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Triphenylmethylphosphonium is an ion channel ligand of the nicotinic acetylcholine receptor

(lipophilic cations/ionophore/potential measurement)

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ABSTRACT **The lipophilic cation triphenylmethylphosphonium (Ph_3MeP^+), which is widely used as a sensor for membrane potential with cells, organelles, and membrane vesicles, is shown also to accumulate in membranes rich in nicotinic acetylcholine receptor in a voltage-independent way. Evidence is presented that Ph_3MeP^+ in this sytem is bound to a cation-binding site of the ion channel that is part of the acetylcholine receptor complex. Binding is stimulated by cholinergic effectors (K_d = 13 μM in the absence of carbamoylcholine; K_d = 1.5 μM in the presence of 10 μM carbamoylcholine), and this stimulation is blocked by α-bungarotoxin. Ph_3MeP^+ blocks efflux of ^{22}Na from receptor-rich microsacs and appears to compete with the channel ligand phencyclidine for a common binding site. In contrast to the binding of other proven channel ligands, Ph_3MeP^+-binding is not affected by desensitization.**

The lipophilic cation triphenylmethylphosphonium (Ph_3MeP^+) and the related compound tetraphenylphosphonium are widely used as membrane potential sensors for whole cells (1, 2), subcellular fractions such as synaptosomes (3), and membrane vesicles (4). These cations accumulate into the membrane-enclosed compartment, when a potential (interior negative) is generated across the membrane. Using radioactively labeled ions, it is possible to determine the amount of ions accumulated by a simple filtration assay (5). The concentration difference of cations between inside and outside can be used to calculate the membrane potential with the Nernst equation.

We intended to apply this method for the investigation of the potentials across membranes of vesicles prepared from the electric organ of *Torpedo marmorata*. Surprisingly, we found that with these vesicles accumulation of Ph_3MeP^+ not only is voltage dependent but also is stimulated in a voltage-independent manner by a variety of cholinergic effectors.†

MATERIALS AND METHODS

Reagents. The following reagents were obtained from New England Nuclear: [^{3}H]$Ph_3MeP^+Br^-$ (36 Ci/mmol), ^{3}H-labeled 1-(1-phenylcyclohexyl)piperidine (phencyclidine, PCP) (48 Ci/mmol), [^{3}H]acetylcholine (50–100 mCi/mmol), and ^{22}NaCl (200 μCi/ml) (1 Ci = 3.7×10^{10} becquerels). All other reagents were of the highest commercially available purity.

Preparation of Acetylcholine Receptor-Rich Membrane Fragments. Membrane fragments were prepared as described in ref. 8.

[^{3}H]Ph_3MeP^+ and [^{3}H]PCP Binding to Acetylcholine Receptor-Rich Membrane Fragments. The receptor-rich membrane suspension is incubated overnight at 4°C in either Na-rich medium, which contains 160 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, and 10 mM Hepes at pH 7.4, or K-rich medium, which contains 165 mM KCl, 2 mM $CaCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, and 10 mM Hepes at pH 7.4. Binding is measured at 20–22°C. The filters used (EHWP 02500 Celotate filters, pore width 0.45 μm, Millipore, Neu-Isenburg) are soaked in Na-rich medium containing bovine serum albumin at 10 mg/ml for at least 2 hr prior to use. Binding reaction is started by 1:10 dilution of the membrane suspension in either Na- or K-rich medium containing bovine serum albumin at 1 mg/ml and additional reagents and ligands as described in *Results*. At the indicated time intervals, the binding reaction is stopped by diluting 100 μl of reaction mixture with 3 ml of ice-cold Na-rich medium containing bovine serum albumin at 1 mg/ml followed by rapid filtration. The filters were washed twice with 3 ml of ice-cold Na-rich medium containing bovine serum albumin at 1 mg/ml and radioactivity is measured with 0.2 ml of H_2O and 5 ml of Supertron (Kontron, München) in a liquid scintillation counter.

RESULTS

Voltage-Dependent and Carbamoylcholine-Stimulated [^{3}H]Ph_3MeP^+ Accumulation. Receptor-rich membrane fragments incubated overnight in K-rich medium were diluted into Na-rich medium and a K diffusion potential was generated that stimulated Ph_3MeP^+ accumulation (Fig. 1*A*). But we found that accumulation of [^{3}H]Ph_3MeP^+ is not only voltage dependent. Dilution in the presence of the cholinergic agonist carbamoylcholine (10 μM) resulted in even larger amounts of [^{3}H]Ph_3MeP^+ bound to the vesicles (Fig. 1*B*). This is in contrast to theoretical predictions, because one would expect the acetylcholine receptor ion channel to be opened by the agonist, the membrane to be depolarized, and accumulation to be inhibited. The stimulating effect of carbamoylcholine could be antagonized by preincubating the acetylcholine receptor-rich membranes with α-bungarotoxin. [^{3}H]Ph_3MeP^+ binding is specific—i.e., saturable (see Fig. 4)—and displaceable by excess unlabeled Ph_3MeP^+ (Fig. 4*A*) and was not observed with receptor-free crayfish axonal membranes.

