Biochimica et Biophysica Acta 897 (1987) 324–330
Elsevier

BBA 73395

Ca^{2+} channels from brain microsomal membranes reconstituted in patch-clamped bilayers

Peter M. Vassilev, Maria P. Kanazirska and H. Ti Tien

Membrane Biophysics Laboratory, Department of Physiology, Michigan State University, East Lansing, MI 48824 (U.S.A.)

(Received 22 September 1986)

Key words: Membrane reconstitution; Patch clamp; Calcium ion channel; (Bovine brain microsome)

Single Ca^{2+} channels from brain microsomal membranes were reconstituted in bilayers made at the tips of patch-clamp micropipettes. The single-channel conductance was defined to be 107 pS in 50 mM Ca^{2+}. The channel activity was stimulated by nucleotides and inositol 1,4,5-trisphosphate (Ins-P_3), and was inhibited by ruthenium red. Na^+ added asymmetrically to the membrane bilayer induced an increase in the Ca^{2+}-channel activity. The described characteristics of these Ca^{2+} channels suggest that they may be responsible for the Ca^{2+} transport across the membranes of the endoplasmic reticulum system triggering and modulating various neurosecretory and excitatory processes in nerve cells.

Introduction

A variety of cellular functions, such as secretion, contraction, hormonal and neuromediator regulation, depend on the cytosolic [Ca^{2+}] [1–8]. The latter is controlled to a great extent by Ca^{2+} transport process across the membranes of the endoplasmic reticulum and other Ca^{2+}-storing organelles. The mechanism of the Ca^{2+}-release from the endoplasmic reticulum is not well understood. A channel mechanism has been suggested [8–10] and recently Ca^{2+} channels from muscle sarcoplasmic reticulum have been incorporated into bilayer lipid membranes [10,11]. In the last paper the large unit conductance and the fast channel kinetics were interpreted as an indication that these channels may be responsible for the release of Ca^{2+} from sarcoplasmic reticulum during the excitation-contraction coupling process. It is of particular interest to verify if this type of Ca^{2+} channels is specific for the sarcoplasmic reticulum membranes. Bearing in mind the universal role of cytoplasmic Ca^{2+}, triggering many cellular functions, it is reasonable to suggest that Ca^{2+} channels with similar properties may be available in the endoplasmic reticulum membranes of nerve and other cells.

The cytosolic [Ca^{2+}] is of particular importance for the processes accompanying the excitability and neuromediator release in nerve cells [12–15]. Their endoplasmic reticulum is one of the main intracellular Ca^{2+}-storing organelles and the transport of Ca^{2+} across the membranes of the endoplasmic reticulum and other Ca^{2+}-storing compartments has been considered in relation to the synaptic and other functions [16–19].

The bilayer lipid membrane method has been used successfully for studying the channel characteristics of different reconstituted membranes [20–25]. We have examined the properties of brain microsomal Ca^{2+} channels reconstituted in bilayer lipid membranes made from monolayers at the tips of patch-clamp micropipettes.

Correspondence: H.T. Tien, Department of Physiology, Giltner Hall, Michigan State University, East Lansing, MI 48824, U.S.A.

0005-2736/87/$03.50 © 1987 Elsevier Science Publishers B.V. (Biomedical Division)

Materials

Tris, calcium chloride, calcium hydroxide, barium chloride, barium hydroxide, sodium chloride, dimyristoylphosphatidylcholine, adenosine 5′-triphosphate (Tris salt, ATP), guanosine 5′-triphosphate (Tris salt, GTP), inositol trisphosphate (Ins-P_3) and Nifedipine were purchased from Sigma. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) was purchased from Calbiochem; ruthenium red from Aldrich and ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) from Fluka.

Methods

Preparation of the membrane forming solutions. The endoplasmic reticulum (microsomal) membranes were prepared from fresh bovine forebrains as described in Ref. 34. The lipid and the proteolipid components were isolated from these membranes according to the methods described in Refs. 35 and 36, respectively. They were dissolved in *n*-pentane at a concentration of 20 mg/ml, the lipid/proteolipid ratio being 3 : 1 (w/w).

Formation of the monolayers and the patch clamp bilayers. Monolayers were formed by spreading 2 μl of this solution on the surface of a compartment filled with a buffer solution containing 0.1 mM $Ca(OH)_2$, 0.1 mM EGTA, 10 mM Tris, 10 mM Hepes (pH 7.4). The free [Ca^{2+}] was 2.5 μM.

