PERMEABILITY PROPERTIES OF CHICK MYOTUBE ACETYLCHOLINE-ACTIVATED CHANNELS

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ABSTRACT The acetylcholine-(ACh-) activated channels of chick myotubes were studied by the patch-clamp method. Single-channel amplitudes were measured over a wide range of potentials in solutions of cesium, arginine, and three small amines. Symmetrical, isotonic cesium solutions gave a linear I-V relationship with the single-channel conductance, γ, of 42 pS at 11°C. Dilutions of cesium by mannitol shifted the reversal potential 23.9 mV per *e*-fold change in internal cesium concentration. Selectivity, as defined by reversal potential criteria, depended on the molecular size of the permeant cation. The Q_{10} of γ for the symmetrical isotonic cesium solutions as well as internal isotonic methylamine was 1.3–1.4. These properties are qualitatively similar to those seen at the ACh-activated channel of the frog neuromuscular junction. Partially substituting arginine for internal cesium depressed outward currents. 80 mM arginine acted equally well from the inside or the outside, as if arginine transiently blocks the ACh-activated channel in a current dependent way. Diluting internal cesium almost 10-fold, from 320 to 40 mM, increased the permeability of the channel calculated from Goldman-Hodgkin-Katz equations by almost threefold. Thus, cesium itself appears to block with a dissociation constant of 135 mM. Methylamine blocked the channel approximately as well as did cesium. Ammonia and ethylamine blocked the channel somewhat more than cesium. We conclude that (*a*) the channel is qualitatively similar to that of frog neuromuscular junction, (*b*) cations bind within the channel, and (*c*) arginine decreases channel conductance equally whether applied from the inside or the outside.

INTRODUCTION

The acetylcholine-(ACh-) activated channel is not as selective as the sodium and potassium channels of nerve (Dwyer et al., 1980), although there are certain restrictions placed on ion movements through the channel. The first is charge; cations are permeant and anions are effectively excluded (Adams et al., 1981); small neutral molecules are also permeant (Huang et al., 1978). The second restriction is size; only molecules smaller than 6.5 Å are permeant (Dwyer et al., 1980). Some compounds cannot pass through the channel but can block it, including QX222 (Neher and Steinbach, 1978), long-chain *n*-alkylguanidines (Farley et al., 1981) and many other charged compounds (Masukawa and Albuquerque, 1978; Peper et al., 1982).

Further information about the channel can be gained by examining the permeability of the channel to various ions and the interaction of these ions with the channel. How easily an ion passes through a channel can be described in two ways. The first, termed selectivity, is determined by how much the ion shifts the reversal potential. The second is the rate at which ions pass through the channel, and is most directly measured by the single-channel conductance. Alternatively, a permeability can be calculated from the Goldman-Hodgkin-Katz (GHK) current equation, assuming independent movement of ions across a constant electrical field (the Independence Principle, Hodgkin and Katz, 1949; Hille, 1977). Saturation of the channel by a wide variety of externally applied cations (Adams et al., 1981) and internal sodium (Horn and Patlak, 1980) has been reported. This saturation indicates that the Independence Principle does not hold and suggests that ions bind within the channel. In such a case, the channel is considered blocked by the permeant ion, if only transiently.

When applied externally, many drugs interact strongly with the ACh-activated channel. However, the ACh-activated channel is known to be asymmetric with respect to the binding of pharmacological agents. del Castillo and Katz (1957) demonstrated that *d*-tubocurarine is ineffective when applied to the inside of the muscle cell at the frog neuromuscular junction. A quaternary local anesthetic (QX314 — Horn et al., 1980) and a long chain derivative of guanidine (octylguanidine, Farley and Narahashi, 1983) are ineffective in blocking the ACh-activated channel after internal application. Both compounds blocked ACh-induced currents when applied externally in low (millimolar or less) concentrations. Similarly, arginine was reported to block if applied externally, but to relieve block when applied internally (Adams et al., 1981).

How does this pharmacological block of the ACh-activated channel compare with that caused by permeant ions? One difference is the rate at which the channel is blocked; many local anesthetics cause brief closings that

can be demonstrated in single-channel records (Neher and Steinbach, 1978). Permeant ions interact with the channel at much faster rates, and any resulting block is seen as a smaller single-channel amplitude (Lewis, 1979). An unresolved question is whether saturation of the channel by permeant ions occurs at the same site as block of the channel by pharmacological agents. Indeed, increasing the concentration of external sodium reduces the efficacy of local anesthetics (Redmann, 1982). To examine this question further, we studied how internal ions modify the current carrying ability of the ACh-activated channel. Single channel currents were recorded from inside-out patches of chick myotubes; the selectivity of the channel is similar to that in frog. All ions tested blocked currents through the ACh-activated channel. Arginine was the ion that bound most tightly to the ACh-activated channel; internally applied arginine depressed outward currents as much as externally applied arginine depressed inward currents. Thus, chick ACh-activated channels are symmetrical in their ability to carry cesium and arginine ions into and out of the cell. Preliminary reports have appeared elsewhere (Dwyer and Farley, 1983 *a*, *b*).

METHODS

Currents were recorded from inside-out patches of membrane plucked from 10–21 d old chick myotubes. In all, 96 patches were used to yield over 6,000 single-channel currents; the experiments were performed from July through September 1982.

Patch-Clamp Technique

These experiments followed the procedure described by Hamill et al. (1981). Briefly, a portion of a plastic coverslip bearing chick myotubes, grown according to the method of Fischbach (1972), was placed in the test chamber normally maintained at 10.5° to 12°C by a Peltier device. The bath potential was driven via a silver-silver chloride pellet that sat in a beaker containing the outside solution. The beaker was connected to the bath by a salt bridge. Sharply tapered microelectrodes with tips of 1 or 2 μm in diam were lightly firepolished just before use. The electrode was filled with the external solution plus 0.5 to 1 μM AChCl, placed in a lucite holder and connected to the clamp electronics by a silver-silver chloride pellet electrode embedded in an agar bridge. Our patch-clamp circuit maintained the inside of the pipette at virtual ground and drove the bath to the desired potential. The headstage responded with a time constant of 0.23 ms to an imposed square current pulse. A seal was judged acceptable if the resistance was 5 GΩ or higher. An inside-out patch was formed and the currents were examined for series resistance artifacts. Series resistance was revealed either by a time constant of current growth of 0.4 ms or greater or by an inward or outward rectification that was markedly greater than usually seen in the particular test solution. The chamber was perfused with 8–10 ml of the test solution by a push-pull syringe, a volume sufficient for a fivefold change of the bath solution. Current records were collected on an FM tape recorder (3 dB frequency response at 2 kHz) after being amplified 10-fold and actively filtered at 2 kHz. Only rarely was the patch used for more than a single test solution. Frequently, the series resistance increased, as measured by either the appearances of rise times >0.4 ms or by a sudden decrease in single-channel amplitude. Presumably, a vesicle formed at the tip to cause this. The frequency of single-channel events generally declined with time, apparently because of desensitization.

