A METHOD FOR THE RAPID EXCHANGE OF SOLUTIONS BATHING EXCISED MEMBRANE PATCHES

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ABSTRACT In this communication we describe a technique for rapidly exchanging solutions bathing excised membrane patches, and present examples of its implementation using both outside-out and inside-out patches. The ability to make step changes in the concentration of channel-activating ligands (e.g., acetylcholine, calcium) offers a novel and direct means of measuring kinetic processes in the 10–100-ms range. The responses to step ligand concentration changes are well suited to ensemble variance analysis, yielding estimates of the number of channels in a patch, and testing assumptions of channel independence and homogeneity. Kinetic analysis of the pseudomacroscopic currents obtained by averaging large numbers of responses can be compared and correlated with analysis of the microscopic behavior of single channels, using the same membrane patch for both approaches. Practical and theoretical limitations associated with the method are briefly discussed.

INTRODUCTION

Since the introduction in 1981 of high-resolution techniques for recording the state transitions of single ion channels in patches of cell membranes (Hamill et al., 1981), the opportunity to analyze the kinetics of voltage-gated channels at the molecular level has been seized in a number of laboratories, sometimes with strikingly novel results. Although a similar opportunity, in principle, exists for the kinetic analysis of ligand-gated channels, direct measurement of their time- and concentration-dependent open probability has been seriously hampered by the technical difficulty of rapidly changing the solutions bathing membrane patches. Here we present a conceptually simple method that allows the solutions perfusing excised patches to be exchanged within 5–15 ms, and describe two applications currently being pursued in our laboratories.

METHODS

The essential feature of the perfusion apparatus, which has existed in a variety of slightly different configurations (Dilger and Adams, 1984; Brett et al., 1984), is a Y-shaped piece of plumbing consisting of two inflow tubes and a single outflow tube, immersed in a tissue culture dish (Fig. 1). A small hole in the outflow tube, several millimeters downstream from the junction of the inflow tubes, admits the tip of a patch pipette to a depth of about half the tubing diameter. Alternatively, the outflow tube may be cut on a bevel and angled upward (Fig. 1, *lower inset*), so that a pipette can be lowered midway into the stream directly through the open end. The solution level in the culture/recording dish is then controlled by a siphon. The former arrangement minimizes contamination of the dish with agonist solution; the latter is technically simpler and seems to allow longer survival of excised patches. Once positioned in the center of the flowing solution, patches often will tolerate a large number (e.g., several hundred) of timed switches between control and experimental solutions, triggered by a solenoid-driven pinch valve (part number 225P09; Neptune Research, Inc., Maplewood, NJ). For construction of the perfusion apparatus, we used Micro-Line tubing (internal diameter 1.27 mm; Cole Parmer, Chicago, IL), which can be readily altered using a scalpel and a heated filament, but other materials may be equally satisfactory. The solution flow rate was typically ~0.2 ml/s, resulting in a linear speed of ~16 cm/s.

The performance of the perfusion system was assessed by switching the solution bathing an open patch pipette from 150 to 75 mM NaCl. The resulting change in the electrode junction potential caused a sudden change in the current measured by the patch-clamp amplifier. With proper adjustment of the flow rate and pipette tip position, this current jump was complete within 1–2 ms.

Clonal BC3H1 cells were cultured and prepared for patch-clamp recording using the methods described by Sine and Taylor (1979) and Sine and Steinbach (1983). The result shown here was obtained during study of cells that had been maintained for 3 d in medium containing 0.5% fetal calf serum to inhibit cell division and promote differentiation. Extracellular solutions (pH 7.3) contained the designated concentration of acetylcholine as agonist and the following concentrations of salts in millimolars: 150 NaCl, 5.6 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES. An artificial intracellular solution (pH 7.3) containing 140 KCl, 5 $MgCl_2$, 5 EGTA, and 10 HEPES was used to fill the patch pipette.