The volume of the Teflon compartment was 0.5 cm^3. The bilayers were made from monolayers on the tips of patch clamp micropipettes according to Refs. 37 and 38. The tips of the micropipettes prepared from Blue-tip microcapillaries were fire polished and had a diameter of less than 1 μm. The pipette solution contained 50 mM Ca^{2+} (47 mM $Ca\,Cl_2$ and 3 mM $Ca(OH)_2$) or other [Ca^{2+}] or [Ba^{2+}] where indicated, and 10 mM Hepes (pH 7.4).

In order to create conditions for asymmetric incorporation of the channel molecules, in some experiments we used asymmetric bilayer lipid membranes formed by passing the pipette tip initially through a monolayer of dimyristoylphosphatidylcholine followed by reintroduction of the micropipette tip through a monolayer containing the isolated lipids and proteolipids. However, we did not find any significant differences between the channel characteristics of these bilayers and of those containing only symmetrically distributed native membrane components.

In some experiments, the monolayers were formed by adsorption of isolated membrane vesicles on the air/water interface after adding them to the aqueous subphase to a final concentration of 0.5 mg vesicular protein per 1 ml and incubating for 15 min at room temperature. The single-channel characteristics of the bilayers made by the different methods were found to be identical. However, the stability of the bilayer patches assembled from isolated membrane lipids and proteolipids was the best. Seals with resistances of 4 GΩ formed readily during the first trial and this was the method which was mainly employed in the described experiments.

Single-channel measurements. The electrical measurements were performed according to the patch-clamp technique [39] by using two Ag/AgCl electrodes connected to a 8800 Dagan Total Clamp system. One of the electrodes was exposed to the pipette solution referred to as the *trans* side of the bilayer, while the other (reference) electrode was immersed in the aqueous subphase facing the *cis* side of the bilayer. According to our convention the *trans* side is considered as 'intravesicular' and the *cis* side as 'cytoplasmic' side. The polarity of the applied potentials (E_a) refers to the trans side of the bilayer. The single-channel current traces were analyzed after recording them at a cut-off frequency of 1 kHz by using Hitachi VCR connected to Neurocorder Model DR-484 (Neuro Data Instr. Corp.) and a chart recorder. Seals with a resistance of more than 10 GΩ were used for the single-channel measurements. The probability of the open state of the channels (P_o) was defined by using the following equation:

$$P_o = I/i \cdot n$$

where I is the time-averaged current flowing through the open channels during a defined time period; i is the mean single open channel current and n is the number of channels functioning in the bilayer. All the experiments were performed at room temperature (20°C).

Results and Discussion

Current-voltage relations and single-channel conductances

We found large current displacements accompanying the opening of the Ca^{2+} channels (Fig. 1A). A tendency for bursting activity was observed. Brief as well as relatively long opening and closing events occurred at different applied voltages. The slope conductance of these Ca^{2+} channels calculated from the current-voltage curve (Fig. 1B) was 107 pS, with 50 mM Ca^{2+} as the charge carrier. An equilibrium potential of -122 mV was obtained, which was close to the theoretically calculated value of the Nernst potential, $E_{Ca^{2+}} = -125$ mV. According to our sign convention the other equilibrium potentials are quite different: $E_{Hepes} = 0$, and E_{Cl^-} and $E_{Tris} = +\infty$. In this study Tris was used at relatively low concentration and it did not influence significantly $E_{Ca^{2+}}$. In 50 mM Ba^{2+} the single-channel conductance was 138 pS. The permeability ratio $P(Ba)/P(Cs)$ was 10.8.

The large unit conductance and the adequate single-channel resolution permitted us to perform experiments at lower [Ca^{2+}]. We registered well resolved single-channel current fluctuations even at 2.5 mM Ca^{2+} or Ba^{2+}. The single-channel conductances at these divalent cation concentrations were 6.3 pS for Ca^{2+} and 9.2 pS for Ba^{2+}.

Effects of Ca^{2+} antagonists and of ATP

We have tested the effect of the typical inhibitor of the endoplasmic reticulum membrane Ca^{2+} transport, ruthenium red and found that 1 μM ruthenium red inhibited strongly the Ca^{2+}-channel activity reconstituted from brain endoplasmic reticulum membranes in bilayers (Fig. 2). A decrease in the channel activity was observed in about 10 s after addition of the agent to the *cis* side of the bilayer (Fig. 2) and in approximately one minute a complete block occurred (data not shown).

