control (Fig. 2*a*) and is associated with a smaller Ca^{2+} transient. (Differences in the Ca^{2+} transients are exaggerated by the non-linear relationship between $[Ca^{2+}]$ and light output for aequorin.)

Possible explanations for this shortening of the action potential include: (1) indirect actions of caffeine, through inhibition of phosphodiesterase[22], either to reduce the number of activatable calcium channels or to increase the calcium sensitivity of Ca^{2+}-activated K^+ channels[23]; (2) a direct action of the elevated $[Ca^{2+}]_i$ to inactivate calcium channels[24,25]. Although we have not directly tested any of these possibilities, in most experiments following the caffeine-induced rise, $[Ca^{2+}]_i$ fell to below the limit of detection ($\simeq 5\times10^{-7}$ M) well before the action potential regained its control duration. Furthermore, in another series of experiments 0.1 mM isobutylmethyl xanthine (IBMX), a phosphodiesterase inhibitor[22], caused a shortening of the action potential of DRG neurones without apparently increasing $[Ca^{2+}]_i$. Interestingly, in an unidentified neurone from the abdominal ganglion of *Aplysia*, IBMX prolongs the action potential[26]. Since there is no evidence that IBMX releases Ca^{2+} from microsomes it seems that an inhibition of phosphodiesterase might underlie the effect of caffeine on action potential duration in these conditions. Figure 2*d* shows that the caffeine effect is reversible.

Figure 3 shows how the caffeine-sensitive intracellular store, once discharged, can be reprimed following electrical activation of the cell. The top traces show that, after an initial prolonged exposure of a neurone to 10 mM caffeine, a subsequent exposure causes negligible Ca^{2+} release from the store. Note also that when there is no release of calcium, there is no membrane depolarization or decrease in input resistance as previously observed. The next traces, contiguous with the last, show that following three action potentials, each associated with significant Ca^{2+} influx, caffeine once more causes an elevation of $[Ca^{2+}]_i$ and associated changes in membrane potential and input resistance. We suggest that caffeine releases Ca^{2+} from an intracellular store which can be replenished by Ca^{2+} moving into the cell during an action potential. That this repriming phenomenon is related to inwardly-moving Ca^{2+} associated with voltage activation and is not simply a function of time to allow equilibration of intracellular calcium stores or influx of Ca^{2+} through a 'leak', is shown in the next contiguous record. The preparation remained quiescent for a period of time similar to that over which the three previous action potentials had been elicited. Quite clearly, following this protocol, caffeine was unable to elicit any response. Caffeine was again tested after a single action potential had been elicited. There was a small but detectable increase in the output of light, indicating that a significant contribution of calcium to the intracellular store is made during a single action potential in these conditions. A further three action potentials once more returned the level of intracellular Ca^{2+} release by caffeine to around control levels.

The results described here indicate that there is rapid buffering of intracellular Ca^{2+} in rat DRG neurones. The calcium transient associated with an action potential declined with a time constant of 100–200 ms from a peak concentration considerably lower than that predicted from previously reported voltage-clamp experiments in chick DRG neurones[12]. While the relative contributions of intracellular Ca^{2+} buffering, active pumping and sodium–calcium exchange to the maintenance of a low $[Ca^{2+}]_i$ remain unclear, there can be little doubt that these mechanisms could all play a part in the regulation of $[Ca^{2+}]_i$ (ref. 27). From the observation that caffeine causes a release of Ca^{2+} from intracellular stores and that these stores are refilled during electrical activity of the neurone, we suggest that the endoplasmic reticulum in mammalian neurones can have an important role in the control of $[Ca^{2+}]_i$ similar to the role of the sarcoplasmic reticulum in muscle.

We thank the British Council, the Nuffield Foundation and the Constantine bequest for travel grants to I.R.N. We also thank G. Storey and P. Hutchinson for technical assistance and Dr B. E. Argent for reading the manuscript.

Received 13 October 1983; accepted 13 February 1984.

