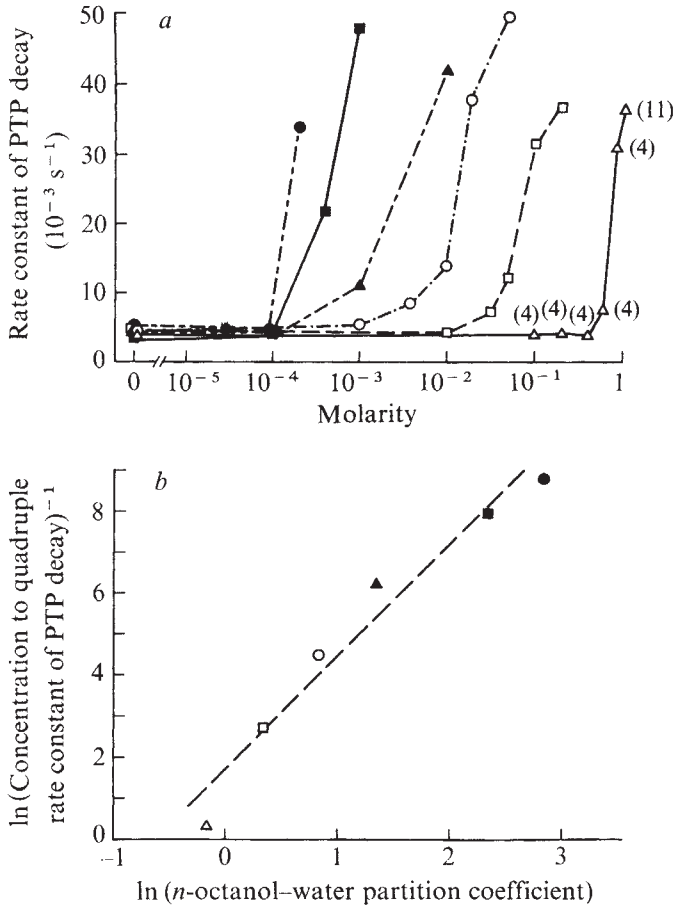

Fig. 2 *a*, Potency of aliphatic alcohols in increasing the rate constant of PTP decay. ●, C_8; ■, C_7; ▲, C_5; ○, C_4; □, C_3; △, C_2. The size of the carbon chain is indicated by the number (C_2 is ethanol; C_8 is *n*-octanol). An aliphatic alcohol (in fortified artificial seawater) was perfused for 30 min, beginning 30 min after a control train; and then a train was given in the presence of the alcohol. In contrast with ethanol, the higher alcohols reduced the amplitudes of all e.p.s.p.s. PTP still decayed, however, with a single exponential time course with a rate constant which was readily determined. Each data point represents the average value obtained from three different preparations, except for ethanol where the number of preparations at each data point is indicated. Note that the effect of each alcohol increases markedly over a narrow range of concentration. *b*, The potency of the effect of the different alcohols (measured as the concentration, *C*, needed to increase the rate of decay of PTP fourfold) correlates with the lipophilicity of the alcohol (as measured by the *n*-octanol: water partition coefficient, *P*). The data points were obtained from (*a*) by interpolating the concentration of each alcohol which would give a rate constant of PTP decay of $20\times10^{-3}\ s^{-1}$. A plot of $\ln(1/C)$ against $\ln P$ gives a straight line with the equation $\ln(1/C) = 2.71\ \ln P + 1.66$. Other biological systems susceptible to alcohols similarly give straight lines in these coordinates[6]. Symbols as in (*a*).

change in presynaptic membrane organisation (for example, a change in the number or effectiveness of "vesicle attachment sites"[9]). In this latter case, the relaxation of this state of organisation would correspond to the decay of PTP, and would be directly affected by the fluidity of components of the presynaptic membrane. Evidence that this possibility is consistent with other known properties of PTP will be presented later. Regardless of the steps connecting membrane fluidity to the rate of PTP decay, our findings suggest that nerve terminal membrane fluidity is a potential physiological regulation point for the duration of PTP.