The observed channel-opening behavior and the inhibitory effect of ruthenium red permitted us to suggest that the Ca^{2+} channels of the brain endoplasmic reticulum membranes and of muscle sarcoplasmic reticulum membranes [11] share some common properties which differ significantly from those of the Ca^{2+} channels in the peripheral cyto-

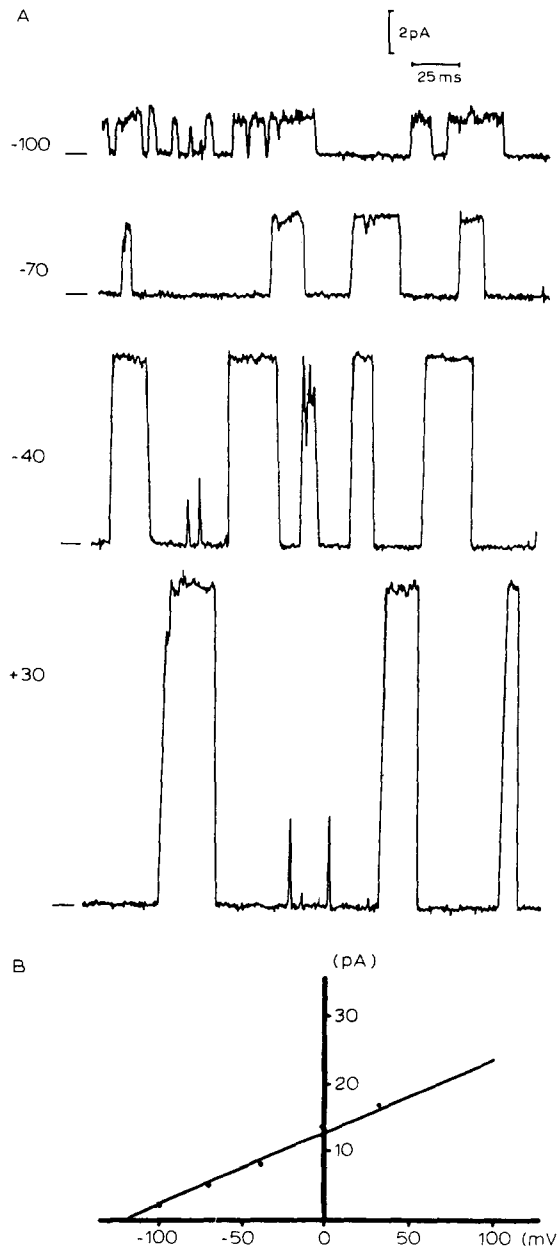

Fig. 1. Since Ca^{2+} channels from brain microsomal membranes reconstituted in bilayers which were made at the tips of patch-clamp micropipettes in 50 mM Ca^{2+} as a current carrier. (A) Representative single-channel current traces at four different applied voltages their magnitude being indicated on the left side of each trace. The horizontal bars on the left side indicate the channel closed state for each record. The vertical and the horizontal calibration bars on the right side above the top current trace are valid for all the records shown. (B) Single-channel current-voltage relations. The equilibrium potential was -122 mV and the conductance determined from the slope of the regression line was 107 pS.