Data Analysis

Single-channel currents were captured from the FM tape by a digital oscilloscope (Tektronix 5223; Tektronix, Inc., Beaverton, OR) at a later time. A square wave, attenuated by a ten-turn potentiometer, was superimposed on the single-channel current displayed on the oscilloscope screen. By selecting a square wave of the appropriate size, the amplitude of the single-channel current could be read directly from the dial of the potentiometer. The reversal potential was obtained by interpolation after the mean values of current amplitudes were fit to a polynomial by a derivative-free, nonlinear, least-squares fit (Dixon, 1981) and reported as mean ± asymptotic standard deviation. Permeabilities were calculated at each test voltage from the mean current amplitudes and reported as mean ± standard error of the mean. Concentrations were used throughout the analysis, except for the calculation of K_D in the discussion, where activities were used. In this case, the value of K_D was converted back to a concentration.

Solutions

The solution bathing the outside membrane face normally contained 108 mM CsCl, 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), and 2 mM $BaCl_2$. This solution was slightly hypotonic to facilitate seal formation. The barium ions were included to block calcium-activated potassium channels (Meech, 1978). In one set of experiments, the external solution was diluted with 120 mM arginine chloride (ArgCl), 5 mM HEPES, and 2 mM $BaCl_2$. In a second set, the outside solution was made hypertonic by the addition of mannitol. The internal solution was made of 120 mM XF and 5 mM HEPES, where X is the test ion. When 120 mM CsF was used inside, the solutions are termed symmetrical even though the external cesium concentration is 108 mM. In the case of cesium, methylamine, ethylamine, and arginine the base form of the test ion was neutralized with a known amount of HF to make up the solution. In all cases, the pH was adjusted to 7.2 using the test ion as the base, and HF or HCl as the acid. The osmolarity was measured between 210 and 240 mOsm for the isosmotic solutions and 710 mOsm for the hypertonic solutions. The reagents were obtained as follows: Cerac, Inc. (Milwaukee, WI): NH_4F, CsOH; Sigma Chemical Co. (St. Louis, MO): CsCl, $BaCl_2$, mannitol; Eastman Kodak Co. (Rochester, NY): ethylamine, methylamine; Research Organics Inc. (Cleveland, OH): HEPES; Mallinekrodt Inc. (St. Louis, MO): HF; Fisher Scientific Co. (Pittsburgh, PA): NH_4OH.

The liquid junction potentials were measured between a calomel reference electrode (476109; Corning Glass Works, Corning, NY) and a silver-silver chloride pellet by a pH meter accurate to 0.1 mV (130; Corning Glass Works). The pellet was grounded, placed in the 108 mM CsCl external solution and connected to the test solution by an agar bridge. The agar bridge had previously been equilibrated with the CsCl solution. This value was added to the holding potential before data analysis.

RESULTS

Measurement of Single-Channel Currents

Individual single-channel currents are shown in Fig. 1. The patch was made such that the inside surface was exposed to the bath and the outside surface was within the electrode. In this experiment, the external or pipette solution contained 108 mM CsCl and the internal solution contained 360 mM CsF. Events as small as 1/4 pA were detected and accurately measured. These records were obtained from myotubes cultured from 10-d old chick embryos. The electrode-patch seal in this case was 10 to 20 GΩ, a seal resistance commonly obtained. Patches of membranes with

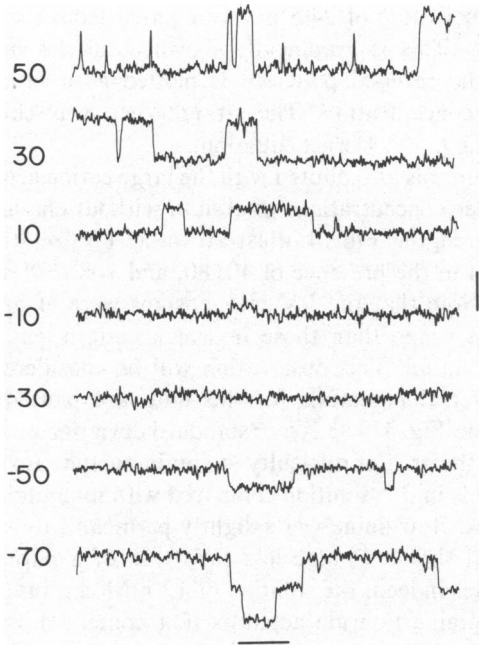

FIGURE 1 Single-channel acetylcholine-activated currents. Examples of single-channel currents elicited from inside-out patches of chick myotube by 1 μM AChCl. The inside solution was 360 mM CsF, 5 mM HEPES; outside was 108 mM CsCl, 5 mM HEPES, 2 mM $BaCl_2$. Calibrations are 2 pA and 10 ms. The channels are open longer at negative potentials than at positive. More than one channel was present in this patch since two channels opened simultaneously in the record obtained at -70 mV. Brief closings, as illustrated in the $+50$ mV record, were present at all voltages.