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1 Gage, P. W., and Hubbard, J. I., *J. Physiol., Lond.*, **184**, 353–375 (1966).
2 Schlapfer, W. T., Tremblay, J. P., Woodson, P. B. J., and Barondes, S. H., *Brain Res.* (in the press).
3 Schlapfer, W. T., Woodson, P. B. J., Smith, G. A., Tremblay, J. P., and Barondes, S. H., *Nature*, **258**, 623–625 (1975).
4 Paterson, S. J., *et al.*, *Biochim. biophys. Acta*, **266**, 597–602 (1972).
5 Schlapfer, W. T., Woodson, P. B. J., Tremblay, J. P., and Barondes, S. H., *Brain Res.*, **76**, 267–280 (1974).
6 Hansch, D., and Anderson, S. M., *J. med. Chem.*, **10**, 745–753 (1967).
7 Rosenthal, J., *J. Physiol., Lond.*, **203**, 121–133 (1969).
8 Wilson, D. F., and Skirboll, L. R., *Am. J. Physiol.*, **227**, 92–95 (1974).
9 Pfenninger, K. H., and Rovainen, C. M., *Brain Res.*, **72**, 1–23 (1974).

Single-channel currents recorded from membrane of denervated frog muscle fibres

THE ionic channel associated with the acetylcholine (ACh) receptor at the neuromuscular junction of skeletal muscle fibres is probably the best described channel in biological membranes. Nevertheless, the properties of individual channels are still unknown, as previous studies were concerned with average population properties. Macroscopic conductance fluctuations occurring in the presence of ACh were analysed to provide estimates for single channel conductance and mean open times[1–3]. The values obtained, however, depended on assumptions about the shape of the elementary conductance contribution—for example, that the elementary contribution is a square pulse-like event[2]. Clearly, it would be of great interest to refine techniques of conductance measurement in order to resolve discrete changes in conductance which are expected to occur when single channels open or close. This has not been possible so far because of excessive extraneous background noise. We report on a more sensitive method of conductance measurement, which, in appropriate conditions, reveals discrete changes in conductance that show many of the features that have been postulated for single ionic channels.

The key to the high resolution in the present experiments lies in limiting the membrane area from which current is measured to a small patch, and thereby decreasing background membrane noise. This is achieved by applying closely the tip of a glass pipette, 3–5 μm in diameter, on to the muscle surface, thus isolating electrically a small patch of membrane (Fig. 1). This method has been applied previously in various modifications and mostly with larger pipette tips to muscle[4], molluscan neurones[5,6], and squid axon[7]. The pipette, which has fire-polished edges, is filled with Ringer's solution and contains the cholinergic agonist at micromolar concentrations. Its interior is connected to the input of a virtual-ground circuit, which clamps the potential inside the pipette to ground and at the same time measures current flowing through the pipette, that is, through the patch of membrane covered by the pipette opening. The interior of the muscle fibre is clamped locally to a fixed value by a conventional two-microelectrode clamp[8]. Thus, voltage-clamp conditions are secured across the patch of membrane under investigation. Since current