Primary cultures of neonatal rat hippocampal cells were prepared using methods similar to those of Deborah Barnes (personal communication; see also Barnes and Dichter, 1984). Briefly, tissue from the brain of rat pups (gestational day 17) was dissociated by trituration and cells plated on glass coverslips at a density of 60,000 per 35-mm dish. Enzymes were not used. Intracellular solutions (i.e., those perfusing the cytoplasmic surface of the excised patch) contained the indicated (Fig. 3) concentrations of free calcium, using the concentrations of total calcium and EGTA specified by Barrett et al. (1982), and 140 or 70 KCl, 0 or 70 NaCl, 1

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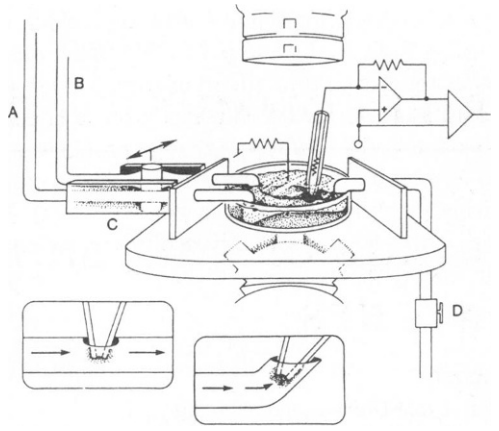

FIGURE 1 An illustration of the rapid perfusion apparatus used to change solutions bathing excised patches of membrane; its operation is described in the text. *A* and *B* are the inflow tubes that are connected to separate reservoirs containing either control or experimental solutions. The position of a solenoid-driven pinch valve *C*, triggered by the analog output of the computer, determines which solution flows past the patch. Valve *D* controls the rate of flow through the outflow tube. The inset on the lower left is an expanded view of the position of the patch pipette in the tubing while the other inset shows an alternative configuration (see text).

$MgCl_2$ and 10 HEPES. The pipette contained 140 KCl, 10 NaCl, 2 $MgCl_2$, 1 EGTA and 25 HEPES. Membrane currents were amplified using a patch-clamp amplifier (model EPC-7; List Electronic, Darmstadt, FRG), and either digitized on line using a Concept 2000 analog interface and stored by a minicomputer (model PDP-11/23; Digital Equipment Corp., Maynard, MA) on a Winchester disc drive, or recorded initially on FM magnetic tape and later digitized using the same system. The current signal was filtered at 2,000 Hz before acquisition or sampling at 200 (acetylcholine data) or 732 (calcium data) μs per point. All experiments were performed at room temperature.

RESULTS

Fig. 2 presents examples of current records showing the activation of nicotinic acetylcholine receptors by consecutive 200-ms applications of 3 μM acetylcholine (*left*) or 100 μM acetylcholine (*right*), followed by the ensemble means of 20 (*left*) or 12 (*right*) such sweeps. The membrane patch was excised in the outside-out configuration from BC3H1 cells at room temperature. Nonstationary ensemble variance analysis (Sigworth, 1980, 1984), of 20–60 sweeps at various concentrations of acetylcholine indicated the presence of 44 activatable channels in this patch. A rapid component of the desensitization process, described approximately by a first-order rate constant of