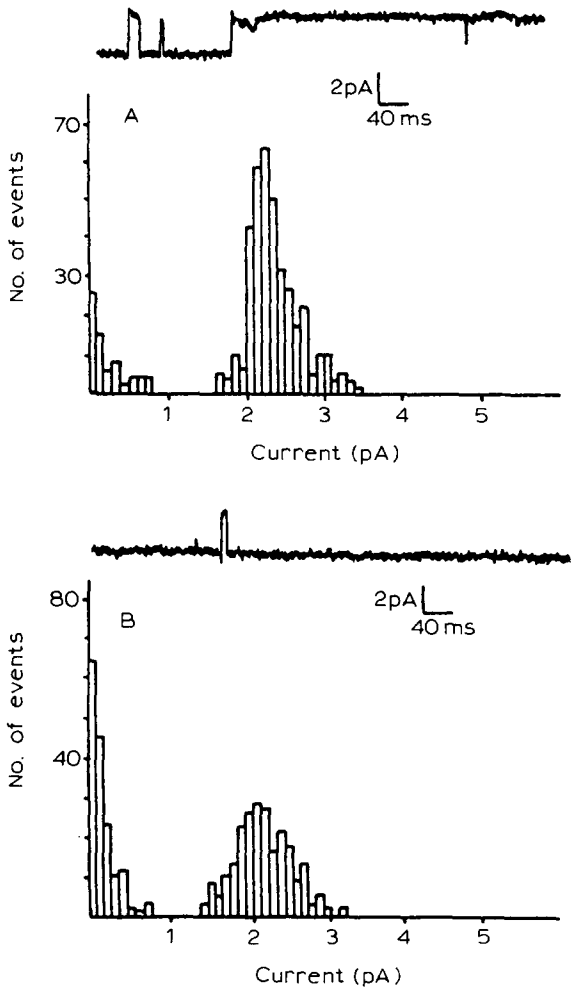

Fig. 2. Ruthenium red-induced inhibition of the brain microsomal Ca^{2+} channels. The patch contains one functioning channel. (A) Current amplitude histogram at -100 mV in the presence of 1 mM ATP before the addition of ruthenium red, and a representative single-channel current trace showing that most of the time the channel resides in the open state. (B) Current amplitude histogram, and a representative current trace at -100 mV in the presence of 1 μM ruthenium red. The data were taken 8–12 s after addition of the agent by analyzing a 4 s segment of the single-channel record.

plasmic membranes [2,3,46–30]. Ca^{2+} channels from sarcolemmal vesicles have been incorporated in bilayer lipid membranes [31] and their kinetic and other characteristics were shown to be similar to those of the L-type channels previously characterized in cell-attached patches [27,28]. The slope conductance of the reconstituted channels was 23 pS in 100 mM Ba^{2+} and 7 pS in 100 mMCa^{2+} [31]. Recently Ca^{2+} channels from brain plasma membranes have been incorporated into bilayer lipid membranes [24]. Their unit conductance was 5 pS in 250 mM Ca^{2+}, which is much lower than the conductance of the channels described in this study. Micromolar concentrations of dihydropyridines and other Ca^{2+} antagonists were found to abolish completely the activity of reconstituted sarcolemmal channels [31–33].

In control experiments we could not find any significant blocking effect of the dihydropyridine agent Nifedipine (25 μM) on the brain endoplasmic reticulum Ca^{2+} channels confirming the evidence that their properties differ from those of plasma membrane channels.

Similarly to the sarcoplasmic reticulum channels [11], we observed also a stimulating action of nucleotides on the reconstituted brain endoplasmic reticulum Ca^{2+} channels. The open time histograms in the absence and presence of ATP are presented in Fig. 3. The data are fitted by a sum of two exponentials, showing the presence of two open-state lifetimes, one with relatively short and another with longer duration. In the presence of 1 mM ATP both time constants increase substantially indicating larger mean open times.

Effect of Na^+

It is reasonable to consider the eventual func-

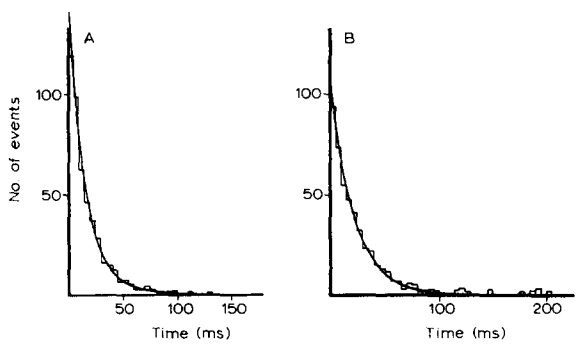

Fig. 3. Stimulation of the brain microsomal Ca^{2+} channel activity by addition of 1 mM ATP to the *cis* side of the bilayer (A) Open-time histogram of data recorded at -100 mV in the absence of ATP. A sum of two exponentials with time constants $\tau_1 = 14$ ms, and $\tau_2 = 39$ ms, fitted the obtained data. (B) Open-time histogram of data recorded at -100 mV in the presence of 1 mM ATP. The two time constants in this case were: $\tau_1 = 21$ ms, and $\tau_2 = 108$ ms.