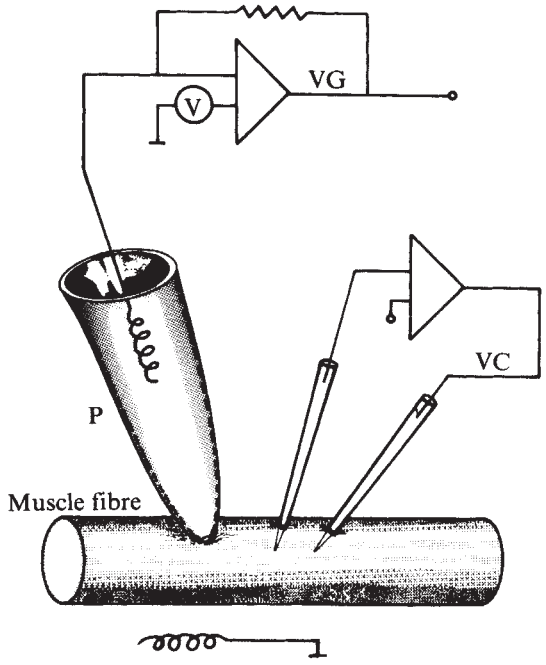

Fig. 1 Schematic circuit diagram for current recording from a patch of membrane with an extracellular pipette. VC, Standard two-microelectrode voltage clamp circuit to set locally the membrane potential of the fibre to a fixed value. P, Pipette, fire polished, with 3–5 μm diameter opening, containing Ringer's solution and agonist at concentrations between 2×10^{-7} and 6×10^{-5} M. d.c. resistance of the pipette: 2–5 MΩ. The pipette tip applied closely on to the muscle fibre within 200 μm of the intracellular clamp electrodes. VG, Virtual ground circuit, using a Function Modules Model 380K operational amplifier and a 500 MΩ feedback resistor to measure membrane current. The amplifier is mounted together with a shielded pipette holder on a motor-driven micromanipulator. V, Bucking potential and test signal for balancing of pipette leakage and measuring pipette resistance.

densities involved are very small, a simple virtual ground inside the pipette is preferable to more complicated arrangements for stabilising potential described previously[6].

The dominant source of background noise in these measurements was the leakage shunt under the pipette rim between membrane and glass. It was constantly monitored by measuring the electrical conductance between pipette interior and bath. Discrete conductance changes could be resolved only when the conductance between pipette interior and bath decreased by a factor of four or more after contact between pipette and membrane. To minimise the leakage conductance, the muscle was treated with collagenase and protease[9]. This enzyme treatment digested connective tissue and the basement membrane, thereby enabling closer contact between glass and membrane. At the same time, however, it made the membrane fragile and more sensitive to damage by the approaching pipette. It did not, however, change the ACh sensitivity of the fibre or alter the properties of ACh-induced conductance fluctuations (E.N. and B.S., unpublished).

All experiments were carried out on the extrasynaptic region of denervated hypersensitive muscle fibres. The uniform ACh sensitivity found over most of the surface of these fibres greatly enhanced the probability of the occurrence of agonist-induced conductance changes at the membrane patch under investigation. Extrasynaptic ACh channels of denervated muscle fibres have mean open times which are about three to five times longer than those of endplate channels[1,10–12]. The longer duration facilitated the detection of conductance changes. Additional measures were taken which are known to either increase the size of the elementary current pulse or prolong its duration: the membrane was hyperpolarised up to −120 mV; suberyldicholine (SubCh) was used as an agonist in most of the experiments; the preparation was cooled to 6–8 °C.

Figure 2 shows a current recording taken in the conditions outlined above. Current can be seen to switch repeatedly between different levels. The discrete changes are interpreted as the result of opening and closing of individual channels. This interpretation is based on the very close similarity to single-channel recordings obtained in artificial membrane systems[13]. The preparation under study is, however, subject to a number of additional sources of artefact. Therefore it is necessary to prove that the recorded events do show the properties which are assigned to ionic channels of the cholinergic system. These are: a correlation with the degree of hypersensitivity of the muscle membrane; an amplitude dependent on membrane potential as predicted by noise analysis; a mean length or channel open time, which should depend on voltage in a characteristic manner[2]; pharmacological specificity with different mean open times for different cholinergic agonists[14,15]. The experiments bore out all of the above-mentioned points as outlined below.

The frequency of occurrence of single blips depended on the sensitivity of the patch under investigation. A plot of the number of current pulses per second against the iontophoretically measured sensitivity of the membrane region, determined either immediately before or after the pipette experiment, revealed a distinct correlation between both quantities with a correlation coefficient of 0.91 for a linear regression (Fig. 3*b*). Student's *t* test assigned a significance better than 0.1% to the relationship.