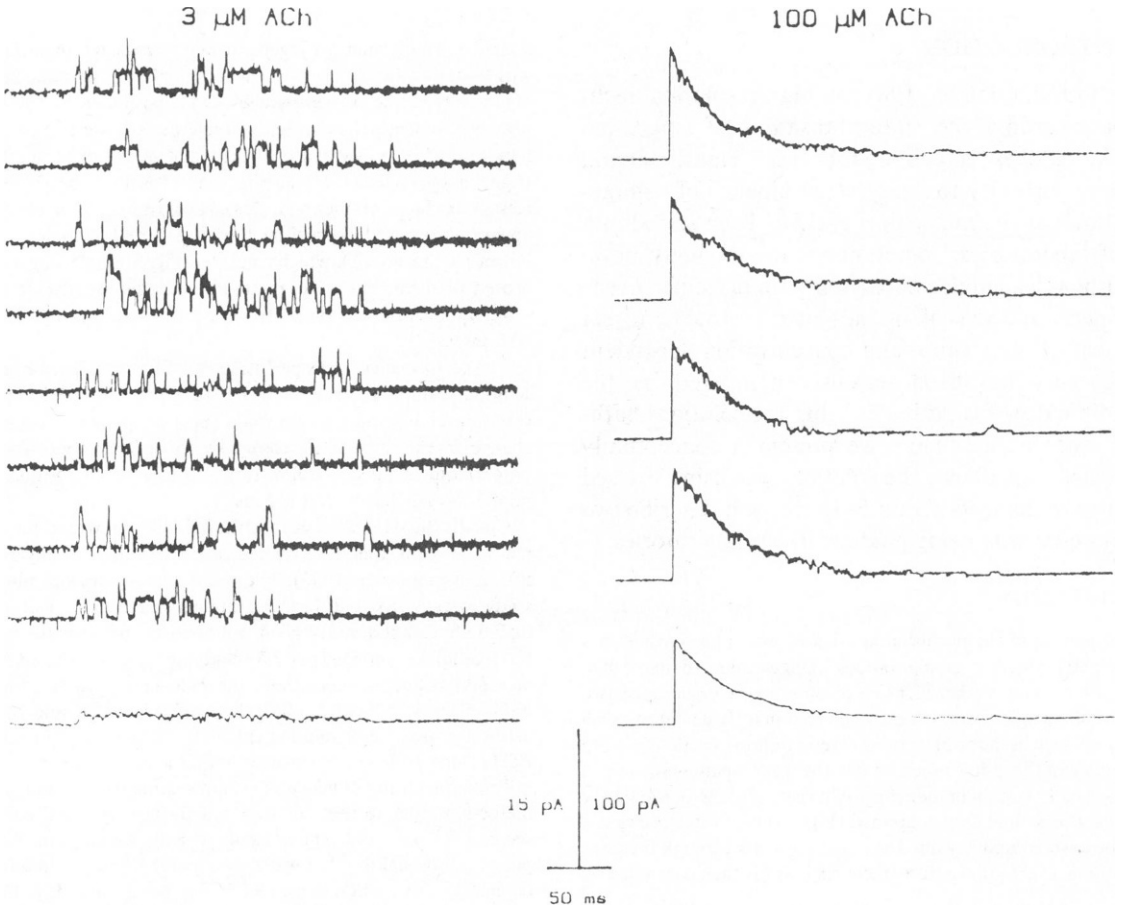

FIGURE 2 Representative current traces obtained when an outside-out patch of membrane from BC3H1 cells was rapidly perfused with 3 μM (*left*) or 100 μM (*right*) acetylcholine. The individual records at each concentration are followed by the ensemble means of 20 (*left*) or 12 (*right*) records. The holding potential was −50 mV.

50 ms, was observed at high concentrations (Dilger, J. P., R. S. Brett, and P. R. Adams, manuscript in preparation [a fuller account of these findings]). At low concentrations, the peak current response was not obscured by the onset of desensitization, and it was possible to set limits on the time required for the effective solution exchange near the membrane. This time was somewhat variable from experiment to experiment, depending on the velocity of solution flow and the configuration of the perfusion apparatus (see Fig. 1, *insets*) and approached 4–6 ms under optimal conditions. A limit in this range is likely to be imposed by diffusion of the ligand through an unstirred layer adjacent to the membrane surface. Following rupture or deterioration of the patch, the solution-exchange profile was usually assessed by making step changes in the NaCl concentration flowing past the open pipette (see Methods). When a slow (i.e., >10–15 ms) effective solution exchange was apparent during the experiment, the post-facto assessment

