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Ion channels induced in lipid bilayers by subvirion particles of the nonenveloped mammalian reoviruses

(planar bilayers/infectious subviral particles)

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ABSTRACT **Mechanisms by which nonenveloped viruses penetrate cell membranes as an early step in infection are not well understood. Current ideas about the mode for cytosolic penetration by nonenveloped viruses include (*i*) formation of a membrane-spanning pore through which viral components enter the cell and (*ii*) local breakdown of the cellular membrane to provide direct access of infecting virus to the cell's interior. Here we report that of the three viral particles of nonenveloped mammalian reoviruses: virions, infectious subvirion particles, and cores (the last two forms generated from intact reovirus virions by proteolysis), only the infectious subvirion particles induced the formation of anion-selective, multisized channels in planar lipid bilayers under the experimental conditions used in this study. The value for the smallest size conductance varied depending on the lipid composition of the bilayer between 90 pS (Asolectin) and 300 pS (phosphatidylethanolamine:phosphatidylserine) and was found to be voltage independent. These findings are consistent with a proposal that the proteolytically activated infectious subviral particles mediate the interaction between virus and the lipid bilayer of a cell membrane during penetration. In addition, the findings indicate that the "penetration proteins" of some enveloped and nonenveloped viruses share similarities in the way they interact with bilayers.**

Studies with animal viruses have provided insights into many features and functions of eukaryotic cells. An example is the use of the influenza virus hemagglutinin as a model for how proteins induce fusion of adjacent cellular membranes, a process central to the endocytic and exocytic activities of cells (1, 2). From the perspective of viral function, fusion represents the mechanism by which influenza and other enveloped viruses penetrate membranes to access the interior of cells for viral replication. Another class of viruses, the nonenveloped viruses, must also penetrate a cell membrane as an early step in their replication cycles but cannot use fusion as a mechanism since they lack the lipid envelope and associated proteins of enveloped viruses. Molecular mechanisms for membrane penetration by any of the nonenveloped viruses are not yet defined, but this phenomenon might be expected to provide models for how cells transport large protein or nucleoprotein complexes across membranes. Proposed mechanisms for penetration by nonenveloped viruses include (*i*) formation of a membrane-spanning pore through which viral components enter the cell and (*ii*) lysis of the adjacent cellular membrane to provide direct access of infecting virus to the cell interior (3–5).

We have undertaken studies to determine how mammalian reoviruses, prototype members of the double-stranded RNA (dsRNA) virus family Reoviridae taken as representative nonenveloped viruses, penetrate cell membranes. Mammalian reoviruses have a genome comprising 10 unique segments of dsRNA, which encode one or two viral proteins each and are encased within mature virion particles by two concentric, icosahedrally organized protein capsids. The outer capsid proteins of reoviruses include those that mediate interactions with cells during the early stages of viral infection: σ1, which binds to cell-surface receptors (6, 7); σ3, which is sensitive to proteolytic degradation and plays a role in stabilizing the outer capsid (8); and μ1, which occurs in virions primarily in cleaved form as fragments μ1N and μ1C (5) and which plays a role in determining interactions with cell membranes (9) and activation of the viral transcriptase (10, 11).

The double-capsid structure of reoviruses makes it possible for intact virion particles to be converted into two distinct types of icosahedral subvirion particles by exposure to proteases *in vitro* or *in vivo*. These subvirion particles—infectious subvirion particles (ISVPs) and cores—differ from each other and from virions primarily by lacking subsets of the outer capsid proteins. ISVPs lack σ3 because of its proteolytic degradation, contain a cleaved form of μ1C (12), and have σ1 in an altered conformation from that in virions (13); cores lack not only σ3 but also σ1 and the fragments of μ1, and they contain the core spike protein λ2 in an altered conformation different from that in both virions and ISVPs (13). Importantly, these three viral forms—virions, ISVPs, and cores—are postulated to play specific, sequential roles during the early stages of reovirus infection as follows: virions, a maximally stable infectious form, is competent for attachment to cells and allows for passage of virus between hosts and between tissues within the host; ISVPs, a proteolytically activated infectious form, remains competent for attachment but is also immediately competent for penetrating cell membranes; and cores, a more completely uncoated noninfectious form, mediates transcription, capping, and export of the 10 viral mRNAs (8). ISVPs can be generated either by extracellular proteolysis, such as in the small intestinal lumen upon infection of mice by the enteric route (14), or by proteolysis inside late endosomes or lysosomes, such as when virions are used to initiate infection of cells in culture (15).