To estimate the size of the current pulses amplitude histograms were calculated from the current recordings (Fig. 3*c*). The histograms show a prominent peak of gaussian shape around zero deviation, the width of which is a measure of the high frequency background noise of the current trace. Multiple, equally spaced peaks at larger deviations represent the probabilities that either one, two, or three channels are open simultaneously. The peak separation gives the amplitude of the single-channel contribution, which was 3.4 pA for the histogram shown in Fig. 3*c*.

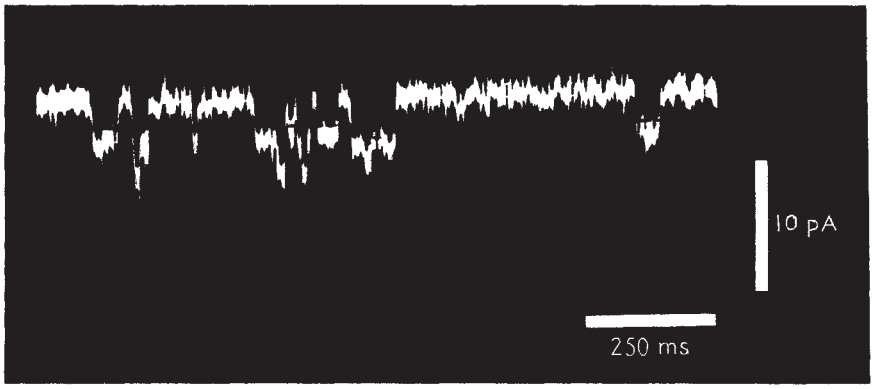

Fig. 2 Oscilloscope recording of current through a patch of membrane of approximately 10 μm². Downward deflection of the trace represents inward current. The pipette contained 2×10^{-7} M SubCh in Ringer's solution. The experiment was carried out with a denervated hypersensitive frog cutaneus pectoris (*Rana pipiens*) muscle in normal frog Ringer's solution. The record was filtered at a bandwidth of 200 Hz. Membrane potential: −120 mV. Temperature: 8 °C.