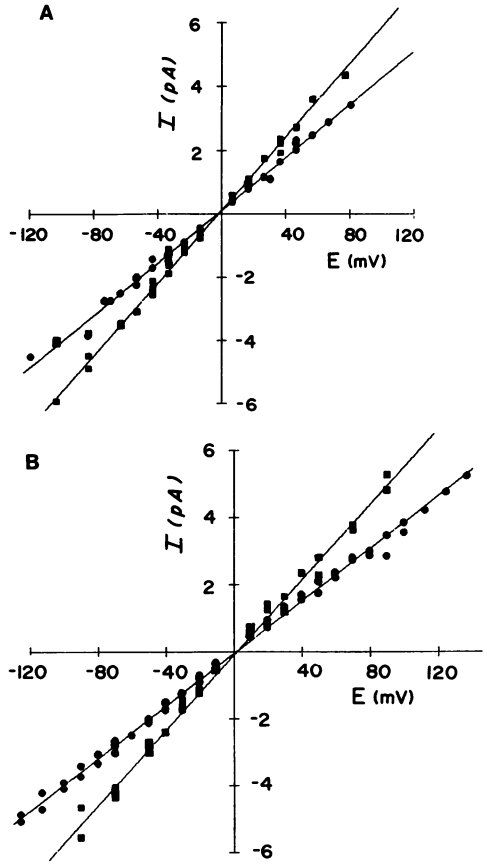

FIGURE 2 Temperature dependence of currents carried by cesium and methylamine. Inside-out patches with a pipette solution of 108 mM CsCl, 5 mM HEPES, and 2 mM $BaCl_2$ were bathed by 120 mM cesium flouride (*A*) or methylamine fluoride (*B*). Currents recorded at either 11°C (•) or 23°C (■) are plotted against the holding potential. At least eight currents were recorded at each patch; data from four to seven patches were averaged for each point. In all cases the standard error is smaller than the symbols.

the best seals would last well over half an hour, often outlasting the occurrence of ACh-activated channels.

The ACh-activated channel does not discriminate between cesium at its inside or its outside mouth when there are nearly symmetrical solutions inside and outside the membrane. Fig. 2 demonstrates this symmetry since the ACh-activated current increases linearly with voltage at 11°C and 24°C. Outside was a buffered solution of 108 mM CsCl plus 2 mM $BaCl_2$ and inside was buffered solution of 120 mM CsF. At 11°C the slope is 42.0 pS and the reversal potential of -2.7 ± 1.0 mV is close to the predicted value (-2.6 mV) for these two concentrations of cesium. This linearity contrasts with the sublinearity found by Gage and Van Helden (1979) and Horn and Brodwick (1980), but is very similar to that seen by Horn and Patlak (1980).

Temperature Dependence. Increasing the bath temperature increases permeability of the ACh-activated channel to such ions as cesium and methylamine in the way expected from the behavior of those ions in bulk solution. Fig. 2 *a* shows the current-voltage relationships for cesium currents at both 11°C and 24°C. The single-channel conductance (γ) for cesium inside increased from 42.0 ± 2.7 to 57.3 ± 4.4 pS. Thus, the Q_{10} for this process was 1.3. The Q_{10} for the diffusion coefficient of cesium over this temperature range is also 1.3 (Robinson and Stokes, 1970). The value of γ for the more bulky ion methylamine inside increased to a similar extent, from 40.2 ± 5.5 to 56.4 ± 5.2 pS, for a Q_{10} of 1.4 (Fig. 2 *b*). For comparison, the Q_{10} for the diffusion coefficient of ammonia and tetramethylamine is 1.2 (Robinson and Stokes, 1970).

Cation Selectivity. Dilutions of cesium by isotonic mannitol shifted the reversal potential by the amount predicted by the Nernst equation. In Fig. 3, current-voltage (I-V) relationships are shown at three different cesium concentrations, 360, 120, and 40 mM. The experiments with 360 mM CsF inside and 108 mM CsCl outside were done in two ways. At first, no effort was made to balance osmotic forces. In some other experiments, mannitol was added to the standard outside solution to raise the osmotic pressure to 710 mOsm. In principle, streaming potentials caused by increasing the internal osmotic pres-

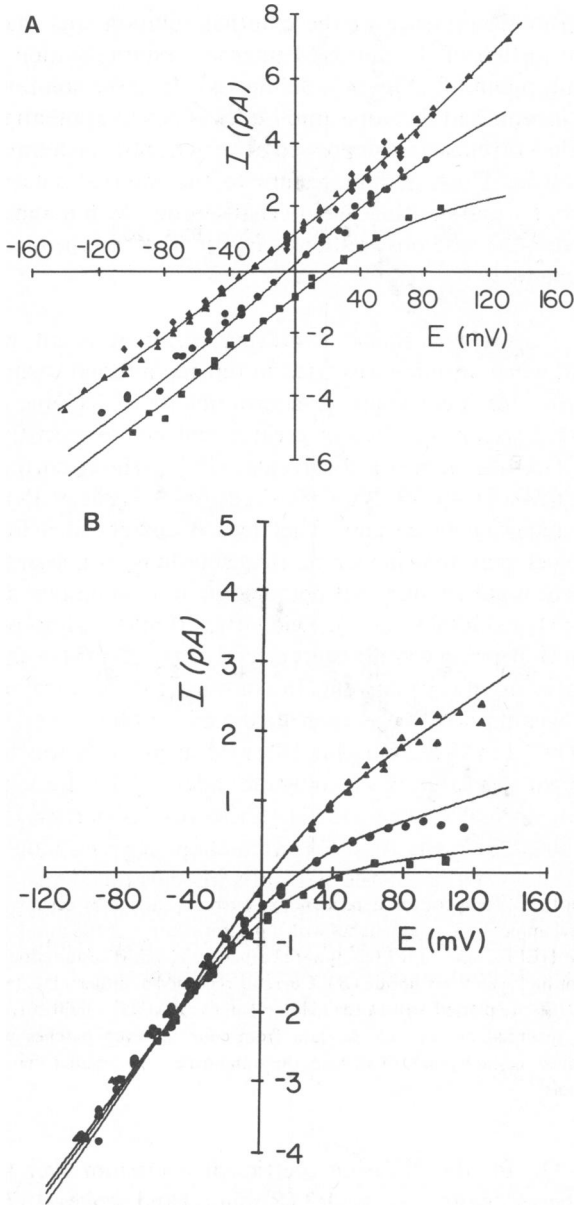

FIGURE 3 (*A*) Current-voltage curves for cesium. Three concentrations of internal cesium were examined: 40 mM cesium plus 160 mM mannitol (■), 120 mM cesium (•) and 360 mM cesium (♦ and ▲). The pipette solution contained 108 mM cesium on all experiments. The osmotic pressure across the membrane in 360 cesium was balanced by mannitol in several experiments (♦). (*B*) Dilutions of 120 mM cesium by arginine. The current-voltage relations are shown for the acetylcholine-activated channel in cesium concentrations of millimolars: 12 (■), 40 (•), and 80 (▲) the remainder being arginine. The numbers of patches for each concentration ranged from 3–9. The points are the mean of at least eight single-channel current amplitudes. The standard errors are smaller than the points. The temperature was 11°C.

sure could make the reversal potential slightly more negative. The small difference observed (-24.9 ± 3.0 vs. -26.6 ± 0.6 mV) was not significant. In the case of 40 mM cesium, the osmotic pressure was maintained by mannitol. There was a 23.9-mV shift in reversal potential per *e*-fold change in cesium concentration, a value close to the theoretical value of 24.5 mV calculated from the Nernst equation. This is graphically shown as circles in Fig. 4 where the reversal potential is plotted as a function of cesium concentration. The straight line was calculated according to the Nernst equation.