1. Blinks, J. R., Rudel, R. & Taylor, S. R. *J. Physiol., Lond.* **277,** 291–323 (1978).
2. Neering, I. R. & Morgan, K. G. *Nature* **288,** 585–587 (1980).
3. Ashley, C. C. & Ridgway, E. B. *J. Physiol., Lond.* **209,** 105–130 (1970).
4. Katz, B. *The Release of Neural Transmitter Substance* (Liverpool University Press, 1960).
5. Baker, P. F. in *Recent Advances in Physiology* (ed. Linden, R. J.) 51–86 (Churchill Livingstone, London, 1974).
6. Meech, R. W. *A. Rev. Biophys. Bioengng* **7,** 1–18 (1978).
7. Schwarz, W. & Passow, H. *A. Rev. Physiol.* **45,** 359–374 (1983).
8. Gorman, A. L. F. & Thomas, M. V. *J. Physiol., Lond.* **308,** 259–285 (1980).
9. Gorman, A. L. F. & Thomas, M. V. *J. Physiol., Lond.* **308,** 287–313 (1980).
10. Smith, S. J., MacDermott, A. B. & Weight, F. F. *Nature* **304,** 350–352 (1983).
11. Dichter, M. A. & Fischbach, G. D. *J. Physiol., Lond.* **267,** 281–298 (1977).
12. Dunlap, K. & Fischbach, G. D. *J. Physiol. Lond.* **317,** 519–535 (1981).
13. Allen, D. G. & Blinks, J. R. in *Detection and Measurement of Free Ca^{2+} in Cells* (eds Ashley, C. C. & Campbell, A. K.) 159–174 (Elsevier, Amsterdam, 1979).
14. Brinley, F. J. *A. Rev. Biophys. Bioengng* **7,** 363–392 (1978).
15. Baker, P. F. & Schlaepfer, W. W. *J. Physiol., Lond.* **276,** 103–125 (1978).
16. Tillotson, O. & Gorman, A. L. F. *Nature* **286,** 816–817 (1980).
17. Ransom, B. R., Neale, E., Henkart, M., Bullock, P. N. & Nelson, P. G. *J. Neurophysiol.* **40,** 1132–1150 (1977).
18. Fabiato, A. & Fabiato, F. *Circulation Res.* **40,** 119–129 (1977).
19. Herbette, L., Messineo, F. C. & Katz, A. M. *A. Rev. Pharmac. Tox.* **22,** 413–434 (1982).
20. Yoshida, S. & Matsuda, Y. *J. Neurophysiol.* **42,** 1134–1145 (1979).
21. Colquhoun, D., Neher, E., Reuter, H. & Stevens, C. F. *Nature* **294,** 752–754 (1981).
22. Montague, W. & Cook, J. R. *Biochem. J.* **122,** 115–120 (1971).
23. De Peyer, J., Cachelin, A. B., Levitan, I. B. & Reuter, H. *Proc. natn. Acad. Sci. U.S.A.* **79,** 4207–4211 (1982).
24. Standen, N. B. *Nature* **293,** 158–159 (1981).
25. Plant, T. D. & Standen, N. B. *J. Physiol., Lond.* **321,** 273–285 (1981).
26. Klein, M. & Kandel, E. R. *Proc. natn. Acad. Sci. U.S.A.* **75,** 3512–3516 (1978).
27. Blaustein, M. P., Ratzlaff, R. W. & Schweitzer, E. S. *Fedn Proc.* **39,** 2790–2795 (1980).
28. Alvarez-Leefmans, F. J., Gamino, S. M. & Rink, T. J. *J. Physiol., Lond.* (in the press).

Conductances of single ion channels opened by nicotinic agonists are indistinguishable

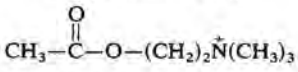
P. Gardner, D. C. Ogden & D. Colquhoun

Department of Pharmacology, University College London, Gower Street, London WC1E 6BT, UK

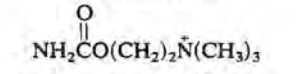
Hypotheses concerning the mechanism by which acetylcholine-like agonists cause ion channels to open often suppose that the receptor–ionophore complex can exist in either of two discrete conformations, open and shut[1–3]. On the basis of noise analysis it has been reported that certain agonists open ion channels of lower conductance than usual[4–8], though many potent agonists give similar conductances[9–13], and hence that differences in the conductance of ion channels opened by different agonists may contribute to differences in efficacy[14]. Here we have reinvestigated this question by recording single ion channel currents[15] evoked by acetylcholine-like agonists on embryonic rat muscle in tissue culture and on adult frog muscle endplate. Ten different agonists (Fig. 1) were tested, including several that noise analysis has suggested have a low conductance[4,5]. The single-channel conductance was found to be the same, within a few per cent, for all 10 agonists. It seems that noise analysis has given erroneously low conductances in some cases. Therefore efficacy differences do not depend on differences in single-channel conductances evoked by various agonists but presumably on the position of the open–shut equilibrium of the agonist–channel complexes[16].

