Table 1. Formation and decomposition of H^{2-} ions. Abbreviations: M, third body; *, excited species; k, specific rate constant; and ΔV, voltage gradient.

Reaction	Description
	Formation
$M + e \longrightarrow M^+$	Ionization of neutral gas in the ion extraction region, pressure dependent
$H^- + M^+ \longrightarrow H^{-*}$	Excitation by collision
$H^{-*} + e + M \longrightarrow H^{2-} + M$	Three-body process, pressure dependent
	Decomposition
$H^{2-} \xrightarrow{k} H^- + e$	Spontaneous dissociation
$H^{2-} + M \rightleftarrows H^- + M + e$ $H + M + 2e$	Collisional dissociation
$H^{2-} \xrightarrow{\Delta V} H^- + e$	Field-induced dissociation

hydrogen. The currents increased with the second power of pressure for both hydrogen and helium, but somewhat more slowly for argon.

All the experiments carried out with a hydrogen plasma were corroborated with parallel experiments with deuterium plasma; the existence and behavior of analogous fast D^- ions were demonstrated in every instance.

This diversity of experimental results is consistent qualitatively and quantitatively with the sequence of processes shown in Table 1. Our suggested mechanism for the formation of H^{2-} is that an excited H^- ion attaches an electron to give the doubly charged atomic ion. The excited H^- can be produced efficiently by collisions with positive ions, formed in our experimental setup by electron impact ionization in the extraction region and accelerated in the opposite direction. Under our conditions, a thousandfold higher electron current (milliamperes) accompanies the H^- ion current (microamperes). The formation of H^{2-} from $e + H^{-*}$ requires removal of the excess energy by a third body. This mechanism explains the second power dependence on the pressure of hydrogen or noble gases in the extraction regions.

Under our experimental conditions (*5*), the contribution of collisional dissociation of H^{2-} to give H atoms was not studied. Also, H^{2-} undergoes field-induced dissociation to give H^- or H. This phenomenon, which contributes to the attenuation of the H^{2-} beam in our experimental setup, will be described elsewhere (*10*).

In summary, we have experimental evidence for the existence of a relatively long-lived H^{2-} ion. Since H^{2-} is isoelectronic with He^-, Li, or Bi^+, it represents an extreme case of the three-electron system (*11*). We believe that this species may play an important role in hydrogen plasmas. We do not know the lifetime of the excited H^- that is presumably the precursor of H^{2-}. If this is a long-lived state and if it can be produced by photoexcitation, then there is room to believe that an equilibrium concentration of H^{2-} may exist in interstellar space. The photoionization of this species, which is expected to take place in the infrared, may thus be observable in the astronomical infrared spectrum. The autodetachment of H^{2-} leaves H^- in an excited state and may also result in single or multiple photon emission. Since we have no information about the electronic structure of H^{2-} at present, the assignment of possible absorption or emission lines will have to wait until appropriate theoretical calculations are performed. We hope that this report will stimulate theoretical work leading to a utilization of our experimental findings to their full capacity.

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12. The mass spectrometer used in this study was constructed as part of a program sponsored by the National Science Foundation under grant GA-37729.

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Reversal of Acetylcholine Potentials in Eel Electroplaque

Abstract. *Although the eel electroplaque is a major source of purified acetylcholine (ACh) receptor, the electrophysiological properties of the receptor have not been studied in detail. In particular, the reversal potential for the action of ACh on the postsynaptic membrane has not been measured directly. In order to obtain the reversal potential, ACh was applied iontophoretically from a micropipette onto the innervated membrane. The resulting depolarization (ACh potential) decreased in amplitude as the cell was depolarized, reached zero at a reversal potential of about –4 millivolts, and then reversed in sign as the inside of the cell was made increasingly more positive. The relation between ACh potential amplitude and membrane potential was nonlinear because of a decrease with depolarization of the peak conductance change produced by the drug.*