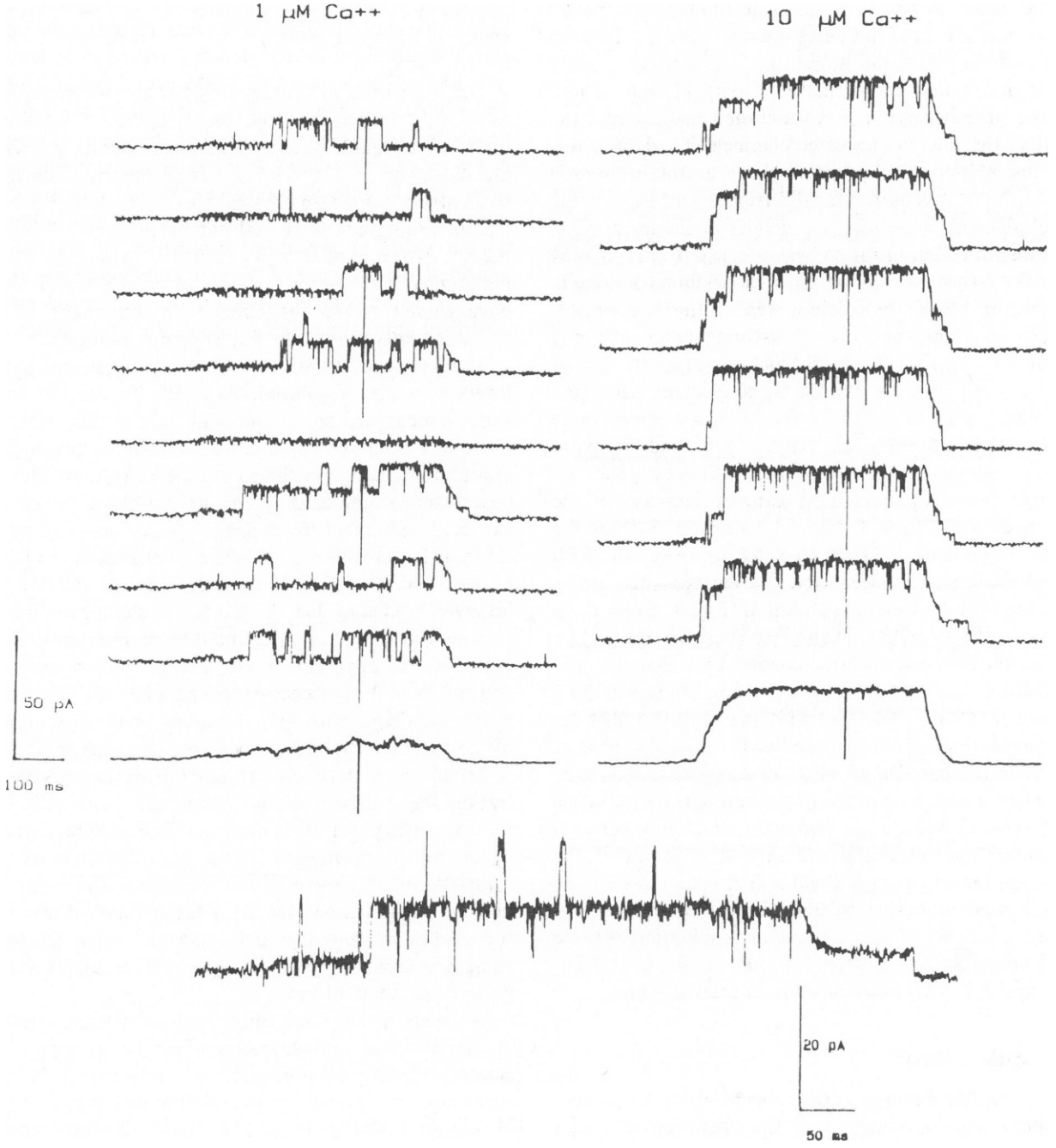

FIGURE 3 Representative current traces obtained when an inside-out patch of membrane from cultured hippocampal cells was rapidly perfused with 1 μM (*left*) or 10 μM (*right*) calcium. The columns of individual records at each concentration are followed by the ensemble means of 16 (*left*) or 25 (*right*) records. The holding potential was +50 mV. The bottom shows a sweep similar to those on the left, at higher gain.

often revealed irregularities in the solution flow after a concentration step, which could sometimes be corrected by a minor adjustment of the pipette's position.