Cesium was also diluted with the large cation arginine to test lower concentrations of cesium without changing the ionic strength. Fig. 4 illustrates the I-V relationships obtained in the presence of 40, 80, and 108 mM arginine inside. Note that the I-V relationships were of markedly different shape than those in which cesium was diluted with mannitol. This observation will be considered later. The reversal potentials for this data are plotted as the squares in Fig. 4. The larger standard deviation of the data reflects the greater difficulty in obtaining and also keeping good seals in this solution compared with mannitol-cesium solutions. If arginine were slightly permeant, the reversal potential should change less than that of a pure cesium electrode. Indeed, the solution of 12 mM cesium and 100 mM arginine flouride acted as if it contained 14.4 mM CsF. Thus, arginine was 0.022 as permeable as cesium. In conclusion, the reversal potential changes can be predicted over two and a half decades of cesium concentration using the Nernst equation.

In addition to cesium and arginine, the reversal potentials of three other ions were measured. Table I shows that ammonia was the most and ethylamine the least permeant of the ions directly tested. Ammonia was almost twice as

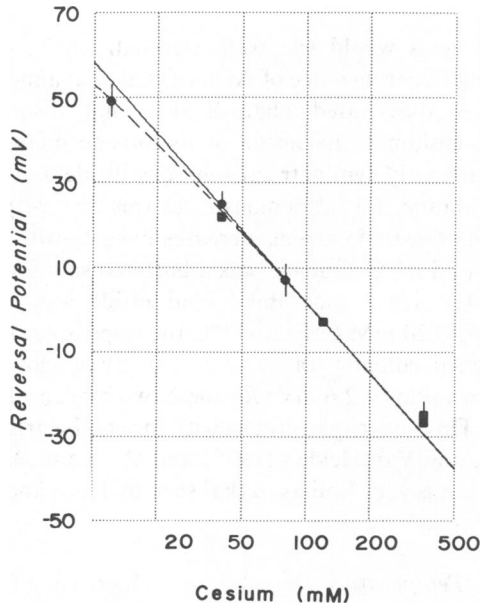

FIGURE 4 A plot of the variation of reversal potential with cesium concentration. The reversal potentials were obtained by interpolation using a polynomial fit to the I-V data (Figs. 3 *a* and *b*) by a nonlinear least squares. Osmotic balance between the internal and external solutions was maintained by mannitol (■) or arginine (•). The lines are drawn according to the Nernst equation, assuming first, that the channel is selective for cesium (—) and second, a small permeability for arginine (---). The channel behaves as if it has a small permeability to arginine.

permeant as cesium and ethylamine was about two-thirds as permeant as cesium. Methylamine was about as permeant as cesium.

Ionic Currents Through the ACh-activated Channel

Cesium Permeability. The currents through ACh-activated channels bathed by nearly symmetrical solutions can be fitted by a variety of models, including Ohm's law and the Goldman-Hodgkin-Katz constant field equation (GHK, Hodgkin and Katz, 1949). The GHK equation gives a fit to this data that is a straight line (P_{Cs} = 22 ± 1 × 10^{-3} cm/s, referred to the 6.5 × 6.5 Å cross-sectional area of the selectivity filter) and offers the advantage of predicting how the current amplitude changes as the concentration of the permeant ion is changed. ACh-activated channel currents obtained after diluting internal cesium to 40 mM by either mannitol (squares) or arginine (triangles) are shown in Fig. 5. The current predicted by the GHK equation is also shown by the solid line. This I-V relation was calculated using the mean cesium permeability calculated for 120//108 mM cesium solutions inside and out, respectively. Replacing cesium with the uncharged molecule, mannitol, yielded currents uniformly larger than predicted by GHK, as if P_{Cs} had increased to 32 ± 2 × 10^{-3} cm/s. Conversely, increasing internal cesium to 360 mM decreased P_{Cs} to 13 ± 2 × 10^{-3} cm/s (Fig. 3 *a*, triangles). In six of these experiments, the osmotic pressure of the external solution was made equal to that of the 360 mM internal cesium solution by adding mannitol (Fig. 3 *a*, diamonds). In these solutions, the currents had the same amplitudes at positive potentials, but the currents were depressed at very negative potentials by ~15%. Thus, adding cesium to the internal solution lowers P_{Cs} and diluting the internal solution with mannitol raises P_{Cs}. These observations suggest that cesium blocks the channel.

Arginine Block. A very different result was found when arginine was used to replace internal cesium. Substituting increasingly greater amounts of arginine for internal cesium resulted in greater and greater rectification, or block, of outward currents. Fig. 3 *b* shows currents recorded with 40, 80, and 108 mM arginine inside, with the remainder being cesium. The inward current was little changed from that in symmetrical solutions, although the current was less than that obtained in 40 mM cesium and 80 mM mannitol (Fig. 5). Evidently, 40 mM cesium plus 80 mM arginine was about as effective as 120 mM cesium in carrying inward current. In contrast, the outward current was markedly less than predicted by GHK. For the case of 12 mM cesium plus 108 mM arginine, γ was less than half the value expected by the Independence Principle (Hodgkin and Huxley, 1952). These results suggest that arginine blocks the ACh-activated channel even though it carries a fraction of the net outward current. Thus, the GHK model, which assumes that ions permeate independently of one another, is of limited value in describing ion transport through the ACh-activated channel.

