A K^+-SELECTIVE CONDUCTANCE SENSITIVE TO CHOLINERGIC ANTAGONISTS OBTAINED BY THE FUSION OF AXONAL MEMBRANE VESICLES TO PLANAR BILAYERS

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1. Introduction

In several preparations, electric parameters of axon and (or) Schwann cell may be affected by cholinergic ligands [1–4]. It has also been demonstrated that axonal membranes contain binding sites for a variety of cholinergic ligands [5–7]. Visualization of these binding sites proved possible by the use of a conjugate of α-bungarotoxin and horseradish peroxidase [8,9]. An important question that arises is whether the cholinergic binding sites of axons are associated with specific receptor channels. Here we address this question in an in vitro membrane system readily accessible to electrical measurements: a hybrid membrane formed by the fusion of axonal vesicles to a planar phospholipid bilayer. Using conditions shown to enhance fusion of native or reconstituted membrane vesicles to planar bilayers [10–12], we demonstrate here the incorporation of a K^+-selective conductance from crustacean axonal membranes and the effect of cholinergic antagonists on this conductance.

2. Materials and methods

Axonal membrane vesicles prepared from lobster (*Homarus americanus*) walking nerve bundles, following [13], were equilibrated in 0.4 M sucrose, 5 mM Tris–HCl (pH 7.4). Vesicle suspensions were shipped packed in ice. Bilayers of the Mueller-Rudin type [14] were formed on Teflon cups having an aperture of 0.8 mm in diameter. The phospholipid mixture (Avanti Chemicals) was composed of 60% phosphatidylethanolamine (PE), and 40% acidic phospholipid (phosphatidylserine (PS), phosphatidylinositol (PI), diphosphatidyl glycerol (DPG) at a molar ratio of PS:PI:DPG = 1:1:0.4). Lipids were dissolved in *n*-decane and applied to the cup at 35 mg/ml. The aqueous phase was composed of 100 mM KCl, 0.1 mM EGTA, 5 mM Hepes–Tris (pH 7.4). Measurements were carried out at 21–23°C under voltage-clamp conditions as in [10]. *cis* defines the side of the bilayer to which axonal vesicles are added while *trans* (opposite side) is the zero-voltage chamber.

3. Results and discussion

Incorporation of axonal membranes to planar bilayers was accomplished by Ca^{2+}-dependent fusion of vesicles with bilayers containing a large amount of negatively charged phospholipids [10]. As shown in fig.1A, addition of vesicles to the aqueous phase of a bilayer in the presence of Ca^{2+}, leads to an increase in membrane conductance by 2–3 orders of magnitude. Increase in conductance is stopped by Ca^{2+} chelation or by removing the non-incorporated vesicles from the *cis* chamber. As demonstrated in other systems [10,11], no massive fusion of vesicles ocurred in neutral membranes containing PE as the only phospholipid or if Ca^{2+} is not present (not shown).

Fig.1B shows that increase in conductance proceeds by discrete jumps, the conductance of single jumps was variable, ranging from 100–300 pS in 0.1 M KCl. The large variation in conductance of the single jump suggests that each individual jump represents the fusion of a single vesicle with a variable number of channels [10–12,15]. This was confirmed in experiments (car-

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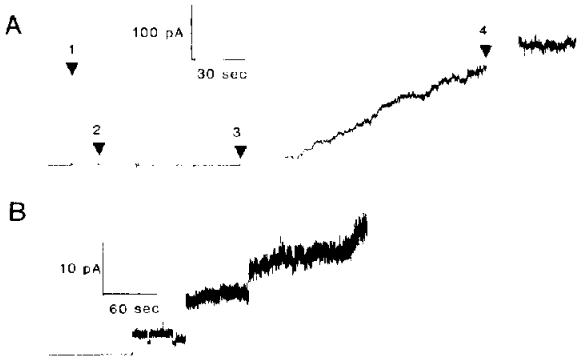

Fig.1. (A) After formation of the bilayer in 100 mM KCl buffer the bilayer was voltage-clamped to +30 mV and this potential was maintained constant during the recording (arrow at 1); (2) $CaCl_2$ was added to the *cis* chamber to a final concentration of 1.8 mM; (3), an aliquot of axonal vesicles was added to the *cis* side; Protein in the chamber was 20 μg/ml; (4) incorporation was stopped by addition of 2 mM EDTA to the *cis* side. Interruptions in current trace correspond to the time that the *cis* solution was stirred. (B) Fusion events were recorded at high gain immediately after the addition of axonal vesicles (20 μg protein/ml). $CaCl_2$ was 1.8 mM and the applied voltage, +30 mV.