In this paper, we report experiments designed to determine whether intact reovirus or its two types of subviral particles can interact directly with planar lipid bilayers. We found that of the three viral forms, only ISVPs induce anion permeable, multisized channels when added to a membrane in the presence of 300 mM CsCl buffered to pH 7.4 (room temperature). These findings are a direct demonstration that a

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Abbreviations: ISVPs, infectious subvirion particles; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine.

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specific form (ISVP) of a nonenveloped virus interacts with planar lipid bilayers.

EXPERIMENTAL METHODS

Preparation of Virions, ISVPs, and Cores. Virions of mammalian reovirus strain type 1 Lang (T1L) were purified from infected mouse L cells by a procedure involving sonication, Freon extraction in the presence of 0.1% sodium deoxycholate, and isolation in CsCl gradients as described (12). Virions were stored at 4°C in virion/ISVP buffer (150 mM NaCl/10 mM $MgCl_2$/10 mM Tris, pH 7.5) prior to use.

ISVPs were generated under standard conditions (12), starting with 1–1.5 × 10^{13} T1L virions per ml in virion buffer. The virions were exposed to 200 μg of α-chymotrypsin (Sigma) per ml at 37°C for 20–30 min, at which time digestion was ended by addition of phenylmethylsulfonyl fluoride (Sigma) and cooling. ISVPs were purified by isolation in CsCl density gradients (ρ, 1.25–1.45) at 5°C and then were dialyzed into virion buffer for storage at 4°C until use.

Cores were generated and purified by a similar procedure as for ISVPs, except that (*i*) T1L virions were added at an initial concentration of 5 × 10^{13} per ml, (*ii*) digestion was allowed to proceed for 3 hr, (*iii*) cores were purified by isolation in CsCl gradients (ρ, 1.25–1.45), and (*iv*) purified cores were dialyzed into core buffer (1 M NaCl/100 mM $MgCl_2$/20 mM HEPES, pH 8) for storage (16). Identification of particles as virions, ISVPs, and cores was generally confirmed by observing their characteristic complements of proteins in SDS/polyacrylamide gels.

Bilayers and Viral Particle Interaction. Lipid bilayers were formed by apposition of two lipid monolayers spread in the presence of 300 mM CsCl in 20 mM Tris/Mops, pH 7.4, from a solution of lipids dissolved in pentane at a concentration of 20 mg/ml [either asolectin or a 1:1 mixture of phosphatidylethanolamine (PtdEtn) and phosphatidylserine (PtdSer) as indicated in the text]. Recording of the steady-state current was performed with a 3900 patch-clamp amplifier (Dagan Instruments, Minneapolis), and the applied voltage corresponds to the potential applied to the cis chamber (locus of addition of viral particles) with Ag–AgCl electrodes. The voltage pulses were applied for 4 min, followed by 3 min at 0 V. Averaged conductances ⟨*G*⟩ were calculated from the mean value of the digitized currents (⟨*I*⟩) determined over a fixed period of time after the voltage pulse was applied (2.5 min) and dividing by the applied potential. Unless otherwise noted, reported results were obtained with three separate membranes, adhering to the same protocol each time. The results as reported correspond to mean values of the separate experiments, with the conductance of each membrane normalized for any difference in area by using a measured value of capacitance (directly proportional to area). Changes in CsCl concentrations were made by addition of an appropriate-sized aliquot of concentrated, buffered CsCl to each compartment, under vigorous stirring. Determinations of reversal potentials due to a concentration gradient were made after addition of an aliquot of concentrated CsCl to only one chamber.

Single-channel analysis from idealized records was done by using modified pClamp software (Axon Instruments, Burlingame, CA). Events that were <2.5 ms were excluded from histograms. The total number of events was between 2000 and 4000, representing 3–5 min of continuous recording, depending on whether openings ≥ 1000-pS conductance were present. The voltage pulse was turned off if these openings were present for a period of time > 2s, since under these conditions, the bilayers would either break or their conductance would increase severalfold without recovery.