Fig. 3 illustrates application of the rapid perfusion method to the study of calcium-activated potassium channels in cultured hippocampal neurons. The membrane patch was excised in the inside-out configuration and perfused with a nominally calcium-free Ringer's solution (see Methods). Steps in the calcium concentration from 0 to 1 μM (*left*) or 10 μM (*right*) caused the opening, after a variable delay, of one to three large-conductance potassium channels. Part of this delay is contributed by the dead volume of the perfusion apparatus, and can be readily measured; to a first approximation, it should be no longer than the time between the valve-closing artifact and the closure of the calcium-activated channels. To identify this delay unambiguously, and particularly to obtain an estimate of the time required for diffusional exchange of the two solutions in the immediate vicinity of the membrane, the potassium concentration was stepped from 70 mM (with 70 mM sodium) to 140 mM (no sodium) and back, simultaneously with the calcium step. In this experiment, the fortuitous presence of a low-conductance (50 pS) potassium channel in the patch, flickering rapidly on and off without any obvious relationship to calcium, permitted direct observation of the solution exchange as an increase in unit current amplitudes. At the gain and temporal resolution shown in the top, the diffusional solution exchange shows up only as a distinct increase in the apparent baseline noise, followed by a decrease where the solution is exchanged again (see, for example, the fifth trace on the left). The rate of the solution exchange can be seen more clearly at higher resolution (Fig. 3, *bottom*). In this sweep, the solution exchange offset occurred during a long opening of one of the large-conductance channels, and the resulting decrease in its current amplitude was 90% complete in 15 ms. The fact that the apparent solution exchange occurs somewhat more slowly for inside-out than for outside-out patches (cf. Fig. 1) suggests that factors related to the geometry of the membrane surface influence the effective diffusion rate. The additional delay between the solution exchange and the earliest openings of the calcium-activated channels could reflect a step (or steps) in the activation process, slow diffusion of calcium in the immediate vicinity of the patch, or a combination of these factors (Brett and Lancaster, 1985; Brett, R. S., B. Lancaster, and J. P. Dilger, manuscript in preparation).

DISCUSSION

Fleeting, steplike changes in the concentration of a neurotransmitter are fundamental to the operation of many vertebrate synapses. In the vertebrate neuromuscular junction, the acetylcholine concentration rises from near zero to the millimolar range within a few hundred microseconds after depolarization of the nerve terminal, and falls as rapidly again to its resting level (Land et al., 1980). Similarly, transient changes in the concentration of neuronal intracellular second messengers mediate a wide variety of modulatory currents, although there is considerable uncertainty in estimates of their magnitude and time course. The concentration of calcium in the space immediately adjacent to the internal membrane surface probably varies between 10^{-7} and 10^{-3} in a variety of cell types (Smith and Zucker, 1980; Ahmed and Connor, 1979; Meldolesi et al., 1984; Augustine et al., 1985) perhaps changing as rapidly as 0.5×10^{-3} mol/l per s (Simon and Llinas, 1985).

Our experimental strategy reflects the wish to duplicate, as closely as possible, the physiological circumstances under which ligand-gated ion channels operate (cf. Kuffler and Yoshikami, 1975) while retaining control of the ligand concentration. Certain physical constraints limit the extent to which this goal can be realized, particularly in regard to the effective rate of solution exchange. Although we have not studied each factor systematically, the experience we have gained during the effort to optimize the solution exchange rate permits the following observations.

The intrinsic fragility of excised membrane patches limits the rate of solution flow. We found a dramatic improvement in patch survival when this rate was decreased from 160 to 16 cm/s, without, surprisingly, an appreciable change in the solution exchange profile. This observation suggests that, in our plumbing system, the expected decrement in exchange speed due to a low flow rate was largely offset by diminished mixing at the juntion of the two inflow tubes. Further decreases in the flow rate, however, tended to slow the solution exchange profile.

At a constant flow rate, there were systematic differences in the apparent speed of the solution exchange, depending on the presence or absence of a membrane patch at the micropipette tip, and, if present, on the configuration of the patch. With optimal positioning, the current signal recorded from a bare micropipette during an ionic concentration step was essentially rectangular, with rising and falling phases of ~1 ms in duration. The solution exchange in the immediate vicinity of outside-out patches, assessed using the BC3H1 membrane's response to low concentrations of acetylcholine, was never faster than ~4 ms and in the case of inside-out patches, assessed using the hippocampal neuronal membrane's response to calcium, it was never faster than ~15 ms.