To test the symmetry of the ACh-activated channel, we

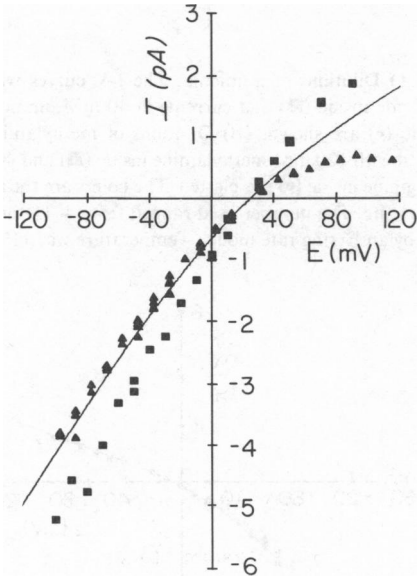

FIGURE 5 Dilution of internal cesium to 40 mM with arginine or mannitol. Current amplitudes measured from an inside-out patch, bathed by 108 mM CsCl, 5 mM HEPES, and 2 mM $BaCl_2$ outside and either 40 mM cesium and 80 mM arginine (▲) or 40 mM cesium and 160 mM mannitol inside (■) are plotted against the holding potential. The current predicted by the GHK current equation, with P_{Cs} derived from the amplitude of currents in 120//108 cesium (inside and outside), is given as a solid line (—).

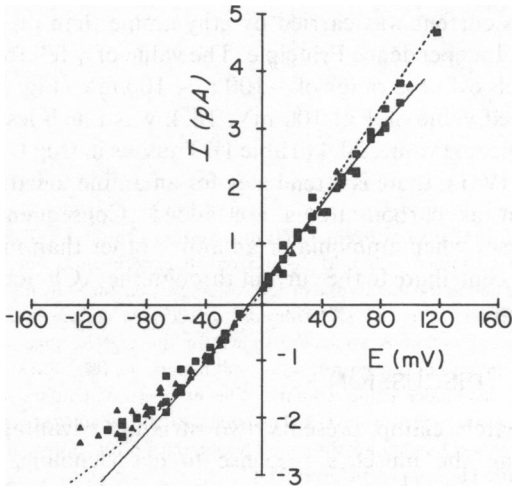

FIGURE 6 Symmetrical solutions of 40 mM arginine. The I-V for the ACH-activated channel obtained with 80 mM cesium, 40 mM arginine, 2 mM $BaCl_2$, and 5 mM HEPES outside and 120 mM cesium, 5 mM HEPES inside (■) are plotted against the holding potential. The I-V obtained with 120 mM cesium outside and 80 mM cesium plus 40 mM arginine inside are shown as ▲. The data, with arginine inside, are plotted with the currents and holding potential negated each point. Note that the data points superimpose.

measured the extent of channel block by arginine when 40 mM internal cesium or when 40 mM external cesium was replaced by arginine. If the channel is symmetrical, γ should decrease as a function of the fraction of current carried by arginine, regardless of whether arginine is added externally or internally. This was found to be the case, as illustrated in Fig. 6. To facilitate this comparison, the amplitude and holding potentials of the data obtained in 40 mM arginine inside were negated before plotting. The data obtained with arginine inside (triangles) and outside (squares) overlap, indicating that the channel interior that arginine encounters as it passes through the membrane must in reality be quite symmetrical.

Currents Carried by Amines. To test the effect of steric hindrance on permeation, we examined the family of ammonia, methylamine, and ethylamine (Figs. 7 and 8). Ammonia had a larger γ, as expected from the selectivity ratio (P_{NH_4}/P_{Cs}) of 1.87. γ averaged 50.4 ± 4.3 pS, one-quarter less than predicted by the Independence Principle. Moreover, the permeability calculated from the GHK equation decreased as the membrane potential became more positive (Fig. 7 *a*), as if ammonia carried current less well than cesium.

Methylamine, which has a diffusion coefficient similar to cesium (Robinson and Stokes, 1970), behaved similarly to cesium when permeating the channel (Fig. 7 *b*). Methylamine (120 mM) had a reversal potential near 0 mV and a γ of 40.2 ± 5.5 pS, both similar to cesium. Note that the stability of the patch in methylamine was very good compared with other solutions. The records also had very low noise. The reasons for this are unknown, but it was our consistent observation.

Less current was carried by ethylamine than predicted by the Independence Principle. The value of γ fell from 40 to 20 pS over the range of -100 to $+100$ mV (Fig. 8); the observed value of γ at 100 mV, 16.4, was much less than the expected value, 24.4 (Table I). Thus, as in frog (Adams et al., 1981), there is a tendency for an amine to carry less current as carbon atoms are added. Consequently, γ decreases when ammonia or an amine other than methylamine contribute to the current through the ACh-activated channel.

DISCUSSION

The patch clamp presents two striking advantages in studying the muscle's response to acetylcholine. First, there is wide latitude in composition of the solutions in which the inner and outer surfaces of the cell membrane can be bathed. Second, the characteristics of individual channels can be determined, uncomplicated by the uncertainty of how many channels are open. We will first discuss how our findings compare with others. Then we will consider the implications of our data in terms of channel function.