The eel electroplaque is one of the major sources of purified acetylcholine (ACh) receptors for chemical studies and for immunological studies on the interaction of ACh receptor antibodies with postjunctional receptors in vertebrate skeletal muscle (*1*). The fact that antibodies to electroplaque ACh receptors cross-react with ACh receptors in skeletal muscle suggests that the structures of the two receptors are similar. However, the electrophysiological properties of the electroplaque receptor, as far as they have been studied, appear to be somewhat different from those of the receptors at the motor end plates of skeletal muscle. For example, it is known that the major effect of ACh at the muscle end plate is to produce an increase in membrane conductance to Na and K, with the conductance ratio of the two ions being such that the end-plate current, and hence the end-plate potential, reverses at a membrane potential of about –15 mv (*2*). In the electroplaque, on the other hand, the reversal potential has not been measured directly; indeed, with bath-applied ACh agonists, the agonist-induced current through the postsynaptic membrane does not reverse at any potential (*3*), apparently because the overall conductance of the drug-activated channels is reduced to zero by extreme membrane depolarization. Because of this, the reversal potential has been estimated only by extrapolation of

the relation between agonist-induced current and membrane potential obtained in the region of membrane potential where the conductance channels are still open. Similarly, extrapolation of a nonlinear relation has been used to estimate the reversal potential of the synaptic potentials evoked by nerve stimulation (*4*). The experiments to be reported here were carried out to specify more completely the action of ACh on the electroplaque in order to determine to what extent the behavior of the postjunctional receptors is analogous to that of the end-plate receptors in skeletal muscle. Specifically, we have been able to reverse the potential produced by application of ACh from a micropipette onto the synaptic membrane and have shown that the reversal potential is of the order of –4 mv.

The electroplaque was mounted horizontally between two chambers, with its innervated face exposed to the upper chamber through a small (0.43 mm^2) hole. Both chambers contained solution of the following composition (in millimoles): NaCl, 160; KCl, 2.5; $CaCl_2$, 2.0; $MgCl_2$, 2.0; tris, 2.0, buffered to *p*H 7.4 with concentrated HCl. Tetrodotoxin (1 μg/ml) was added to the upper chamber to prevent initiation of action potentials. All experiments were done at room temperature, which was about 20°C. The transmembrane potential of the innervated membrane was measured with two micropipettes glued together so that their tips were staggered by about 80 μm. One pipette was inserted through the membrane, and the other remained just outside the cell; the difference in potential between the two tips was taken as the membrane potential. The potential across the innervated face was altered by passing constant current pulses between the two chambers or, in some experiments, by voltage-clamping. Acetylcholine was applied to the membrane iontophoretically from a third micropipette filled with 4*M* ACh and placed over an ACh-sensitive spot close to the recording pipettes. Acetylcholine pulses were 50 to 60 msec in duration.

Records of synaptic potentials produced by ACh application are shown in Fig. 1A. The records were taken at the resting membrane potential (–94 mv) and at various levels of depolarization up to +33 mv. The relation between the amplitude of the ACh potential (E_s) and membrane potential (E_m) is shown in Fig. 2A. The response decreased with membrane potential, reached zero amplitude at about –3 mv, and reversed in sign as the membrane potential was made more positive. These results were typical of those obtained in a total of nine cells, except that the amplitude of the reversed response was usually somewhat smaller and, in some experiments, just barely measurable. In most experiments the amplitude of the reversed ACh potential was virtually independent of the magnitude of the positive membrane polarization. The average reversal potential for the series was -3.8 ± 4.2 mv (mean $\pm$ standard deviation).

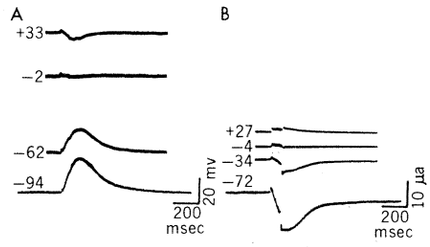

Fig. 1. Synaptic potentials (A) and synaptic currents (B) produced by iontophoretic application of ACh to eel electroplaque. Responses were recorded at the resting membrane potentials [–94 mv in (A), –72 mv in (B)] and at various levels of depolarization as indicated. Depolarization was by constant current pulses in (A), with voltage clamp circuit in (B). Tetrodotoxin was added to the bathing solution to prevent action potentials.

In other experiments the voltage clamp circuit was used to obtain the relation between synaptic current and membrane potential. Figure 1B shows records from one such experiment. At the resting membrane potential (–72 mv), application of ACh resulted in inward current across the synaptic membrane which reached a maximum amplitude of about 15 μa. As the membrane was clamped to progressively smaller levels of polarization the inward current was reduced, reaching zero at about –4 mv and reversing to outward current at more positive membrane potentials. The results of this experiment are plotted in Fig. 2B, showing the relation between synaptic current (I_s) and membrane potential. The results are qualitatively similar to those obtained with constant current pulses (Fig. 2A).