tional importance of the brain endoplasmic reticulum Ca^{2+} channels. Similarly to sarcoplasmic reticulum channels, they may be involved in the Ca^{2+} release from the neuron endoplasmic reticulum system in connection with the excitatory and neurosecretory processes. The cytosolic [Ca^{2+}] is the main factor controlling the neuromediator release from the presynaptic terminals. Its increase during the excitation is mainly due to the influx of extracellular Ca^{2+}. The role of the smooth endoplasmic reticulum in the presynaptic terminals is related predominantly to the uptake of Ca^{2+} during the resting period. Nevertheless, there is evidence that the depletion of Ca^{2+} from the intracellular stores may be responsible for some events associated with the neuromediator release under defined conditions [16–19]. There is evidence that Ca^{2+} from the intracellular stores may control the processes of potentiation and facilitation in neuromuscular synapses [17,18]. These events may be triggered by Na^+ entering the cytoplasm during the excitation of the synaptic membrane [17]. The mechanism of this phenomenon remains unknown.

We suggested that Na^+ can eventually provoke Ca^{2+} release from the brain endoplasmic reticulum and we studied the effect of Na^+ added to the *cis* side of the bilayer on the Ca^{2+} channel activity. Fig. 4 shows the amplitude histograms of the brain microsomal Ca^{2+} channels reconstituted in bilayers in the absence and in the presence of asymmetrically added 5 mM NaCl. Na^+ induced a significant increase in the Ca^{2+}-channel activity. When [Na^+] was increased from 0.5 mM to 5 mM the channel-opening probability rose substantially (Table I). In control experiments, no channel activity was observed at these Na^+ concentrations in the absence of Ca^{2+}.

These results permit us to suggest that the release of Ca^{2+} through Ca^{2+} channels of the brain endoplasmic reticulum membranes may be stimulated by increases in the cytosolic [Na^+]. This phenomenon may be involved in the process of neuromediator secretion in the synapses.

Stimulation by Ins-P_3 and GTP

Recently it has been demonstrated that Ins-P_3 may play an important role as an intracellular messenger inducing release of Ca^{2+} from the endoplasmic reticulum in different cells [40,41,42]. It has been found that GTP may potentiate this effect of Ins-P_3 [43]. A recent study showed that GTP (10 μM) can also directly activate Ca^{2+} release from neuronal endoplasmic reticulum [44].

In an attempt to find some further physiological implications of the microsomal Ca^{2+} channels

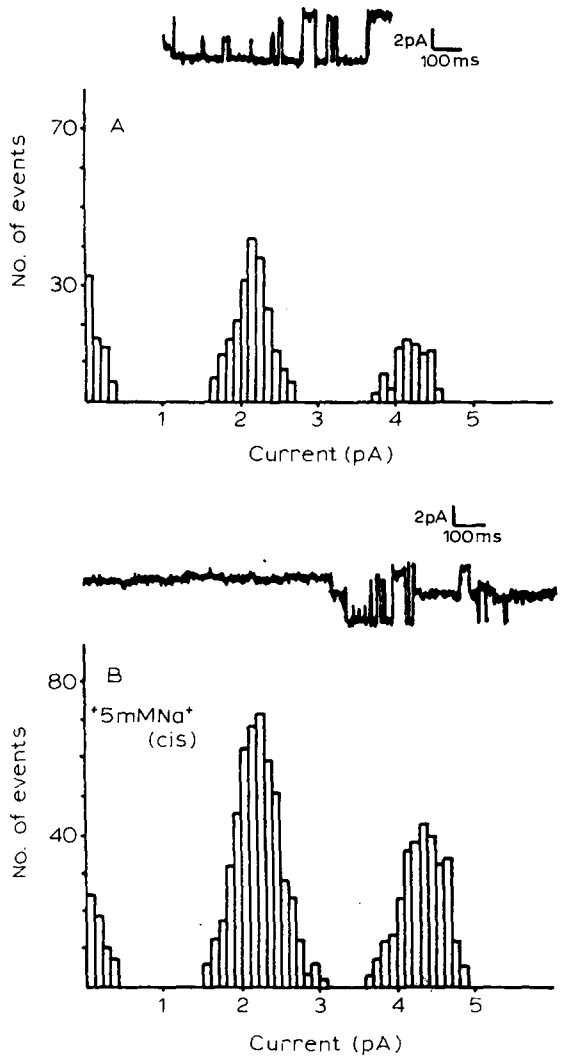

Fig. 4. Stimulation of the brain microsomal Ca^{2+}-channel activity by adding 5 mM Na^+ to the *cis* side of the bilayer. The patch contains two functioning channels. (A) Current amplitude histogram at −100 mV before the addition of Na^+, and a representative current trace from the data which were used to plot the histogram. (B) Current amplitude histogram at −100 mV for data obtained after adding 5 mM NaCl to the cis side of the bilayer, and a representative current trace recorded in the presence of Na^+.