Carbamoylcholine stimulation of [^{3}H]Ph_3MeP^+ binding is dose dependent. Half-maximal stimulation occurs with 1 μM carbamoylcholine (Fig. 2). This value is close to the K_d of the nondesensitized acetylcholine receptor for agonist binding (9). Various cholinergic ligands, both agonists and antagonists, stimulate [^{3}H]Ph_3MeP^+ binding (Fig. 3). Only α-bungarotoxin and the local anesthetic tetracaine (Fig. 3*A*) inhibit; acetylcholine,

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Abbreviations: H_{12}-HTX, perhydrohistrionicotoxin; PCP, 1-(1-phenylcyclohexyl)piperidine (phencyclidine); Ph_3MeP^+, triphenylmethylphosphonium.

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† We presented preliminary results on Ph_3MeP^+ binding to acetylcholine receptor-rich membranes at a meeting held in Berlin, Sept. 1981 (6, 7).

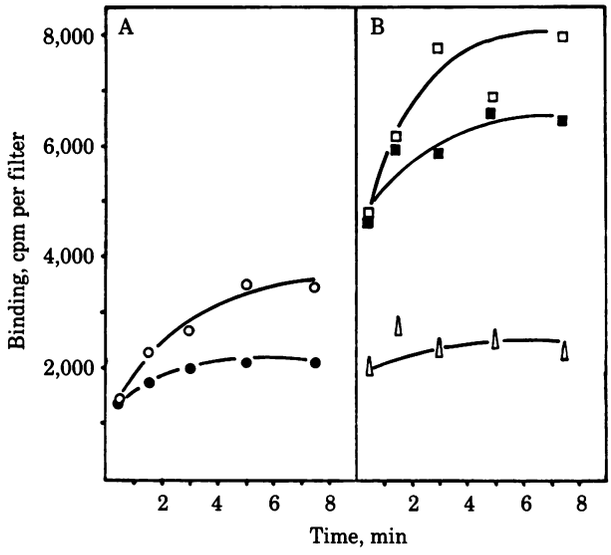

FIG. 1. [^{3}H]Ph_3MeP^+ binding to acetylcholine receptor-rich membrane fragments. Protein concentration was 0.26 mg/ml; membranes were equilibrated in K-rich medium; [^{3}H]Ph_3MeP^+ was 130 nM. (*A*) Membrane suspension was diluted into either K-rich (●) or Na-rich (○) medium. (*B*) Dilution medium contained 10 μM carbamoylcholine. Membrane suspension was diluted into either K-rich (■) or Na-rich (□) medium. △, Membrane suspension was incubated 10 min at room temperature with 10 μM α-bungarotoxin prior to dilution into Na-rich medium.

decamethonium, and *d*-tubocurarine (Fig. 3*B*) stimulate [^{3}H]Ph_3MeP^+ binding. Acetylcholine binding is inhibited only at very high Ph_3MeP^+ concentrations (1 mM). Saturable binding of Ph_3MeP^+ can be shown at much lower concentrations (Fig. 4).

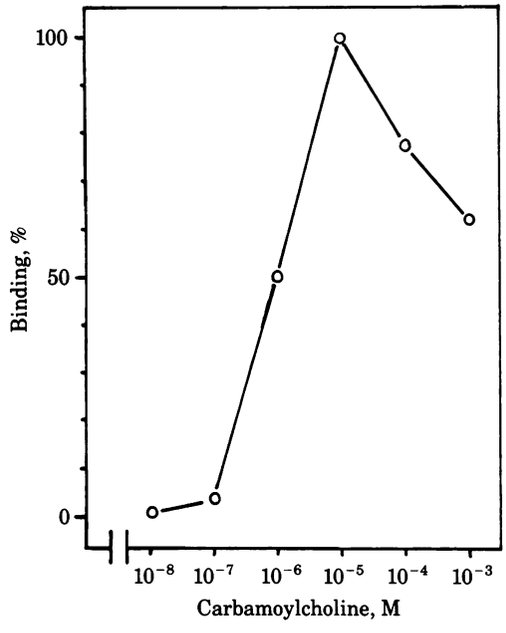

FIG. 2. Dose–response curve for the action of carbamoylcholine. Protein concentration was 0.84 mg/ml; membranes were equilibrated in K-rich medium; [^{3}H]Ph_3MeP^+ was 100 nM. Points represent the plateau values of the time-dependent [^{3}H]Ph_3MeP^+ binding after dilution into Na-rich medium (the plateau is reached in 5–10 min; compare Fig. 1) in the presence of the indicated concentration of carbamoylcholine. Binding in the absence of carbamoylcholine is set as 0%; binding in the presence of 10 μM carbamoylcholine is set as 100%.