The first set of experiments was carried out on dissociated embryonic rat muscle cells in primary tissue culture. Single ion channel currents were recorded by the patch-clamp method from cell-free patches in the 'outside-out' recording configuration, that is, with their external surface facing the bath solution[15]. Several agonists were applied to each outside-out patch. Records of single-channel currents for one outside-out patch, which had been sequentially activated by six agonists, are shown in Fig. 2*a*. The amplitudes of 30–50 unit currents were measured for each agonist at each of four or five membrane potentials, and histograms of amplitudes were plotted (Fig. 2*b*). In most cases the distributions were unimodal at each potential and could be fitted with a simple gaussian curve. However, some patches revealed a bimodal distribution of current amplitudes

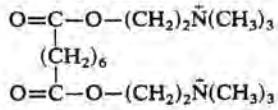
Acetylcholine (ACh)



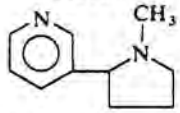
Carbachol (CCh)



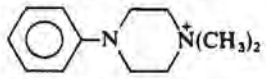
Suberyldicholine (SubCh)



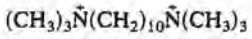
Nicotine



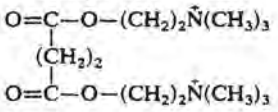
1,1-Dimethyl-4-phenylpiperazinium (DMPP)



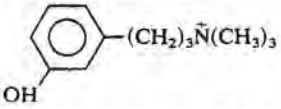
Decamethonium (Deca)



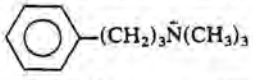
Succinylcholine (SucCh)



3-(*m*-hydroxyphenyl)propyltrimethylammonium (HPTMA)



3-Phenylpropyltrimethylammonium (PPTMA)



3-Phenylethyltriethylammonium (PETEA)

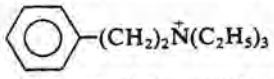


Fig. 1 Structures of the agonists tested.

at each potential; in one case where both conductances were measured, all six agonists tested elicited channels with the same two conductance values as those elicited by acetycholine on the same patch. There was no evidence for preference by any agonist for one or the other channel type. Conductance sublevels of the sort reported in cultured embryonic rat muscle at 5–10 °C (ref. 17) and in cultures of chick myotubes[18] were rarely seen in the present conditions. The current–voltage relationships for the agonists were determined by plotting the mean single-channel current amplitude as a function of patch potential. Figure 2*c* illustrates the current–voltage characteristics obtained for six different agonists applied to the same patch. The relationship is linear over the potential range tested and is very similar for each agonist. The slope conductance and reversal potential for each agonist were obtained from such plots. Extrapolated reversal potentials were close to 0 mV in all cases (mean ± s.e. = 2.78 ± 1.80 mV, $n = 26$). Single-channel slope conductance was quite variable; values of 30–55 pS were seen in different rat myotube patches, and sometimes more than one value was observed in the same patch. Because of this variability, acetylcholine was applied to every patch, and the results were expressed as the ratio of the conductance for each agonist to that for acetylcholine on the same patch (Table 1). The observed conductance ratios for nine agonists relative to acetylcholine