TABLE I

OPEN STATE PROBABILITY (P_o) OF BRAIN MICROSOMAL Ca^{2+} IN THE PRESENCE OF TWO DIFFERENT CONCENTRATIONS OF NaCl ADDED TO THE *cis* SIDE OF THE BILAYER MEMBRANE AT 10 mV

Mean values ± S.E. are presented for P_o. *N*, number of experiments.

	P_o	*N*
Control	0.10 ± 0.03	5
0.5 mM NaCl (*cis*)	0.16 ± 0.02	5
5 mM NaCl (*cis*)	0.25 ± 0.05	4

described in the present study we have examined the changes in their activities under the influences of Ins-P_3 and GTP (Table II). We found that after addition of Ins-P_3 the open-state probability of the channels increased more than two times (Table II). GTP potentiated substantially the action of Ins-P_3 on the channel activity. When used separately at a concentration of 50 μM GTP was also able to induce an increase of P_o, but its effect was less prominent than that of 3 μM Ins-P_3. ATP when used at a concentration of 50 μM did not provoke any significant change in P_o (data not shown). Thus, the obtained results show that both Ins-P_3 and GTP when used separately or in combination can activate the Ca^{2+} channels which may be responsible for the release of Ca^{2+} from endoplasmic reticulum. By participating in the mechanism of Ca^{2+} transport across endoplasmic reticulum membranes in nerve cells these channels may be involved in the regulation of various excitatory and neurosecretory processes.

We suppose that microsomal Ca^{2+} channels with typical characteristics and similar mechanisms of functioning may be responsible for the excitation-contraction coupling in muscle cells, as well as for the excitation-secretion coupling in nerve cells.

Other functions such as regulation of the intracellular transport, control of the configuration of the cytoskeleton, etc., may be also dependent on the activity of the channels described in this study.

The bilayer lipid membrane technique has been shown to be an adequate method for investigating the single-channel activities of intracellular membranes. Further studies on reconstituted bilayers containing components from other intracellular sources should be needed in order to verify the specificity of the described Ca^{2+} channels for the microsomal membranes.

TABLE II

INFLUENCE OF Ins-P_3 AND GUANOSINE 5′-TRIPHOSPHATE (GTP) ON THE OPEN STATE PROBABILITY (P_o) OF BRAIN MICROSOMAL Ca^{2+} CHANNELS AT 10 mV

The agents were added to the *cis* sides of the bilayers. Mean values ± S.E. are presented for P_o. *N*, number of experiments.

	P_o	*N*
Control	0.09 ± 0.02	9
3 μM Ins-P_3	0.27 ± 0.04	4
3 μM Ins-P_3 + 50 μM GTP	0.37 ± 0.06	3
50 μM GTP	0.22 ± 0.03	5

Acknowledgments

Major funding of this work was provided by an NIH grant (GM-14971). Thanks are due to Ms. Jan Reid for typing.

References

1 Hagiwara, S. and Byerly, L. (1981) Annu Rev. Neurosci. 4, 69–125
2 Tsien, R.W. (1983) Annu. Rev. Physiol. 45, 341–358
3 Reuter, H. (1983) Nature 301, 569–574
4 Fabiato, A. and Fabiato, F. (1979) Annu Rev. Physiol. 41, 473–484
5 Rasmussen, H. (1981) Calcium and c-AMP as Synarchic Messengers, John Wiley and Sons, New York
6 Carafoli, E. and Crompton, M. (1978) Curr. Top. Membranes Transp. 10, 152–216
7 De Lorenzo, R.J. (1985) Calcium and Cell Physiology (Marmé, D., ed.), pp. 265–284, Springer-Verlag, Berlin
8 Ebashi, A. (1976) Annu Rev. Physiol. 38, 293–313
9 Endo, M. (1977) Physiol. Rev. 57, 71–108
10 Orozco, C.B., Suarez-Isla, B.A., Froehlich, J.P. and Heller, P.F. (1985) Biophys. J. 47, 57a
11 Smith, J.S., Coronado, R. and Meissner, G. (1985) Nature 316, 446–449
12 Katz, B. and Miledi, R. (1967) J. Physiol. (Lond.) 189, 535–544
13 Llinas, R.R. (1986) Curr. Top. Membranes Transp. 22, 519–547
14 Simon, S.M. and Llinas, R.R. (1985) Biophys. J. 48, 485–498
15 Casteels, R. and Droogmans, G. (1985) in Calcium in Biological Systems (Rubin, R.P., ed.), pp. 453–457, Plenum Publishing Corp., New York
16 Baker, P.F. and Crawford, A.C. (1975) J. Physiol. (Lond.) 247, 209–226