A small volume (5–10 μl) of a suspension of viral particles (strains type 1 Lang or type 3 Dearing) were routinely added to one side of the preformed bilayer, 10–20 min after formation, in the presence of buffered 300 mM CsCl (pH 7.4). This salt was chosen since it is known that the larger monovalent cations (1a series) enhance the capacitance of reovirus ISVPs to interact with membranes in other assay systems (M.L.N., unpublished data).

RESULTS

Conductance Induced by ISVPs. To determine if any of the three forms of reovirus could interact with lipid bilayers, we tested all three forms under the same experimental conditions as described above. Our initial experiments indicated that virions, when added to the cis side of a preformed bilayer, did not change its conductance. In contrast, we found that upon addition of ISVPs, the membrane conductance changed after a lag period. The length of this period (15–90 min) varied inversely with the final concentration of ISVPs added to the bilayer (data not shown). The current response to a constant applied voltage when membranes were modified by ISVPs was found to be "step-like," characteristic of channels opening and closing. In addition, we observed that the current steps seemed to occur in "bursts" (openings separated by relatively long closed times) and were multisized (Fig. 1). This behavior is reminiscent of conductance changes accompanying the incorporation of some peptides in lipid bilayers (17, 18) as well as the cell-fusion events induced by the influenza virus hemagglutinin protein expressed in fibroblasts (19, 20). Further shown in Fig. 1 is the amplitude histogram of the current values obtained at 50 mV, fitted to four Gaussian distributions, even though other, higher values of current were also present with a much lower probability of occurrence. The values obtained for the current at the peak of the distributions ranged from 4.2 to 11.8 pA (corresponding to values of conductance ranging from 84 to 236 pS).

The increased conductance mediated by the ISVPs was further quantitated by averaging the measured current over the time that the voltage was applied. Since it is not clear whether the different sizes observed for the current jumps are derived from independent channels or from different levels of one channel, this analysis is only useful to determine voltage-dependent trends of the ensemble of openings at each volt-

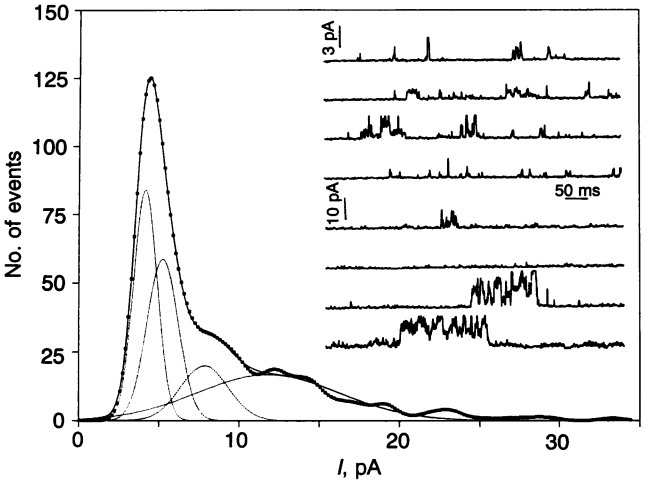

FIG. 1. Amplitude histogram of the single channels induced by ISVPs. (*Inset*) Eight consecutive traces (640 ms each) corresponding to the same voltage pulse. The upper four traces differ from the lower four in their amplitude sensitivity (3 and 10 pA, respectively). Current values were obtained at 50 mV, 40 min after an asolectin bilayer was exposed to 2 × 10^{11} ISVPs per ml. The gaussian fit to the histogram yields the four following peak currents: 4.17, 5.25, 7.84, and 11.8 pA.

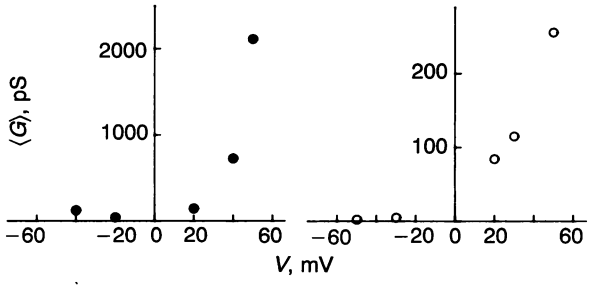

FIG. 2. Dependence of the ISVP-induced averaged conductance on lipid and voltage. The graph shows the results obtained when averaging the conductance of lipid bilayers modified by ISVP. (*Left*) Composite of results obtained in two different PtdEtn/PtdSer bilayers. (*Right*) Data obtained in three different asolectin bilayers. The line is the fit to the points using the expression in the text.

age. The mean conductance ($\langle G \rangle$), calculated from the value of the mean current passing through the bilayer for a given potential, increased an e-fold per 16-mV increase in voltage at potentials positive on the side of the bilayer to which the ISVPs were added ($G \approx \exp[neV/kT]$, where e = electronic charge; k = Boltzmann constant, and n = apparent gating charge) (Fig. 2). This yields an equivalent gating charge of 1.5, which was found to be the same in asolectin and in PtdEtn/PtdSer bilayers and thus was independent of the surface charge of the lipid membranes.