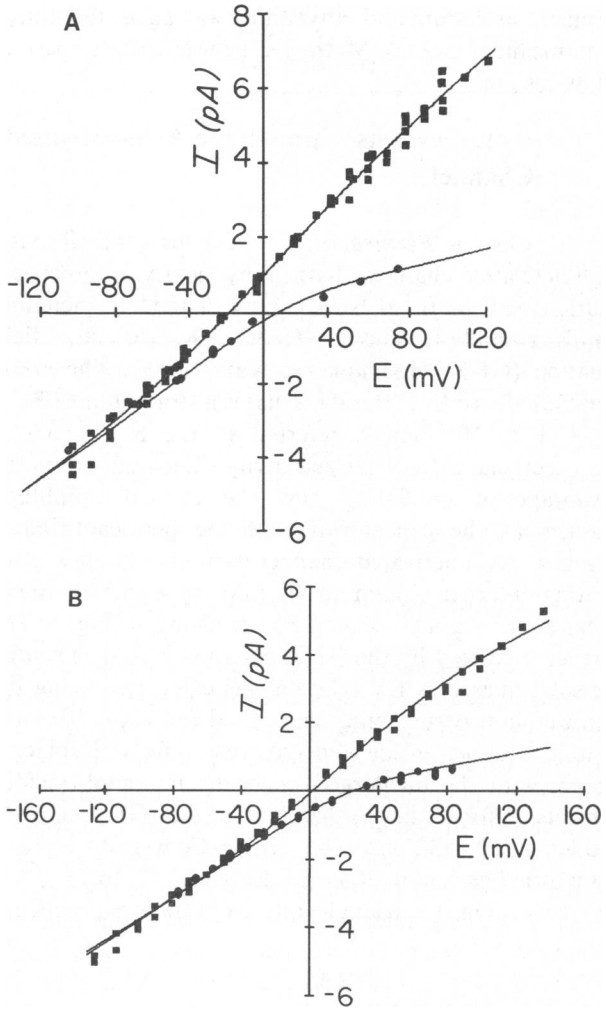

FIGURE 7 (*A*) Dilutions of ammonia. The I-V curves with 120 mM ammonia flouride inside (■) and currents in 40 mM ammonia, 80 mM arginine inside (•) are shown. (B) Dilutions of methylamine. The I-V curves obtained with 120 mM methylamine inside (■) and 40 methylamine plus 80 arginine inside (•) are plotted. The points are the average of at least eight currents. The number used ranged from 4–11 for each curve. The lines are by an Eyring rate model. Temperature was 11°C.

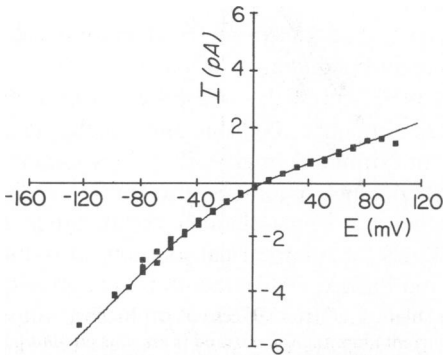

FIGURE 8 Current-voltage curve of ethylamine. Current amplitudes recorded with 120 mM ethylamine inside are plotted against holding potential. Six patches were used to obtain this I-V. Note the marked curvature of the I-V especially at positive potentials. The line is by the model considered in the discussion. Temperature was 11°C.

TABLE I
SELECTIVITY AND SINGLE-CHANNEL CONDUCTANCE

Test ion	Chick				Frog	
	E_r	N	P_X/P_{Cs}	γ	P_X/P_{Cs}	γ
Ammonia	−18.0 ± 0.8	11	1.87	50.4 ± 4.3	1.26	43.7
Cesium	−2.7 ± 1.0	7	1.00	42.0 ± 2.7	1.00	28.9
Methylamine	−0.9 ± 1.1	4	0.93	40.2 ± 5.5	0.94	25.0
Ethylamine	7.9 ± 1.4	6	0.65	16.4	0.80	8.0
Arginine*	49.2 ± 1.3	8	0.02	3.0	<0.03	—

Data were calculated from inside-out patches with the interval reference solution being 120 mM CsF, 5 mM HEPES. γ for ammonia, cesium, and methylamine is the average of all the observations. γ for ethylamine and arginine is the value at 100 mV, obtained from a linear regression of γ *vs.* voltage. External solution was 108 CsCl,2 $BaCl_2$, 5 HEPES. Temperature was 11°C.

*Calculated from mixture of 12 CsF and 108 arginine F plus 5 HEPES.

Comparison with Previous Work

Single-Channel Conductance. Analysis of excess membrane noise observed during exposure to acetylcholine provided the first measure of single-channel conductance (γ, Katz and Miledi, 1972). Values of γ of ~25 pS have been obtained at amphibian neuromuscular junctions bathed in sodium containing Ringer's solution at ~20°C (Anderson and Stevens, 1973; Gage and Van Helden, 1979; Colquhoun et al., 1975; Adams, et. al., 1981). Replacing external sodium with cesium modestly increases the single-channel conductance in most poikilotherms (to 34 pS in toad, Gage and Van Helden, 1979, and to 29 pS in frog, Adams et al., 1981; however, when half the sodium is replaced by cesium, γ at snake ACh-activated channels increases from 26 to 46 pS, Hoffmann and Dionne, 1983). Chick myotube ACh-activated receptors have been reported to have the same conductance as frog (25–30 pS, Fischbach and Lass, 1978). However, there is a systematic underestimation of γ by noise analysis when compared with single-channel measurements (Fenwick et al., 1982).

We report that in symmetrical cesium solutions the single-channel conductance in chick myotubes is 42.0 ± 2.7 pS at 11°C and 57.3 ± 4.4 pS at 23°C. In agreement with these findings, Nelson and Sachs (1979) have reported an estimated single-channel conductance of ~60 pS at 22°C in chick myotubes. Rat muscle ACh-activated channels have a single-channel conductance similar to chick, ~42 pS for symmetrical solutions of sodium (Fig. 5 of Horn and Patlak, 1980) and 48 pS in a growth medium (Jackson and Lecar, 1979). Amphibian muscle ACh-activated channels have a smaller single channel conductance (28 pS, Neher and Steinbach, 1978).

Temperature Dependence of Current Amplitudes. The temperature dependence of single-channel conductance (Fig. 2) is very similar to that of free diffusion for the ions tested, which in turn is the same as the viscosity of water (Robinson and Stokes, 1970). This finding agrees with other reports based on single-channel measurements (for chick: Nelson and Sachs, 1979) or noise analysis (for amphibia: Anderson and Stevens, 1973, and Gage and Van Helden, 1979). A notable exception is the report of a large Q_{10} at snake endplates, calculated from noise analysis (Hoffmann and Dionne, 1983). It seems likely that the mechanisms of ion flux through the snake ACh-activated channel differs from that at the amphibian, chick, and mammalian ACh-activated channels, particularly because only the snake channel shows markedly different γ's for the various alkaline metals (Adams et al., 1981; Dwyer and Farley, unpublished observations; Hoffmann and Dionne, 1983). For a channel that obeys the Independence Principle (i.e., no binding occurs), the Q_{10} of γ for an ion would be very similar to its diffusion coefficient in free solution. For