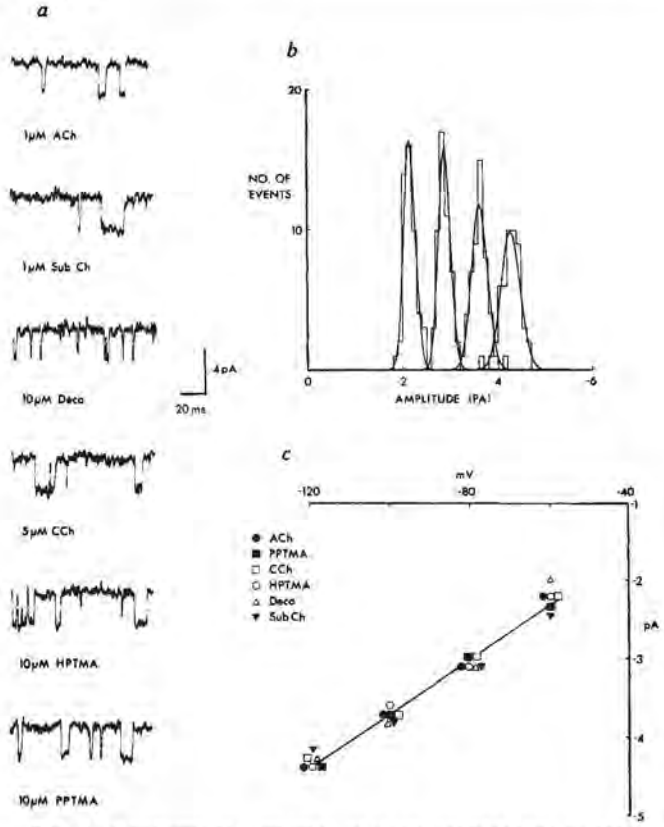


Fig. 2 Conductance properties of single acetylcholine-receptor channels activated by different cholinomimetic agonists. *a*, Single-channel current steps recorded from a cell-free, 'outside-out' patch isolated from rat myotubes. Membrane potential was −80 mV; temperature 20–23 °C. The recording pipette contained the following solution (in mM): KCl 150.0; Na_2EGTA 5.0; $CaCl_2$ 0.5; HEPES 10.0, *p*H 7.0. The bath solution was Eagle's minimal essential medium with HEPES buffer, at *p*H 7.2 (this contains 135 mM Na^+ and 5.4 mM K^+). Six different agonists were sequentially bath-applied as indicated under the individual recordings (ACh, 1 μM; CCh, 5 μM; SubCh, 1 μM; HPTMA, 10 μM; PPTMA, 10 μM; Deca, 10 μM). Application of each agonist was followed by washout during which disappearance of the single-channel currents was confirmed. A downward deflection denotes an inward current. All records are filtered with 2.0–3.0 kHz (−3 dB) low-pass Bessel filter. *b*, Distribution of current amplitudes at membrane patch potentials of −60, −80, −100 and −120 mV, during activation by 1 μM acetylcholine. The distribution at each potential is singly peaked with mean step sizes of −2.1 ± 0.1 pA at −60 mV, −2.9 ± 0.1 pA at −80 mV, −3.6 ± 0.2 pA at −100 mV, and −4.3 ± 0.2 pA at −120 mV. *c*, Current–voltage relation of single-channel currents from one outside-out patch that had been sequentially activated by ACh, CCh, SubCh, Deca, HPTMA and PPTMA. The mean amplitudes obtained from histograms as shown in *b* are plotted as a function of patch potential. The line represents the current–voltage curve obtained from least squares linear regression analysis of the mean current amplitudes and patch potentials obtained with acetylcholine. Similar curves were drawn for each agonist. The slope conductance was close to 34 pS for each agonist tested on this patch.

differ from unity by no more than 3%. These results provide no evidence to support the view that conductance of an ion channel depends on the nature of the agonist that causes it to open.

A second series of experiments was made at endplates of adult frog (*Rana temporaria*) cutaneus pectoris muscle fibres, after enzyme treatment[19]. Single-channel membrane currents were recorded with a patch pipette in the cell-attached configuration[15], with the agonist in the pipette solution. Current–voltage relationships were constructed from unit current measurements at four or five membrane potentials over a 120-mV range, and slope conductances were determined, as described above. The slope conductances obtained with 10 different agonists for adult

Table 1 Single-channel slope conductance ratios for agonists relative to acetylcholine on rat myotubes

CCh ($n=3$)	1.00 ± 0.03	SucCh ($n=2$)	1.01 ± 0.04
SubCh ($n=3$)	1.02 ± 0.05	HPTMA ($n=3$)	1.00 ± 0.03
Nicotine ($n=2$)	0.98 ± 0.02	PPTMA ($n=3$)	1.01 ± 0.05
DMPP ($n=2$)	1.00 ± 0.02	PETEA ($n=2$)	0.98 ± 0.10
Deca ($n=3$)	1.03 ± 0.05		

Values of the ratio of the single-channel conductance of the indicated agonist to that of acetylcholine on the same 'outside-out' membrane patch. Values represent mean ± s.e. (pS) with the number of determinations in parentheses. CCh, Carbachol; SubCh, suberyldicholine; DMPP, 1,1-dimethyl-4-phenylpiperazinium; Deca, decamethonium; SucCh, succinylcholine; HPTMA, 3-(*m*-hydroxyphenyl)propyltrimethylammonium; PPTMA, 3-phenylpropyltrimethylammonium; PETEA, 3-phenylethyltriethylammonium.

frog muscle endplate at 9–12 °C are given in Table 2. The actual conductances, rather than the values relative to acetylcholine, are given because conductance values were much less variable from one membrane patch to another on adult frog muscle endplate than on cultured embryonic rat muscle. The values obtained for each agonist fell within 5% of the weighted mean of all 10, 30.2 ± 0.09 pS (mean ± s.e., $n=10$), a value similar to that found by Anderson and Stevens[2], but rather higher than most other estimates from noise analysis. Reversal potential was approximately 0 mV for all 10 agonists tested (mean ± s.e. = −0.2 ± 1.1 mV, $n=10$). Note that each value given was obtained from a different muscle patch and that several muscles were used in this study.