Characteristics of the Single Channels. Concentrating our analysis of the single-channel behavior to the smallest size detectable under our experimental conditions (300 mM salt, pH 7.4), we found that the conductance was independent of voltage at positive applied voltages (Fig. 3). If bilayers were made from asolectin, when the potential on the cis side was negative, the channels closed at all voltages applied. When the ISVPs were added to bilayers made from PtdEtn/PtdSer, the lowest value of the single-channel conductance was found to be higher than that in bilayers made from asolectin. Further, the probability of opening channels with a negative potential applied was nonzero, but the value of the single-channel conductance was much lower than that obtained when applying positive potentials (Fig. 3).

Effects of Salt Concentration. Addition of increasing salt concentration to both sides of bilayers exposed to ISVPs generated a set of parallel lines in a plot of log $\langle G \rangle$ vs. V (Fig. 4 *Left*). This result indicates that the dependence of conductance on voltage was not changed as the salt concentration was increased, suggesting that increases in salt concentration do not produce significant changes in the current pathway created by ISVPs in the bilayers. Further, increasing salt concentration also resulted in an increase in the single-channel conductance, g, with the same dependence on salt concentration as that found for the mean conductance, $\langle G \rangle$ (at a fixed voltage) (compare Fig. 4 *Right*). From the data shown in this figure, it is possible to infer that the relationship between conductance and salt concentration is $G \approx [CsCl]^{3.5}$. This finding could indicate that there is a salt-concentration-dependent adsorption step for viral components to interact with the bilayers (21), a salt-concentration-dependent aggregation step for viral components associating within the bilayers to form the pores, or a combination of both. At present we have no direct evidence to support one or another of these possibilities.

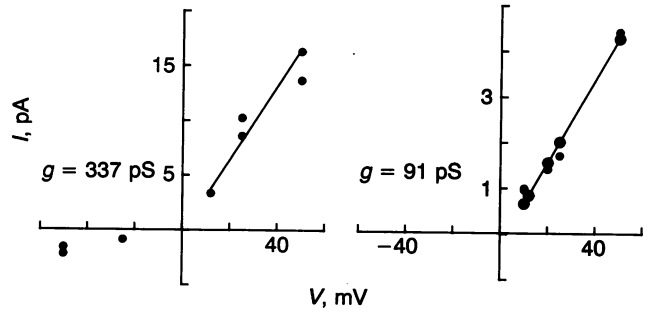

FIG. 3. Current–voltage curves of the single channels induced by ISVPs. The first peak of the gaussian fit is plotted vs. the voltage when PtdEtn/PtdSer bilayers (*Left*) or asolectin membranes (*Right*) were exposed to ISVPs. The points correspond to mean values obtained in at least three separate experiments. The lines correspond to the least-squares fit to the experimental points.

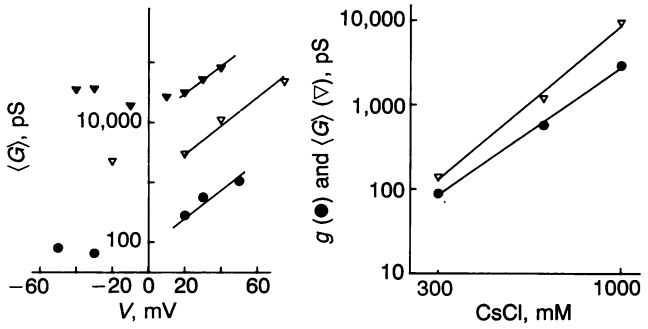

FIG. 4. Dependence of the ISVP-induced conductance on salt concentration. A representative experiment obtained in one membrane. (*Left*) $\langle G \rangle$ vs. V curve at various salt concentrations: 300 mM (●), 600 mM (▽), and 1000 mM (▼). After the membrane was formed in the presence of 300 mM CsCl and ISVP was added, each of the curves was generated by measuring current at various potentials for each salt concentration tested. (*Right*) Single-channel conductance (g) and $\langle G \rangle$ (at 10 mV) as a function of the salt concentration. The single-channel conductance was found to be independent of voltage at the salt concentrations tested. The lines correspond to the least-squares fit to the points in the graph.