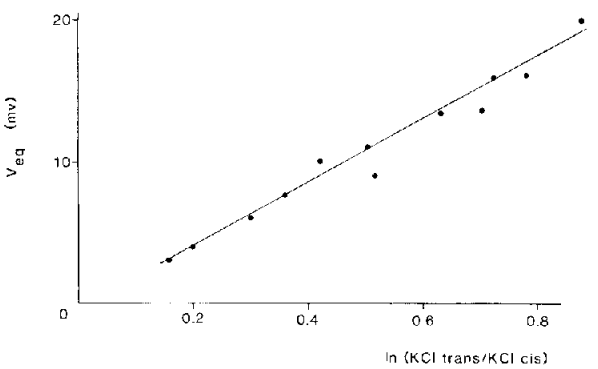

Fig.2. Axonal vesicles were fused as in fig.1. After the membrane conductance reached 3–6 nS, fusion was stopped by perfusing the *cis* side with KCl buffer containing no Ca^{2+}. Equilibrium potentials were measured by increasing the *trans* concentration of KCl. Under these conditions [(KCl) *trans* > (KCl) *cis*], a negative current develops at 0 mV which can be compensated by clamping the bilayer to positive potentials. The potential at which the current is zero (V_{eq}) was measured for several [KCl] *trans*:[KCl] *cis* ratios. V_{eq} was plotted, according to the Nernst equation, as a function in ln (KCl) activity ratio. The slope is 23.0 ± 1 mV (±SE).

ried out in the laboratory of C. Miller, Brandeis University) done on a fast time scale in bilayers in which single fusion jumps were allowed. After the single jumps, 1–3 fluctuating channels were observed. Channels have a conductance of 200 pS in 0.5 M KCl and mean open times in the order of 200 ms (not shown). This observation also indicates that the macroscopic conductance studied here is channel-mediated.

Table 1
Cation selectivity of axonal conductance incorporated in planar bilayers

Cation (X^+)	V_{eq} (mV)		P_X/P_K
Tl^+	15.6 ± 0.3	(3)	2.02
K^+	–		1.00
Rb^+	+10.6 ± 0.2	(11)	0.65
NH_4^+	+30.0 ± 1	(4)	0.30
Cs^+	+33.2 ± 1	(6)	0.23
Na^+	+52.8 ± 2	(5)	0.12
Li^+	+65.5 ± 2	(6)	0.074

Reversal potentials, V_{eq}, were measured under bi-ionic conditions consisting of 100 mM KCl on the *trans* side and 100 XCl on the *cis* side. In case of the pair Tl^+/K^+, the anion was acetate instead of chloride. After fusion of vesicles in symmetrical 100 mM KCl solutions, the *cis* chamber was extensively perfused with the test cation solution, to set the bi-ionic condition and to remove Ca^{2+} and nonincorporated vesicles. Reversal potentials were measured as in fig.2 and text. Permeability ratios were calculated as described [16] and corrections were made for ionic activities. V_{eq} reported corresponds to mean ± SE; numbers in parenthesis correspond to number of determinations.

To determine which ion in KCl contributes to the conductance of bilayers containing axonal membranes, we measured the zero-current potential generated when the *trans* concentration of KCl is increased. The potential at which the membrane current is zero (reversal potential) is found by applying voltage pulses of different amplitude to the bilayer [16]. Fig.2 shows a plot of reversal potentials *vs* ln (KCl *trans*/KCl *cis*) activity ratios. Potentials are positive in sign; the slope of the plot is 23 mV, as compared to a slope of 25.3 mV predicted by the Nernst equation for a bilayer ideally selective for K^+ over Cl^- (see legend fig.2). The transport number for K^+ is 0.9 ± 0.02 and is independent of the ionic strength of KCl within 50–500 mM. Thus, the hybrid membranes are almost perfectly K^+ permeable. The steady-state K^+ conductance is independent of voltage in the range of ± 60 mV (not shown).