We suspect that these differences arise from variations in the thickness and geometry of unstirred layers associated with the micropipette tip, and that such layers constitute an important practical limit on achievable rates of solution exchange in experiments of this kind. Given a diffusion coefficient (D) for acetylcholine of 6×10^{-6} cm^2/s (Dionne, 1976) and our fastest observed response time (using outside-out BC3H1 membrane patches) of ~5

ms, the Einstein relation $r^2 = 2Dt$ predicts an unstirred layer thickness of 2.4 μm. As a comparison, we note that estimates of the unstirred layer thickness around human erythrocytes exposed to a constant, high velocity, turbulent flow of solution range between 1.7 and 15 μm (Brahm, 1983). The fact that our estimate of unstirred layer thickness around a 1–2-μm diameter patch of membrane is of the same magnitude, even though the flow in our perfusion tubing is slower and probably not turbulent[1] suggests that diffusion is likely to be the rate limiting process.

Several previous techniques for making solution changes during patch clamp recording have been described in the literature. These systems effect concentration changes on the time scale of a few seconds (Yellen, 1982), 100 ms (Krishtal and Pidoplichko, 1980; Fenwick et al., 1982) and 10 ms (Akaike and Oomura, 1984). The first two techniques are suitable for certain applications including whole-cell recording (which is not feasible with our current perfusion system), but are not fast enough for the type of experiments outlined here. The details of the third method have not yet been published, so it is impossible to make a comparison of that method with ours. Our perfusion apparatus also bears some resemblance to a system used to perfuse single nodes of Ranvier (Stampfli, 1959; Vierhaus and Ulbricht, 1971) in that both systems employ a piece of tubing with a slit or hole to admit the preparation, and a stopcock or valve to effect changes in the solutions bathing the preparation.

The theoretical appeal of arrangements that would allow kinetic measurements to be made at both the microscopic and macroscopic levels in the same preparation has been previously pointed out (Patlak and Horn, 1982). The method described here meets this requirement, although it does so at the expense of a technically demanding experimental approach. In its current state the technique offers a useful approximation to the conceptually ideal experiment, in which instantaneous ligand concentration steps are applied to a membrane under perfect voltage clamp. Its limitations are essentially those of excised-patch recording per se: the effects of isolation from the cell, of cytoplasmic elements that may remain associated with the membrane, and of patch geometry on channel function and on local diffusion are largely unknown. Although these factors require further investigation, the technique of rapid solution exchange described here should be readily applicable to a wide range of problems in the study of ligand-gated ion channels.

This work was supported by the National Institutes of Health (grants R23 NS21581 and NS18796, to J. P. Dilger and P. R. Adams, respectively), the Foundation of the University of Medicine and Dentistry of New Jersey (a grant to J. P. Dilger), the Wellcome Trust (a travel grant to B. Lancaster), the American Society of Anesthesiologists and the International Anesthesia Research Society (grants to R. S. Brett).

Received for publication 24 February 1986 and in final form 3 June 1986.

[1]Calculation of the Reynolds Number for the conditions of these experiments gives a value of 200, indicating laminar flow, although the presence of the patch electrode may introduce local turbulence.

REFERENCES

Ahmed, Z., and J. A. Connor. 1979. Measurement of Ca^{++} influx under voltage clamp in molluscan neurons using the metallochromic dye Arsenazo III. *J. Physiol. (Lond.).* 286:61–82.

Akaike, N., and Y. Oomura. 1984. GABA activates three types of the receptor-ionophore complex with their relative distribution. *Soc. Neurosci.* 10: 643.

Augustine, G. J., M. P. Charleton, and S. J. Smith. 1985. Calcium entry into voltage-clamped presynaptic terminals of squid. *J. Physiol. (Lond.).* 367:143–162.

Barnes, D. M., and M. A. Dichter. 1984. Development of inhibitory mechanisms in dissociated cultures of rat hippocampus. *Soc. Neurosci.* 10:419.