Knowing the peak synaptic current and the reversal potential (E_r), one can then calculate the peak synaptic conductance (g_s) from the relation $g_s = I_s/(E_m - E_r)$. The curve relating synaptic conductance to membrane potential is shown by the dashed line in Fig. 2B, calculated by taking current and voltage values from the solid line. It can be seen that the peak conductance decreases markedly with depolarization. In most experiments it approached zero asymptotically as the membrane potential was made increasingly more positive, becoming negligibly small at membrane potentials greater than about +40 mv. Qualitatively similar nonlinearity in the voltage-current relation, with conductance decreasing with depolarization, has been observed at the frog neuromuscular junction with iontophoretic application of ACh agonists and can be accounted for by the voltage-dependence of rate constants for opening and closing of the conductance channels (*5*).

The results reported here differ from those obtained previously with bath-applied agonists in two major respects: (i) we have been able to obtain reversal of the synaptic current; (ii) the synaptic channels appear to remain open until the membrane potential reaches +40 mv or more, whereas with bath-applied drugs the channels appear to close at about –10 mv. These differences may be related to the rate at which the activated conductance channels respond to changes in membrane potential and to changes in intracellular ionic composition during prolonged exposure to ACh agonists.

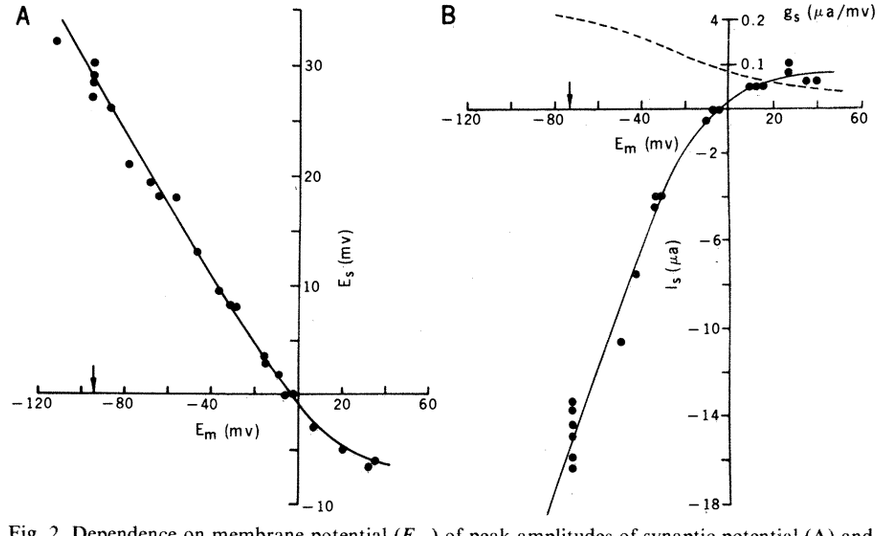

Fig. 2. Dependence on membrane potential (E_m) of peak amplitudes of synaptic potential (A) and synaptic current (B). Same experiments as in Fig. 1. In (A), synaptic potential (E_s) reverses at –3 mv membrane potential. In (B), synaptic current (I_s) reverses at –4 mv. Dashed line in (B) is peak synaptic conductance (g_s) calculated from the relation $g_s = I_s/(E_m - E_r)$, where E_r is the reversal potential.

The reversal potential obtained here (–4 mv) is some 10 mv more positive than that obtained at the vertebrate neuromuscular junction (*2*). This difference may or may not indicate a difference in the specificity of the synaptic conductance channels. Assuming, as at the neuromuscular junction, that the major conductance change is to Na and K, the ratio of the two ionic conductances is given by the relation $\Delta g_{Na}/\Delta g_K = -(E_K - E_r)/(E_{Na} - E_r)$, where Δg_{Na} and Δg_K are the drug-induced conductance changes in the postsynaptic membrane and E_{Na} and E_K are the equilibrium potentials for Na and K. For frog muscle, the conductance ratio is about 1.3 (*2*). If we take E_K for the electroplaque as –90 mv and E_{Na} as +140 mv (*4*), then the conductance ratio $\Delta g_{Na}/\Delta g_K$ is 0.60, slightly less than half that at the neuromuscular junction. However, the estimate of E_{Na} is somewhat uncertain, and if we take instead a value of +62 mv the synaptic conductance ratio would be the same in the electroplaque as at the neuromuscular junction. Consequently, until a value for E_{Na} is established accurately, there is no overwhelming reason to suppose that the electrophysiological characteristics of the ACh receptors in the two preparations are different.