We conclude that the single-channel conductances are the same, within a few per cent, for all 10 agonists tested. In particular this was observed to be the case for 3-(*m*-hydroxyphenyl)propyltrimethylammonium (HPTMA), 3-phenylpropyltrimethylammonium (PPTMA), nicotine and decamethonium, which had been reported on the basis of noise analysis[4,5] to produce lower conductance channels than acetylcholine. The reversal potentials were also closely similar. This suggests that the open ion channel has essentially the same conformation whichever agonist opens it. Thus, it seems that agonist–receptor complexes formed by different agonists show differences in those regions of the macromolecules concerned with opening and shutting probabilities, but not in those regions concerned with ion permeation.

Recent observations have shown that the conventional concept of channel openings of fixed amplitude may be an oversimplification. It has been found that a single channel type may adopt unusual open states (sublevels), which have lower than normal conductance, in cultured rat[17] and chick[18] muscle, and occasionally in adult frog endplates[20]. These discoveries suggest that differences in agonist efficacy could result from different degrees of preference for open-state sublevels of low conductance. This is unlikely, however, since conductance sublevels were rarely seen in this study with any of the 10 agonists.

It seems improbable that errors could cause apparent identity of single-channel currents that are in fact different, though it is possible that the absolute values from single-channel analysis could be erroneously high. Therefore, it appears that noise analysis has given erroneously low relative conductances for some agonists[4,5]. Indeed a survey of the literature shows that noise analysis almost invariably gives lower estimates of the absolute single-channel conductance than those found from single-channel recording, though the size of the discrepancy varies from trivial to quite substantial. For example, acetylcholine-activated ion channels in rat endplate (diaphragm) have been reported to have conductances of about 30 pS (refs 21, 22) from noise studies, while channels of the rat endplate (omohyoid) have been reported to have conductances of approximately 58 pS (ref. 23) by single-channel recording techniques. Similarly it has been found that ion-channel blocking drugs appear to reduce single-channel conductance when tested by noise analysis[24,25], but no reduction is seen in single channel records (ref.

Table 2 Single-channel slope conductance on the adult frog muscle endplate

	γ (pS)		γ (pS)
ACh	30.0 ± 1.0	Deca	30.2 ± 0.1
CCh	29.8 ± 0.4	SucCh	30.0 ± 0.4
SubCh	29.9 ± 1.4	HPTMA	29.0 ± 0.4
Nicotine	31.4 ± 1.2	PPTMA	30.0 ± 0.3
DMPP	30.6 ± 1.2	PETEA	31.9 ± 0.4

Values of the slope conductance (γ) of channels activated by the indicated agonists on the frog muscle endplate. Values are expressed as slope conductance ± s.d. (pS). The recordings were made in the cell-attached configuration on frog muscle endplate at 9–12 °C. The bath and pipette solution contained the following (in mM): NaCl 116.5; KCl 2.5; $CaCl_2$ 1.5; HEPES buffer 10.0; tetrodotoxin 50 nM, at *p*H 7.2. Agonists were added to the pipette solution in the following concentrations: ACh, 100 nM; SubCh, 100 nM; CCh, 500 nM; nicotine, 500 nM; DMPP, 1 μM; Deca, 1 μM; SucCh, 500 nM; HPTMA, 500 nM; PPTMA, 1 μM; PETEA, 10 μM.

26 and D.C., D.C.O. and S. A. Siegelbaum, in preparation). This suggests that one or more of the assumptions that are used in noise analysis may be invalid. If all channels pass the same elementary current, i, then the channel conductance $\gamma = i/(V - V_{rev})$ is given by $\gamma = s^2/[m(V - V_{rev})(1-p)]$ where m and s^2 are mean and variance of the agonist-induced current, V is the membrane potential, V_{rev} is the reversal potential, and p is the fraction of time for which a single channel is open. This expression is usually simplified by assuming that $p \ll 1$. It is valid regardless of whether or not channel block is occurring (as long as blockages are complete)[24,25,27]. Thus the estimate of γ obtained from noise analysis would be too low (1) if the variance were underestimated (for example, by failing to extrapolate correctly the observed spectral components or by missing a spectral component altogether), (2) if the mean current, m, or the driving potential, $(V - V_{rev})$, were overestimated (for example, by imperfect voltage clamp of the active membrane), or (3) if the fraction of channels, p, opened by the agonist were too large to be neglected.