Table 1 presents the cation selectivity of the axonal conductance incorporated in bilayers. K^+ is the most permeable of all alkali cations and there is a particularly high discrimination against Na^+ and Li^+. The overall selectivity sequence is typical of that followed by K^+ channels, of nerve membranes [17,18]. Similar to the findings in nerve K^+ channels, Tl^+ is the only cation in

Table 2

	Half inhibition constants		Potential	% inhibition
	cis	*trans*		
α-Tubocurarine	0.3 mM	1.2 mM	−30 mV	65–80
Atropine	0.7 mM	>3 mM	−20 mV	60–70
Decamethonium	0.15 mM	0.55 mM	+20 mV	50–60
Tetracaine	0.2 mM	>0.85 mM	−20 mV	60–65

cis and *trans* titrations were done on different membranes. Carbamylcholine (1 mM), Acetylcholine (1 mM), Tetrodotoxin (100 μM), Veratridine (200 μM), Cs^+ (30 mM); Tetraethylammonium (5 mM), γ-aminobutyric acid (1 mM) and substance P (1 μM) added to the *cis* or *trans* side, at the concentrations indicated were ineffective

our system more permeable than K^+ [17,18].

While blockers of voltage-dependent K^+ channels such as Cs^+, TEA, 4-amino-pyridine and 3,4-diamino-pyridine have no effect on the K^+ conductance described, blockade can be achieved by some cholinergic antagonists. Fig.3 shows the inhibition by d-tubocurarine. Addition of curare to the *cis* or *trans* side of bilayers yielded different half inhibition constants, the *cis* side being much more sensitive to inhibition. Similar differences in *cis* or *trans* inhibition were seen with decamethonium, atropine and the local anesthetic tetracaine. Table 2 summarizes these data as well as the lack of effect when several other drugs were tested. Inhibition of conductance was also observed when drugs were added at equal concentration at both sides of the bilayer; thus, effects arising from asymmetric

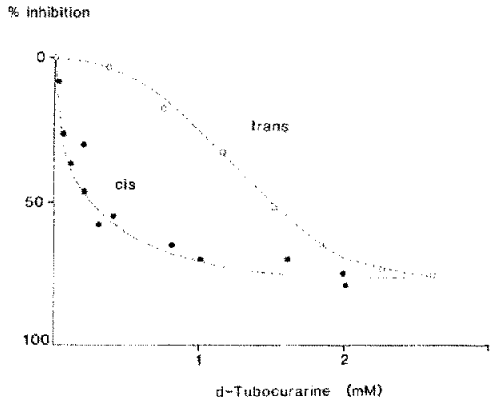

Fig.3. Titrations were done by addition of small aliquots from stock solutions of inhibitor either to the *cis* side (•) or *trans* side (○) on separate membranes. The holding potential was −30 mV. Data points correspond to measurements on 4 different membranes. Segmented lines have no theoretical meaning.

changes in surface potentials [19] can be ruled out. The striking differences in *cis* or *trans* inhibition indicate that the K^+ conductance inserted in the bilayer has a high degree of orientation.

The lack of block by TEA and 4-aminopyridine and the lack of activation by cholinergic agonists indicates that the axonal conductance described is different from the classical K^+ channels of axons and cholinergic receptors of synapses. However, the inhibition observed with cholinergic antagonists and local anesthetics is in qualitative agreement with the binding studies in crustacean membranes [1–3].

At this time, the location of the axonal conductance is not certain since the fused vesicles could have their origin in the axolemma or in the Schwann cell membrane. However, the histochemical evidence in lobster nerve indicates that cholinergic markers bind to the axolemma [4]. Interestingly, the axonal conductance follows the sequence $Tl > K > Rb > NH_4 > Na > Li$, a sequence which is quantitatively identical with that in [18] for the resting conductance of the squid axon. Thus, if present in the axolemma, this conductance might play a part in controlling the resting potential of the crustacean axon or serve as a pathway for K^+ efflux during repolarization. The latter function could be analogous to the TEA, 4-aminopyridine-insensitive K^+ channel which repolarises the Mauthner axon of goldfish [20].

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