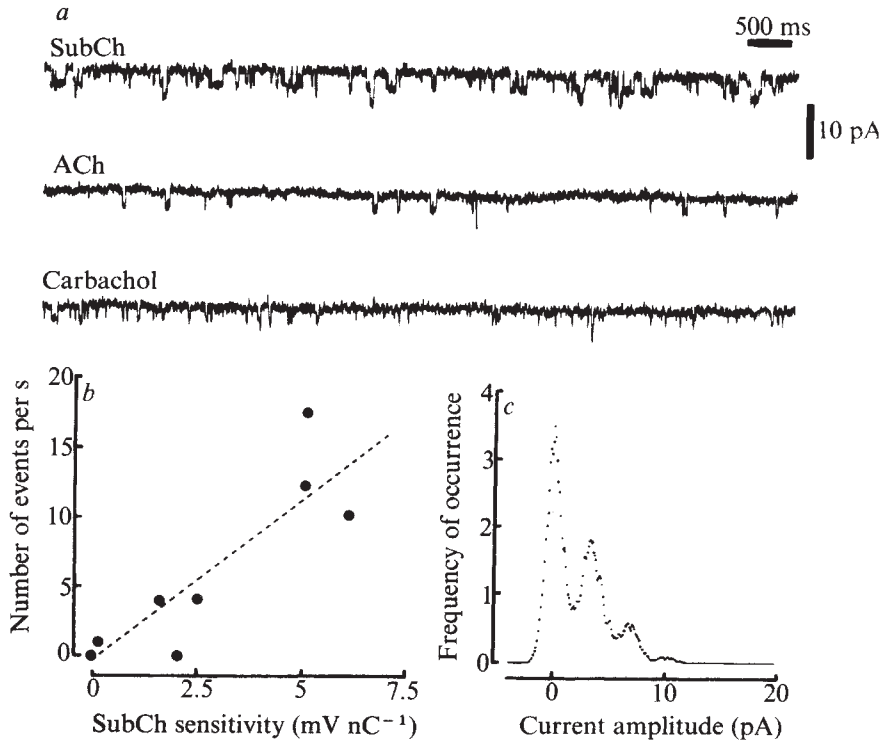

Fig. 3 Characterisation of single-channel currents. *a*, Comparison of current recordings obtained with different cholinergic agonists at concentrations of 2.10^{-7} M(SubCh), 2.10^{-6} M(ACh), and 6.10^{-5} M(carbachol). Downward deflection of the trace represents inward current. Three different experiments. Pen records replayed from analogue tape at a bandwidth of 100 Hz. All experiments at −120 mV membrane potential; 8 °C. *b*, Number of current blips per second is plotted against iontophoretic sensitivity of the membrane region under investigation. Sensitivity was determined by 100-ms iontophoretic pulses delivered from a pipette filled with 1 M SubCh (40 MΩ pipette resistance, 10 nA bucking current, sensitivity measured at resting potential). Pooled data from eight experiments. Broken line represents linear regression. *c*, Amplitude histogram of membrane current. Current traces were digitalised and baselines fitted by eye to data records, each 4 s in length. Frequency of occurrence of deviations from the baseline is shown in arbitrary units. Histograms were calculated on a PDP-11 computer; 2–8 histograms were averaged to obtain curves like the one shown. 8 °C; −120 mV membrane potential.

This was obtained from an experiment at −120 mV membrane potential. A similar histogram from the same muscle fibre obtained at −80 mV yields a current pulse amplitude of 2.2 pA. These two values extrapolate to an equilibrium potential of −7 mV. Channel conductance is estimated as 28 pmhos in this case. It scattered from fibre to fibre with a mean value of 22.4±0.3 pmhos (mean±s.e.; number of determinations=27). This value is somewhat lower than the one derived from noise analysis at normal endplates, which is 28.6 pmhos for SubCh[15]. Higher order peaks in the histograms are not merely scaled images of the zero order peak. They tend to be smeared out due to non-uniformity of current pulse amplitudes. We cannot decide at present whether this is a real feature of the channels or a measurement artefact. Such an effect could arise if not all of the channels are located ideally in the central region of the pipette opening. Current contributions from peripherally located channels would only partially be picked up by the pipette. This source of error is also likely to lead to an underestimate of channel size if the pipette seal is not optimal.

Temporal analysis of the current records was carried out partly by measurement of individual channel length and averaging 40–50 measurements, and partly by calculation of the power spectrum of the current recordings. In the latter case, the cutoff frequency f_0 of the Lorentzian spectrum yielded an estimate of mean channel open time τ (or pulse duration) through the relationship $\tau=1/(2\pi f_0)$. Values of mean open times obtained by the two methods were consistent within ± 30%. For SubCh as an agonist and at a temperature of 8 °C it was 45±3 ms (n=11) at −120 mV and 28±3 ms (n=14) at −80 mV. These values are approximately three times longer than the corresponding mean open times of endplate channels derived from noise analysis[15]. Note, however, that lengthening of channel durations by factors of three to five at extrajunctional sites with respect to endplate values has been measured independently by conventional noise analysis[12]. The voltage dependence of the values given above corresponds to an e-fold change per 80 mV, which is within the range of published values[2].

Channel open times were different when different cholinergic agonists were used (Fig. 3*a*). For −120 mV and 8 °C, mean channel open time was 45±3 ms (n=11) for SubCh, 26±5 ms (n=4) for Ach, and 11±2 ms (n=3) for carbachol. This sequence reflects the well known relationship between the open times of channels induced by these drugs at normal endplates[14,15] and at extrasynaptic membrane of hypersensitive fibres[12].

The results obtained so far, especially the pharmacological specificity, lead us to conclude that the observed conductance changes are indeed recordings of single-channel currents. They are consistent with the conclusions drawn from statistical analysis of endplate current fluctuations, and show that current contributions of individual channels are of the form of square pulses. In addition, analysis of areas under the peaks of histograms like Fig. 3*c* indicates that in our experimental conditions opening of individual channels is statistically independent, since the probabilities of zero, one, or two channels being open simultaneously follow—within the limits of experimental resolution—a Poisson distribution.