Ionic Selectivity. The ionic selectivity of the channels was determined through measurements of the reversal potential established by a salt concentration gradient across the bilayer. The value of this potential, −15 mV for a 2:1 concentration gradient (higher salt on the trans side; see Fig. 5) indicates that the permeability to Cl^- relative to that of Cs^+ is ≈20. Thus, the channels formed in bilayers after interaction with ISVPs are more permeable to anions than to cations.

Other Viral Particles. We were able to document the formation of channels with three different preparations of

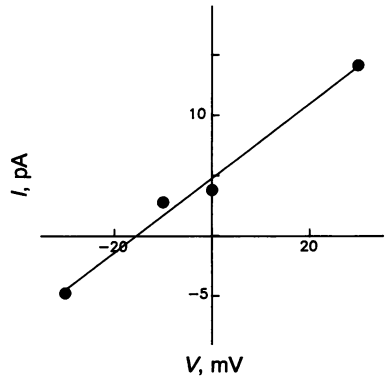

FIG. 5. Determination of the reversal potential. The asolectin bilayer was formed in the presence of 300 mM CsCl and ISVP added. After establishment of the conductance, the CsCl concentration in the trans compartment was increased to 600 mM. The single channel current was obtained from histograms such as the one shown in Fig. 1 and is plotted as a function of the applied voltage. The line through the points corresponds to the least square fit and yields a value of (−15 ± 3) mV for the reversal potential.

T1L ISVPs, representing two different clonal isolates of this strain, as well as when using a chymotrypsin digest containing ISVPs of a second reovirus strain, type 3 Dearing, which belongs to another of the three reovirus serotypes. This suggests that the channel-inducing phenotype is a property shared by the ISVPs of many or all reovirus strains. A fourth preparation of T1L ISVPs representing a third clonal isolate showed no activity, for reasons which we do not now understand. In contrast, three preparations of T1L virions and one preparation of T1L cores showed no channel-inducing activity under the experimental conditions routinely used with ISVPs. The lack of activity with virions and cores was found even when high particle concentrations and voltages were used and when particles were maintained in contact with the bilayer for long periods of time (up to 4 hr; data not shown). Furthermore, these negative results served as an internal control for the possibility of proteolytic products with channel activity in the ISVP preparations, since cores were obtained from virions by using a procedure similar to that for ISVPs. Thus, under the experimental conditions of our experiments, the capacity to induce channels is limited to the ISVP form of reovirus.

DISCUSSION

The data presented in this paper show that an infectious form of a nonenveloped virus, the mammalian reovirus of the family Reoviridae, interacts with planar lipid membranes and promotes the formation of multisized channels. This finding is consistent with related studies showing that ISVPs, but not virions or cores, mediate release of ^{51}Cr from L cells (9) and of hemoglobin from erythrocytes exposed to high concentrations of these particles (M.L.N., unpublished data). These findings support a model for the early steps in reovirus infection in which virions, while capable of attaching to and being taken up into cells by endocytosis, must undergo proteolytic processing of outer capsid proteins $\sigma 3$ and $\mu 1C$ to generate ISVPs, or particles similar to ISVPs, which are the viral form that mediates direct interaction with a lipid bilayer. Although the molecular details of the bilayer-associated structures induced by ISVPs are not understood, data from both genetic (9) and biochemical (5, 12) studies suggest that fragments of the 76-kDa $\mu 1$ protein (encoded by the viral *M2* gene) mediate the interactions with a lipid bilayer that occur during penetration by reoviruses. A direct interaction between one or more viral components and the lipid bilayer is certainly indicated by this study. It is unclear, however, whether these viral components remain attached to ISVPs or whether they are released from particles before interacting with the bilayer.