Thus, we have found closely similar single-channel conductances for 10 agonists. It appears that noise analysis may have underestimated the relative single-channel conductance in some circumstances, but it is not clear which assumptions of the noise analysis were breached.

We thank R. B. Barlow for the gifts of the compounds HPTMA, PPTMA and PETEA[28] and Chris Magnus for tissue culture. P.G. was supported by the William Moseley Foundation of Harvard University. This work was supported by the MRC.

Received 24 October 1983; accepted 17 February 1984.

1. Katz, B. & Miledi, R. *J. Physiol., Lond.* **224,** 665–669 (1972).
2. Anderson, C. R. & Stevens, C. F. *J. Physiol., Lond.* **235,** 655–691 (1973).
3. Changeux, J. P. *Harvey Lect.* **75,** 85–254 (1980).
4. Colquhoun, D., Dionne, V. E., Steinbach, J. H. & Stevens, C. F. *Nature* **253,** 204–206 (1975).
5. Dreyer, F., Walther, C. & Peper, K. *Pflügers Arch. ges. Physiol.* **366,** 1–9 (1976).
6. Spivak, C. E., Waters, J., Witkop, B. & Albuquerque, E. X. *Molec. Pharmac.* **23,** 337–343 (1983).
7. Auerbach, A., del Castillo, J., Specht, P. C. & Titmus, M. *J. Physiol., Lond.* **343,** 551–568 (1983).
8. Stettmeier, H. & Finger, W. *Pflügers Arch. ges. Physiol.* **397,** 237–242 (1983).
9. Gray, P. T. A. & Rang, H. P. *Br. J. Pharmac.* **80,** 235–240 (1983).
10. Jackson, M. B., Lecar, H., Askanas, V. & Engel, W. K. *J. Neurosci.* **2,** 1465–1473 (1982).
11. Peper, K., Bradley, J. & Dreyer, F. *Physiol. Rev* **62,** 1279–1340 (1982).
12. Anderson, C. R., Cull-Candy, S. G. & Miledi, R. *J. Physiol., Lond.* **282,** 219–242 (1978).
13. Cull-Candy, S. G., Miledi, R. & Parker, I. *J. Physiol., Lond.* **321,** 195–210 (1981).
14. Stephenson, R. P. *Br. J. Pharmac.* **11,** 379–393 (1956).
15. Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. *Pflügers Arch. ges. Physiol.* **391,** 85–100 (1981).
16. Castillo, J. Del & Katz, B. *Proc. R. Soc.* B **146,** 369–381 (1957).
17. Hamill, O. P. & Sakmann, B. *Nature* **294,** 462–464 (1981).
18. Auerbach, A. & Sachs, F. *Biophys. J.* **42,** 1–10 (1983).
19. Ogden, D. C. & Colquhoun, D. *Pflügers Arch. ges. Physiol.* **399,** 246–248 (1983).
20. Colquhoun, D. & Sakmann, B. in *Single Channel Recording* (eds Sakmann, B. & Neher, E.) (Plenum, New York, in the press).
21. Sakmann, B. *Fedn Proc.* **37,** 2654–2659 (1978).
22. Colquhoun, D., Large, W. A. & Rang, H. P. *J. Physiol., Lond.* **266,** 361–395 (1975).
23. Sakmann, B., Bormann, J. & Hamill, O. *Cold Spring Harb. Symp. Quant. Biol.* **48,** (in the press).
24. Ruff, R. L. *J. Physiol., Lond.* **264,** 89–124 (1977).
25. Ruff, R. L. *Biophys. J.* **37,** 625–631 (1982).
26. Neher, E. *J. Physiol., Lond.* **339,** 663–678 (1983).
27. Colquhoun, D. in *Cell Membrane Receptors for Drugs and Hormones* (eds Straub, R. W. & Bolis, L. (Raven, New York, 1978).
28. Barlow, R. B., Thompson, G. M. & Scott, N. C. *Br. J. Pharmac.* **37,** 555–584 (1969).