Recordings of single-channel currents finally resolves the third level of quantification in the process of neuromuscular transmission after the discovery of endplate currents and miniature endplate currents. It should facilitate discrimination between factors influencing the properties of single channels and agents creating or modifying different populations of channels.

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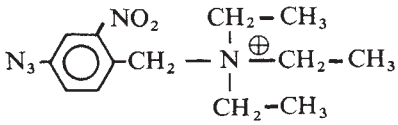
1 Katz, B., and Miledi, R., *J. Physiol., Lond.*, **224**, 665–699 (1972).
2 Anderson, C. R., and Stevens, C. F., *J. Physiol., Lond.*, **235**, 655–691 (1973).
3 Ben Haim, D., Dreyer, F., and Peper, K., *Pflügers Arch. ges. Physiol.*, **355**, 19–26 (1975).
4 Strickholm, A., *J. gen. Physiol.*, **44**, 1073–1087 (1961).
5 Frank, K., and Tauc, L., in *The Cellular Function of Membrane Transport* (edit by Hoffman, J.) (Prentice Hall, Englewood Cliffs, New Jersey, 1963).
6 Neher, E., and Lux, H. D., *Pflügers Arch. ges. Physiol.*, **311**, 272–277 (1969).
7 Fishman, H. M., *Proc. natn. Acad. Sci. U.S.A.*, **70**, 876–879 (1973).
8 Takeuchi, A., and Takeuchi, N., *J. Neurophysiol.*, **22**, 395–411 (1959).
9 Betz, W., and Sakmann, B., *J. Physiol., Lond.*, **230**, 673–688 (1973).
10 Neher, E., and Sakmann, B., *Pflügers Arch. ges. Physiol.*, **355**, R63 (1975).
11 Dreyer, F., Walther, Ch., and Peper, K., *Pflügers Arch. ges. Physiol.*, **359**, R71 (1975).
12 Neher, E., and Sakmann, B., *J. Physiol., Lond.* (in the press).
13 Hladky, S. B., and Haydon, D. A., *Nature*, **225**, 451–453 (1970).
14 Katz, B., and Miledi, R., *J. Physiol., Lond.*, **230**, 707–717 (1973).
15 Colquhoun, D., Dionne, V. E., Steinbach, J. H., and Stevens, C. F., *Nature*, **253**, 204–206 (1975).

Selective inhibition of potassium conductance in node of Ranvier with a photoaffinity label derived from tetraethylammonium

BIOCHEMICAL investigation of membrane components involved in ion-specific conductance changes is difficult because of the lack of a functional assay for the components after membrane solubilisation. Proteins thought to participate in conductivity changes during synaptic transmission[1] have been isolated using a binding assay and affinity chromatography[2,3], because of their high affinity for a variety of ligands; but the connection between such receptor proteins and postulated ion channels remains to be shown. To isolate molecules forming sodium channels of the axon membrane[4–7], tetrodotoxin was used as ligand (binding assay $K_d = 3$ nM). The most efficient known blocking agent of the change in potassium conductance in nerve and muscle known is tetraethylammonium (TEA) but its affinity is too low ($K_d = 0.4$ mM (ref. 8)) for either of the above techniques. We have attempted to label radioactively those molecules associated with the voltage-dependent potassium conductance changes covalently *in situ* with the aim of recovering that radioactivity after solubilisation using classical fractionation procedures. We suggest that it is possible to block selectively and irreversibly potassium conductance in myelinated nerve fibres using a photoaffinity lable. This method may open the way for the isolation of the TEA receptor of excitable membranes, proposed as part of the potassium channel (if not the channel itself).