17 Rahamimoff, R., Erulkar, S.D., Lev-Tov, A. and Meiri, H. (1978) Ann. N.Y. Acad. Sci. 307, 583
18 Erulkar, S.D. (1983) Rev. Physiol. Biochem. Pharmacol. 98, 63–175
19 Adams, D.J., Takeda, K. and Umbach, J.A. (1985) J. Physiol. (Lond.) 369, 145–159
20 Tien, H.T. (1985) Progress in Surface Science (Davison, S.G., ed.), pp. 169–274, Pergamon Press, New York
21 Tien, H.T. (1974) Bilayer Lipid Membranes (BLM); Theory and Practice, 655 pp., Marcel Dekker, New York
22 Akabas, M.H., Cohen, F.C. and Finkelstein, A. (1984) J. Cell Biol. 98, 1063–1071
23 Ehrlich, B.E., Finkelstein, A., Forte, M. and Kung, C. (1984) Science 225, 427–428
24 Nelson, M.T., French, R.J. and Krueger, B.K. (1984) Nature 308, 77–80
25 Vassilev, P.M. and Tien, H.T. (1985) in Structure and Properties of Cell Membranes, Vol. 3, (Benga, G., ed.), pp. 63–101, CRC Press Boca Raton
26 Reuter, H., Stevens, C.F., Tsien, R.W. and Yellen, G. (1982) Nature 297, 501–504
27 Nowicky, M.C., Fox, A.P. and Tsien, R.W. (1985) Nature 316, 440–443
28 Nilius, B., Hess, P., Lansman, J.B. and Tsien, R.W. (1985) Nature 316, 443–446
29 Lux, H.D. and Brown, A.M. (1984) Science 225, 432–434
31 Rosenberg, R.L., Hess, P., Reeves, J.P., Smilowitz, H. and Tsien, R.W. (1986) Science 231, 1564–1566
32 Ehrlich, B.E., Schen, C.S., Garcia, M.L. and Kaczorowski, G.J. (1986) Proc. Natl. Acad. Sci. USA 83, 193–197
33 Affolter, H. and Coronado, R. (1986) Biophys. J. 49, 767
34 Butler, M. and Morell, P. (1982) J. Neurochem. 39, 155–164
35 Bligh, E. and Dyer, E. (1959) Can. J. Biochem. Physiol. 37, 911–917
36 Folchi-Pi, J. and Stoffyn, P.J. (1972) Ann. N.Y. Acad. Sci. 195, 86–107
37 Suarez-Isla, B.A., Wau, K., Lindstrom, T. and Montal, M. (1983) Biochemistry 22, 2319–2323
38 Coronado, R. and Latorre, R. (1983) Biophys. J. 43, 231–236
39 Hamill, O.P., Marty, A., Neher, E., Sakmann, B. and Sigworth, F. (1981) Pfluegers Arch. 391, 85–100
40 Berridge, M.J., Dawson, R.M.C., Downer, C.P., Meslop, J.P. and Irvine, R.F. (1983) Biochem. J. 212, 473–482
41 Berridge, M.J. and Irvine, R.F. (1984) Nature 312, 315–321
42 Gershengorn, M.C., Geras, E., Purrello, V.S. and Rebecchi, M.J. (1984) J. Biol. Chem. 259, 10675–10681
43 Dawson, A.P. (1985) FEBS Lett. 185, 147–150
44 Gill, D.L., Ueda, T., Chuck, S.-H. and Noel, M.W. (1986) Nature 320, 461–464