Barrett, J. N., K. L. Magleby, and B. S. Pallotta. 1982. Properties of single calcium-activated potassium channels in cultured rat muscle. *J. Physiol. (Lond.).* 331:211–230.

Brahm, J. 1983. Permeability of human red cells to a homologous series of aliphatic alcohols. *J. Gen. Physiol.* 81:283–304.

Brett, R. S., J. P. Dilger, and P. R. Adams. 1984. Improved "concentration clamp" for use with membrane patches. *Soc. Neurosci.* 10:241.

Brett, R. S., and B. Lancaster. 1985. Activation of potassium channels by rapid application of calcium to inside-out patches of cultured hippocampal neurons. *Soc. Neurosci.* 11:954.

Dilger, J. P., and P. R. Adams. 1984. Rapid perfuson of excised patches — activation and desensitization of nicotinic receptor channels. *Biophys. J.* 45(2, Pt. 2):386*a*. (Abstr.)

Dionne, V. E. 1976. Characterization of drug iontophoresis with a fast microassay technique. *Biophys. J.* 16:705–717.

Fenwick, E. M., A. Marty, and E. Neher. 1982. A patch-clamp study of bovine chromaffin cells and of their sensitivity to acetylcholine. *J. Physiol. (Lond.).* 331:599–635.

Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. 1981. Improved patch-clamp technique for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* 391:85–100.

Krishtal, O. A., and V. I. Pidoplichko. 1980. A receptor for protons in the nerve cell membrane. *Neuroscience.* 5:2325–2327.

Kuffler, S. W., and D. Yoshikami. 1975. The distribution of acetylcholine sensitivity at the post-synaptic membrane of vertebrate skeletal twitch muscles: iontophoretic mapping in the micron range. *J. Physiol. (Lond.).* 244:703–730.

Land, B. R., E. E. Salpeter, and M. M. Salpeter. 1980. Acetylcholine receptor site density affects the rising phase of miniature endplate currents. *Proc. Natl. Acad. Sci. USA.* 77:3736–3740.

Meldolesi, J., W. B., Huttner, R. Y. Tsien, and T. Pozzan. 1984. Free cytoplasmic Ca^{++} and neurotransmitter release: studies on PC12 cells and synaptosomes exposed to alpha-latrotoxin. *Proc. Natl. Acad. Sci. USA.* 81:620–624.

Patlak, J., and R. Horn. 1982. Effect of *N*-bromoacetamide on single sodium channel currents in excised membrane patches. *J. Gen. Physiol.* 79:333–351.

Sigworth, F. J. 1980. The variance of sodium current fluctuations at the node of Ranvier. *J. Physiol. (Lond.).* 307:97–129.

Sigworth, F. J. 1984. Nonstationary noise analysis of membrane currents. *In* Membranes, Channels and Noise. R. S. Eisenberg, M. Frank, and C. F. Stevens, editors. Plenum Publishing Corp., New York. 21–48.

Simon, S. M., and R. R. Llinas. 1985. Compartmentalization of the submembrane calcium activity during calcium influx and its significance in transmitter release. *Biophys. J.* 48:485–498.

Sine, S. M., and P. Taylor. 1979. Functional consequences of agonist-mediated state transitions in the cholinergic receptor. *J. Biol. Chem.* 254:3315–3325.

Sine, S. M., and J. H. Steinbach. 1984. Activation of a nicotinic acetylcholine receptor. *Biophys. J.* 45:175–185.

Smith, S. J., and R. S. Zucker. 1980. Aequorin response facilitation and intracellular calcium accumulation in molluscan neurons. *J. Physiol. (Lond.).* 300:167–196.

Stampfli, R. 1959. Is the resting potential of Ranvier nodes a potassium potential? *Ann. NY Acad. Sci.* 81:265–284.

Vierhaus, J., and W. Ulbricht. 1971. Effect of a sudden change in sodium concentration on repetitively evoked action potentials of single nodes of Ranvier. *Pfluegers Arch.* 326:76–87.

Yellen, G. 1982. Single Ca^{++}-activated nonselective cation channels in neuroblastoma. *Nature (Lond.).* 296:357–359.