We applied 4-azido-2-nitrobenzyltriethylammonium fluoroborate,



which belongs to a class of photoaffinity labels developed by H.K.[9] and which was first used in the investigation of the structure of the acetylcholine receptor (F.H., P. Layer, H.K. and G. Bandini, unpublished) and the acetylcholine esterase (P. Layer, H.K. and F.H., unpublished). The label was synthesised using the same procedure described for the corresponding trimethylammonium derivative (P. Layer, H.K. and F.H., unpublished). Voltage clamp experiments were carried out with sensory and motor fibres of the sciatic nerve of the frog *Rana esculenta*[10,11]. The Ranvier node was continuously superfused with Ringer or the test solution as indicated in Figs 1–4. Photoaffinity labelling was induced by irradiation with an ultraviolet lamp Typ TL-900 (Camag, Switzerland; emission maximum 254 nm; lamp distance from fibre 5 cm). Without irradiation the photoaffinity label reversibly inhibited the potassium conductance (Fig. 1*c*), whereas the sodium conductance was not affected.

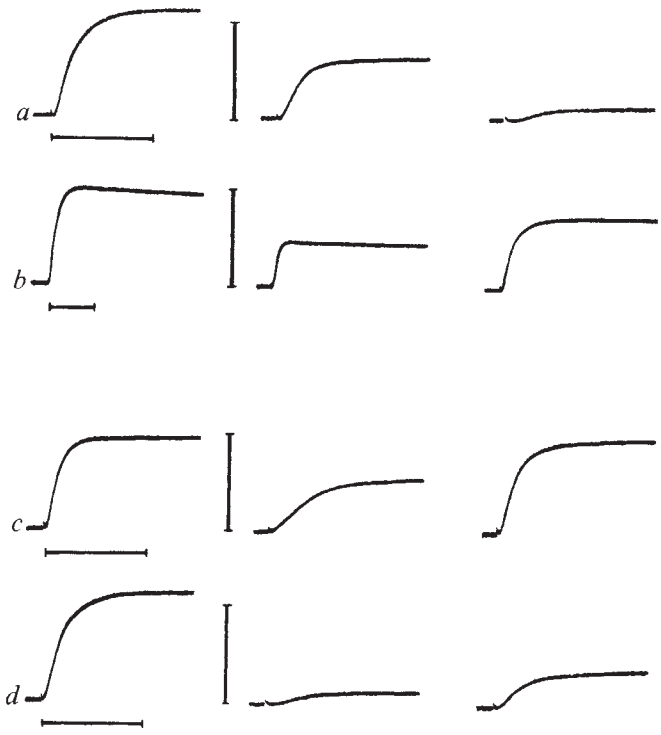


Fig. 1 Potassium current records during 140 mV depolarising pulses from a holding potential of −100 mV. Capacity and leakage currents were compensated analogically. Test node superfused with Ringer's solution (117 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$) plus tetrodotoxin (300 nM). Control, First record in each row. In each experiment photoaffinity label (1 mM) was added either to Ringer's solution (*a*, *c* and *d*) or to isosmotic KCl solution in contact with the cut ends of the fibre (*b*). *a* and *b*, Effects of photoaffinity label applied either externally (*a*) or internally (*b*), before (second records) or after (third records) application of ultraviolet light for 3 min. *c* and *d*, Reversibility for the experiment without (*c*) or with irradiation (*d*). Third record in *c* and *d* was taken 40 min after removal of photoaffinity label from Ringer's solution. Vertical and horizontal calibrations: 10 nA and 10 ms, respectively.

50% inhibition of the potassium current was obtained with 4 mM photoaffinity label (Fig. 2), indicating a much lower affinity than that for TEA ($K_d = 0.4$ mM (ref. 8)). During irradiation in the presence of the label the potassium current decreased markedly and rapidly (Figs 1*a* and 3), whereas the sodium current decreased only slightly (Fig. 3). In a typical experiment, we obtained after about 2 min irradiation an 80% decrease in potassium current and a less than 10% decrease in sodium current. After removal of the

Fig. 2 Dose–response curve for 4-azido-2-nitrobenzyltriethylammonium fluoroborate (without irradiation). Photoaffinity label was added to Ringer's solution used to superfuse test node. Temperature: 11 °C.