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Measurement of Membrane Protein Lateral Diffusion in Single Cells

Abstract. *Fluorescence rapidly returns to spots bleached by a laser beam in the continuous fluorescence of cultured cells labeled on the surface with fluorescein isothiocyanate. The rate of recovery of fluorescence after bleaching can be interpreted as a measure of the lateral diffusion of integral membrane proteins labeled with fluorescein.*

Cell membranes are fluids whose constituent molecules move freely in two dimensions (*1*). This lateral mobility may be important to the action of cell surface receptors in transmitting signals across membranes. Also, changes in relative mobility of membrane proteins have been associated with malignant transformation of cultured cells. However, convincing values for diffusion have been derived only for lipids (*2*). Quantitative data on protein movements in cell membranes are scant. There are rough estimates of diffusion times for antigens of cultured cells (*3*), but the only precise measurements on membrane protein mobility are those by Cone (*4*) and Poo and Cone (*5*) on rotational and translational diffusion of rhodopsin in outer segment disk membranes. Poo and Cone (*5*) measured the return of native rhodopsin to a portion of the disk in which rhodopsin has been bleached. The value calculated for D was about 4×10^{-9} cm^2 sec^{-1} in concordance with previous estimates of lipid viscosity in disk membranes.

Peters *et al.* (*6*) performed an experiment, similar in principle to that of Poo and Cone (*5*), which may be applied to a variety of cell surface membranes. A fluorophore, fluorescein, was introduced into membranes by permitting fluorescein isothiocyanate to react with intact erythrocytes. The cells, covalently labeled largely in their membrane proteins, were made into ghosts and an attempt was made to follow diffusion of the proteins labeled with fluorescein from an untreated hemisphere of each ghost into a hemisphere in which the photolabile fluorescein had been bleached. Recovery of fluorescence in the bleached hemisphere, accompanied by loss of fluorescence from the intact hemisphere, would have indicated diffusion of label from one hemisphere to the other, but in this experiment no diffusion was observed. Nevertheless, the method is attractive because it should be applicable to any cell that can withstand the labeling procedure. We present data on the movement of fluorescein-labeled proteins of cultured mouse fibroblasts (L cells). Our observations on the return of fluorescence to bleached spots on the cell membrane, together with a solution of the diffusion equation applicable to the geometry of the cells, allow calculation of constants for the lateral diffusion of membrane protein.

We labeled cells of L cell–derived line Cl ld (*7*) by brief exposure to fluorescein isothiocyanate at *p*H 9.5 (*8*), at room temperature, approximately 24°C. All cells became labeled at the surface as judged by a ring of fluorescence observable at their periphery. Damaged cells fluoresced throughout their interiors. Labeling in ice in the presence of millimolar concentrations of NaCN and NaF did not affect the distribution or intensity. Freshly prepared reagents and dense healthy cultures were required for successful labeling. When old or sparse cultures were labeled, all cells were damaged by the procedure.

Fluorescence was measured on a Leitz microscope, with excitation from above the specimen. The microscope also has a side window through which the 441-nm beam of a Liconix HeCd laser was introduced, via a ×90 objective with a numerical aperture of 1.32, to bleach a spot about 5 μm in diameter at the cell periphery. The cells, although adhering to cover slips, were nearly spherical; their diameters ranged from 12 to 17 μm (with most being 12 to 15 μm). The laser beam was directed at a tangent to the sphere to form the spot; the half angle θ subtended by the bleached spot was approximately 20°. An image of the center of the spot was passed through a limiting diaphragm to a photomultiplier (*9*). All measurements were made at 22° to 24°C.

Immediately after labeling, the cells appeared to contain some fluorescein in their interiors, as well as being prominently outlined by a ring of fluorescence. When such cells were bleached and examined no recovery of fluorescence was observed within the first 2 or 3 minutes after bleaching, and the recording of fluorescence intensity was a horizontal line which did not shift during the time of observation. Such data were obtained for 42 cells.

In contrast to cells examined immediately after labeling, those cultured for at least 2 hours, or trypsinized and allowed to reattach to cover slips (minimum of 20 hours, maximum of 48 hours), were fluorescent only at the periphery, with no internal stain. All cells in untrypsinized cultures appeared to have shed labeled material collected in aggregates that were often stuck to cells. Bleaching measurements were never made at or near aggregates, but rather at regions of cell periphery that were clearly ring-stained.

When cells that had been cultured for at least 2 hours after labeling were bleached, striking recovery of fluorescence of the bleached spot could be observed (Fig. 1). During bleaching and between measurements the shutter to the photomultiplier was closed so that the trace dropped to baseline. Recovery of fluorescence, measured at intervals during the first few minutes after bleaching, is evident. In particular, immediately after bleaching, a sharp rise in intensity occurs (this is not a recorder artifact since it is not found in cells ex-