem. 262, 1740-1747.
10. Inui, M., Saito, A., and Fleischer, S. (1987) J. Biol. Chem. 262, 15637-15642.
11. Imagawa, T., Smith, J.S., Coronado, R. and Campbell, K.P. (1987) J. Biol. Chem. 262, 16636-16643.

12. Hymel, L., Inui, M., Fleischer, S., and Schindler, H.G. (1988) Proc. Natl. Acad. Sci. USA 85, 441-445.
13. Lai, F.A., Erickson, H.P., Rousseau, E., Liu, Q-Y., and Meissner, G. (1988) Nature 331, 315-319.
14. Hymel, L., Schindler, H., Inui, M., Nagasaki, K., and Fleischer, S. (1988) Biophys. J. 53, 470a.
15. Inui, M., and Fleischer, S. (1988) Methods Enzymol. 157, in press.
16. Nagasaki, K., and Fleischer, S. (1988) Cell Calcium 9, 1-7.
17. Schindler, H., Spillecke, P., and Neumann, E. (1984) Proc. Natl. Acad. Sci. USA 81, 6222-6226.
18. Schindler, H. (1988) in Methods Enzymol. 157, in press.
19. Schindler, H. (1980) FEBS Lett. 122, 77-79.
20. Owen, J.D. (1976) Biochim. Biophys. Acta 451, 321-325.
21. Lewis, C.A. (1979) J. Physiol. (Lond.) 286, 417-445.
22. Chamberlain, B.K., Volpe, P., and Fleischer, S. (1984) J. Biol. Chem. 259, 7547-7553.
23. Rousseau, E., Smith, J.S., Henderson, J.A., and Meissner, G. (1986) Biophys. J. 50, 1009-1014.
24. Meissner, G., and Henderson, J.S. (1987) J. Biol. Chem. 262, 3065-3073.
25. Hymel, L., Striessnig, J., Glossmann, H., and Schindler, H. (1988) Proc. Natl. Acad. Sci. USA, in press.
26. Rios, E., and Brum, G. (1987) Nature 325, 717-720.
27. Schneider, M.F., and Chandler, W.K. (1973) Nature 242, 244-246.
28. Laemmli, V.K. (1970) Nature (London) 227, 680-685.
29. Merril, C.R., Goldmann, D., and Van Keuren, M.L. (1983) Methods Enzymol. 96, 230-239.