Studies with other channel-forming proteins and peptides suggest that amphipathicity is an attribute of the regions of protein that form pores (18, 22, 23), and it has been noted recently that predicted amphipathic α-helices flank the δ-ϕ cleavage junction in the reovirus $\mu 1C$ protein (12). In addition, the $\mu 1$ protein and its fragments $\mu 1N$ and $\mu 1\delta$ are modified with a C_{14} fatty acyl (myristoyl) group at their shared amino terminus, which should be capable of inserting into membranes (5). We therefore favor a model in which portions of $\mu 1$ form the bilayer-associated structure providing conductance changes in this study. The suggested maximum dimension of channels seen here (diameter $\approx$ 3 nm, obtained from the maximum single-channel conductance) is consistent with a large structure formed by several $\mu 1$ subunits.

In summary, this paper reports that subvirion particles from representative nonenveloped viruses—mammalian reoviruses—can induce channels in planar lipid bilayers. These channels are postulated to reflect the function of a viral component(s) that is active in penetrating a cellular membrane during the early stages of reovirus infection. Restriction of channel-inducing activity to proteolytically processed ISVPs is reminiscent of a requirement for cleavage of many membrane-inserting proteins before their activities are expressed (1, 2). Similarities noted between channels induced by reovirus ISVPs and fusion pores formed by the influenza virus hemagglutinin suggest the existence of fundamental mechanistic similarities between the processes of penetration by some enveloped and nonenveloped viruses.

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1. Stegmann, T., Doms, R. W. & Helenius, A. (1989) *Annu. Rev. Biophys. Biophys. Chem.* **18,** 187–211.
2. White, J. M. (1990) *Annu. Rev. Physiol.* **52,** 675–697.
3. Pastan, I., Seth, P., Fitzgerald, D. & Willingham, M. (1986) in *Concepts in Viral Pathogenesis II*, eds. Notkins, A. & Oldstone, M. (Springer, New York), pp. 141–146.
4. Fricks, C. E. & Hogle, J. M. (1990) *J. Virol.* **64,** 1934–1945.
5. Nibert, M. L., Schiff, L. A. & Fields, B. N. (1991) *J. Virol.* **65,** 1960–1967.
6. Weiner, H. L., Ault, K. A. & Fields, B. N. (1980) *J. Immunol.* **124,** 2143–2148.
7. Lee, P. W. K., Hayes, E. C. & Joklik, W. K. (1981) *Virology* **108,** 156–163.
8. Nibert, M. L., Furlong, D. B. & Fields, B. N. (1991) *J. Clin. Invest.* **88,** 727–734.
9. Lucia-Jandris, P. A., Hooper, J. & Fields, B. N. (1993) *J. Virol.* **67,** 5339–5345.
10. Joklik, W. K. (1972) *Virology* **49,** 700–715.
11. Drayna, D. & Fields, B. N. (1982) *J. Virol.* **41,** 110–118.
12. Nibert, M. L. & Fields, B. N. (1992) *J. Virol.* **66,** 6408–6418.
13. Dryden, K. A., Wang, G., Yaeger, M., Nibert, M. L., Coombs, K. M., Furlong, D. B., Fields, B. N. & Baker, T. S. (1993) *J. Cell Biol.* **122,** 1023–1041.
14. Bodkin, D. K., Nibert, M. L. & Fields, B. N. (1989) *J. Virol.* **63,** 4676–4681.
15. Sturzenbecker, L. J., Nibert, M. L., Furlong, D. B. & Fields, B. N. (1987) *J. Virol.* **61,** 2351–2361.
16. Coombs, K. M., Fields, B. N. & Harrison, S. C. (1990) *J. Mol. Biol.* **215,** 1–5.
17. Tosteson, M. T., Alvarez, O. & Tosteson, D. C. (1985) *Regul. Pept. Suppl.* **4,** 39–45.
18. Tosteson, M. T., Auld, D. S. & Tosteson, D. C. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 707–710.
19. Spruce, A. E., Iwata, A., White, J. M. & Almers, W. (1989) *Nature (London)* **342,** 555–558.
20. Spruce, A. E., Iwata, A. & Almers, W. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 3623–3627.
21. Gordon, L. G. M. & Haydon, D. A. (1976) *Biochim. Biophys. Acta* **436,** 541–556.
22. Menestrina, F., Voges, K. P., Jung, G. & Boheim, G. (1986) *J. Membr. Biol.* **93,** 111–128.
23. Tosteson, M. T., Caulfield, M. P., Levy, J. J., Rosenblatt, M. & Tosteson, D. C. (1988) *Biosci. Rep.* **8,** 173–183.