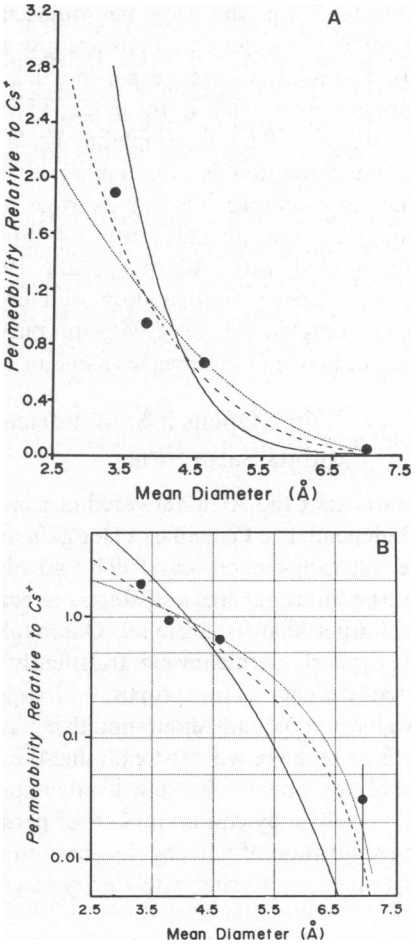

FIGURE 9 Selectivity and molecular dimensions. (*A*) Selectivity of the ACh-activated channel to ammonia, methylamine and ethylamine was obtained by measuring the shift of the reversal potential (E_r) caused by replacing cesium (inside) with the test ion. The permeability ratio is given by ln $E_r/RT/F$, where R, T, and F have the usual thermodynamic meanings. The permeability of arginine was calculated from the reversal potential of a solution of 108 mM arginine plus 12 mM cesium. The molecular size was determined from Corey-Pauling-Koltum space filling models. (*B*) Data are replotted semi-logarithmically for clarity.

this reason it is interesting that conductance has a low Q_{10}, equivalent to that of free diffusion, even though we demonstrate the binding of permeant ions in the channel, as have others (Lewis, 1979; Adams et al., 1980).

Selectivity. Fig. 9 shows that the larger the ion as measured from CPK models, the smaller the permeability ratio. Steric hindrance alone predicts the greater selectivity against larger ions for both the frog and chick ACh-activated channel (Dwyer et al., 1980, and Fig. 9, this paper). To approach more closely the behavior expected for a diffusing ion, we can include a simple term for drag through the medium (dashed line). If the viscous drag is included, the discrimination between a smaller and a larger ion becomes even more steep, as shown by the solid line. Although the amplitudes of these lines are arbitrary, note that these three fits are, respectively, (*a*) equal to, (*b*) three-quarters of, and (*c*) one-half of the amplitude of the corresponding lines for the frog neuromuscular ACh-activated channel; the exact equations are given in Dwyer et al. (1980). The relations are steeper in chick than frog, as if the channel were smaller in the chick. Thus, in chick, P_{EtN}/P_{NH_4} is 0.35 vs. 0.63 in amphibia (Adams et al., 1981). This interpretation is confounded by the larger single-channel conductance. On the average, the single-channel cesium currents in chick are 1.4 times that of amphibia (compared with Adams et al., 1981). This discrepancy is a further confirmation that conductance measurements complement selectivity in our complete understanding of how an ion traverses a channel.

Acetylcholine Opens a Symmetrical Pore That Exhibits Saturation

Fig. 5 illustrates that the ACh-activated channel fails one test for the Independence Principle of Hodgkin and Huxley (1952). The outward current carried by 40 mM cesium depends on the diluting species, since the currents in mannitol are larger than in arginine. One explanation is that arginine, a poorly permeant ion, transiently blocks the ACh-activated channel. This proposal is supported by calculations given below that show similar behavior for all permeant ions tested. We will first examine the symmetry of the action of arginine. Next, we will calculate the block by arginine, as well as by cesium and other permeant ions two ways: by saturation of current flow assuming a single binding constant or by Eyring rate theory (Eyring et al., 1949).

Symmetry of the ACh-activated Channel. The symmetry of the ACh-activated channel with respect to arginine block is strikingly demonstrated by the overlap of data points in Fig. 6. If arginine were to bind more tightly when applied to the outside than to the inside (Adams et al., 1981), the inward currents in the presence of arginine-out should be smaller than the outward currents in the presence of arginine-in. This is certainly not the case, as the currents carried by 40 cesium plus 80 arginine into a solution of 120 cesium are equal in size, regardless of the direction taken. The symmetry we have observed for arginine block of single-channel currents is in contrast to that block of macroscopic currents, where external application of arginine was more effective than internal application (Adams et al., 1981). Such macroscopic currents are the sum of the currents through many single channels. Thus external arginine must act to markedly decrease the number of open channels during the iontophoretic pulse, compared with the control or with the internal arginine experiments. Similar interpretations have been given for the action of a variety of pharmacological agents. For example, QX314, octylguanidine, and D-tubocurarine are known to be ineffective when applied internally at concentrations much higher than those needed to block macroscopic ACh-activated currents externally (Horn et al., 1980; Farley et al., 1981; del Castillo and Katz, 1957).

Saturation of Current and Permeability. The binding constant of an ion can be estimated directly by examining current amplitudes at different ion concentrations. Plotting the current amplitudes from Fig. 3 *A* at +100 mV against cesium ion activity gives a binding constant of ~150 mM, with a saturating current of 9.0 pA. This is similar to the case of sodium at the rat ACh-activated channel (Horn and Patlak, 1980). An improved estimate of channel occupancy by an ion uses permeability, as calculated by the GHK current equation, rather than current as an estimate of channel occupancy. This approach automatically accounts for both the increase in driving force with the increase of internal cesium concentration and also for the curvature of the I-V relationship. Applying this method for values at +100 mV, the binding constant, K_D, is ~135 mM and the limiting value for permeability, P_{Cs}, is 66×10^{-3} cm/s.

Relative Binding Constants. If a channel obeys the Independence Principle and if we know the relative selectivities of two ions calculated from reversal potentials, we can predict the relative size of the currents in these solutions (Hodgkin and Huxley, 1952), assuming that the solution on only one side of the membrane varies. Large deviations from these predictions were observed. Assuming that these deviations are due to channel block by the permeant ion, we can calculate a value for K_D, relative to cesium. Adams et al. (1981) gave the case for substituting one external ion for another. If the internal ion is varied, the single-channel conductance, γ, for a test ion, X_I, relative to γ for the standard ion, S, at a given voltage, E, is predicted to be

$$\frac{\gamma_X}{\gamma_S} = \frac{P_X}{P_S} \cdot \frac{[X_I]}{[S_I]} \cdot \frac{\exp E'_X - \exp E'}{\exp E'_S - \exp E'} \cdot \frac{E - E_S}{E - E_X},$$

where P_X/P_S is the relative permeability as given by the reversal potential. E' is E divided by RT/F. E_X and E_S are the reversal potentials for the test solution and the standard solution of cesium. As long as the external solution composition is constant, here 108 mM CsCl, a deviation between the predicted and the observed ratio indicates a departure from independence. Following the convention of Adams et al. (1981), Q is the ratio of the observed over the predicted. The values for Q are given in Table II, all relative to cesium.

If we first examine binding at −100 mV where most of the current is inward and is carried by cesium, the value of Q is within 10% of the expected value for all ions tested. That is, all inward currents tend to the same value (Fig. 1 *A*). This result differs from the analysis of ACh-activated noise at the frog neuromuscular junction (Adams et al., 1981), where γ at −73 mV increased by 10% when 90% of the internal cesium was replaced by arginine. We found the chord conductance fell by 54% at −80 mV when cesium was diluted by this amount of arginine.

At +100 mV, most of the current is carried by the test ion, and Q should be a measure of the overall binding constant for that ion, relative to cesium. Measured in this way, the binding sequence is Cs MeN < NH_4 < EtN < Arg (Table II). Despite the fact that Q is approximately the same for ammonia and ethylamine, the I-V for ethylamine curves more than that of ammonia. The increased curvature arises because of the difference in selectivity of the channel for ethylamine and ammonia ions ($P_{EtN}/P_{NH_4} = 0.35$). Thus, there appears to be only 0.35 times as many permeant ions in the internal solution when ethylamine is compared with an equimolar ammonia ion solution. The lower apparent concentration decreases single channel current.

Eyring Rate Theory. Lewis (1979), Horn and Brodwick (1980), and Lewis and Stevens (1979) have proposed Eyring models to describe ion permeation through endplate channels. The model has two barriers and one binding site. We have used a modified form of this model to fit our I-V data. The lines drawn through the I-V's of Figs. 3 *a*, *b*, 6–8, were generated according this scheme. We used a nonlinear least-square fit to the data. Well depths were calculated by the assumptions of Hille (1975). We first fit the 40 mM cesium I-V because cesium binding should be smallest at this concentration. Then the remaining cesium I-V curves were fit using these values for barrier heights and well depths. As more ions were added into the model, the values determined for any ion before the one being fitted were fixed at the values previously determined. The values for barrier heights and well depths are given in Table II. The well was located 43% of the way through the membrane field from the outside. The barriers were located 10 and 80% of the way into the membrane field.

TABLE II
CHANNEL BINDING AND BARRIER CONSTANTS

	Cs	NH_4	MeN	EtN	Arg
Q	1.0	0.76	0.98	0.53	0.51*
K_D	135	81	130	47	33
$Q_{(frog)}$	1.0	1.28	0.91	0.33	0.42*
G_1	6.2	5.5	6.4	6.6	11.2‡
G_2	5.6	5.1	5.3	6.0	9.5
G_3	−1.1	−1.5	−1.0	−1.4	−1.2

The fractional binding (Q) is relative to cesium. Q is obtained by the Hodgkin-Huxley Independence test; Q_{frog} is similarly calculated except that these values refer to the outside of the ACh-activated channel (Adams et al., 1981). Binding to the center well, K_D, is given as millimoles per liter. The outer and inner energy barriers, G_1 and G_2, and the energy well, G_3, of the model are given in kT units. Binding to the surface sites inside and outside of the voltage field is set to 500–750 mM, depending on the ion species.

*Calculated for mixtures of 80 mM arginine and 40 mM cesium (Q), or 57 mM arginine and 67 mM sodium (Q_{frog}).

‡Fixed at 5 kT greater than cesium to account for the shift in reversal potential.

This analysis yields two important results. First, no binding site selective for metal ions over organic ions need be postulated. If a distinct metal ion binding site existed (Adams et al., 1981), the mixtures of an organic plus a metal cation, i.e., arginine plus cesium, would give a more curved I-V than mixtures of two organic ions, i.e., arginine plus methylamine. This is not the case (see Figs. 6 and 7). Second, the "constant offset assumption" of Hille (1975) is true for the four most permeant cations within 0.5 kT, as shown by Table II.

The two barrier-one site model has three limitations in predicting our data adequately. First, the observed I-V in symmetrical cesium solutions is linear. This type of I-V can only be approximated by a model of this type, as shown in Fig. 3 *a* (circles). Moreover, to obtain a relatively straight line for the symmetrical cesium data, it was necessary to have the barriers and the well asymmetrical located in the membrane field, and the outer barrier slightly larger than the inner. Thus the model is inconsistent with our experimental result that arginine blocks the channel equally well from either membrane surface; this indicates that the channel is in fact symmetrical. Second, the model does not predict the degree of block by arginine at very positive potentials with arginine inside, or at very negative potentials with arginine outside. A third limitation of the model is that the current amplitudes are overestimated as the internal cesium concentration is increased from 40 to 360 mM. For example, if a fit is made of the 108//40 mM cesium data by the model and the energy well depth, barrier heights and locations are used to fit the 108//360 mM data, the currents are overestimated by 20% at all potentials. Thus, our model could be made to fit by multiplying the predicted currents by a constant (0.8). The discrepancy is unlikely to be a surface potential effect

(Lewis, 1979). A second possibility is that there are binding sites at the inner and outer channel mouth that are not in the membrane field (Adams et al., 1981). If these sites are in equilibrium with the bulk solution, then the currents will be reduced by a constant factor related to the K_D of this site for the ion. Taken together, these discrepancies suggest that an improved Eyring rate model for the ACh-activated channel should be symmetrical and contain at least three binding sites.

CONCLUSION

The chick myotube ACh-activated channels behave qualitatively like frog ACh-activated channels, that is, as if they are large water-filled pores. The chick myotube channels are apparently smaller in diameter but with a larger conductance than in frog. Cesium, arginine, methylamine, ethylamine, and ammonia all bind within the channel, indicating that the GHK current equation does not hold since independence is violated.

The channel is symmetrical with respect to the action of arginine applied either inside or out, suggesting that the energy profile of the channel is in fact symmetrical for permeant ions. The simple two barrier-one binding site model cannot account for all of the data presented here. We suggest that the ACh-activated channel may have more than one ion binding site.

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