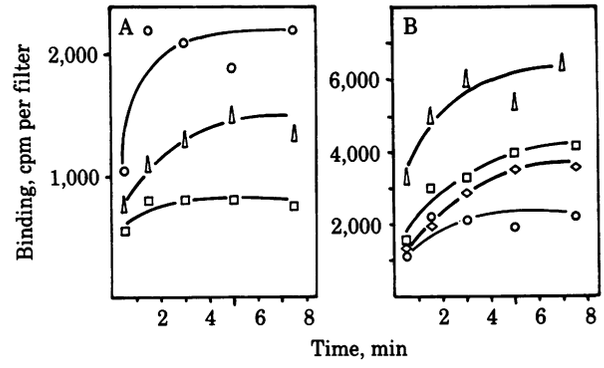

FIG. 3. Effect of cholinergic ligands on [^{3}H]Ph_3MeP^+ binding. Protein concentration was 0.26 mg/ml; membranes were equilibrated in K-rich medium; [^{3}H]Ph_3MeP^+ was 90 nM. (*A*) Inhibiting effectors. Binding after dilution into Na-rich medium with no additions (○) or in the presence of 100 μM α-bungarotoxin (△) or 100 μM tetracaine (□). (*B*) Stimulating effectors. Binding after dilution into Na-rich medium with no additions (○) or in the presence of 100 μM decamethonium (◇), 100 μM *d*-tubocurarine (□), or 100 μM acetylcholine (△).

Analysis of the binding data (Fig. 4*B*) shows that carbamoylcholine causes a decrease in K_d to about 1/10th (K_d = 13 μM in the absence of carbamoylcholine; K_d = 1.5 μM in the presence of 10 μM carbamoylcholine) without altering the maximal

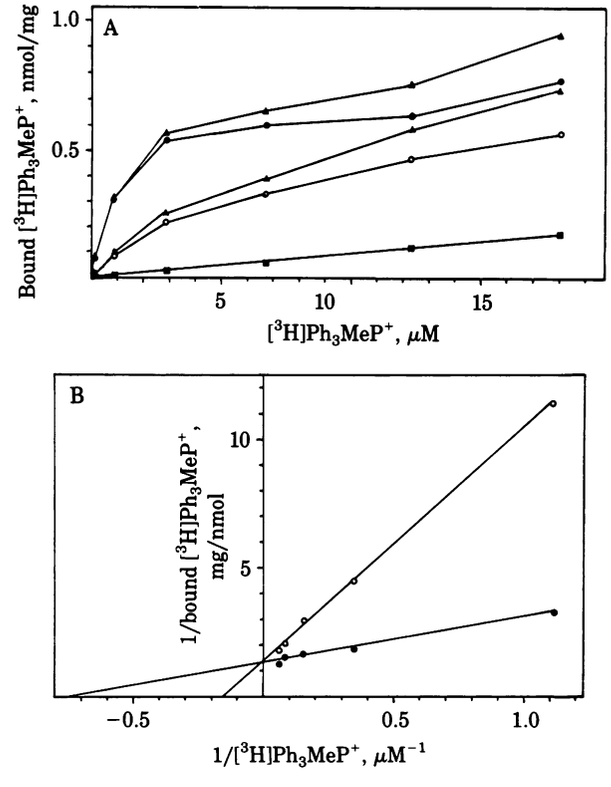

FIG. 4. Binding of [^{3}H]Ph_3MeP^+ to acetylcholine receptor-rich membrane fragments. Protein concentration was 0.23 mg/ml; membranes were equilibrated in Na-rich medium. (*A*) Direct representation of binding data. Each point is the mean value of two determinations 5 and 5.5 min after dilution into Na-rich medium. Filled symbols: binding in the presence of 10 μM carbamoylcholine. Open symbols: unstimulated binding, no further additions. ■, Nonspecific binding in the presence of 5 mM Ph_3MeP^+, amounts to the same value with and without carbamoylcholine. △ and ▲, total binding; ○ and ●, specific binding (nonspecific binding has been subtracted from total binding). (*B*) Double reciprocal plot of the data for specific binding. Binding in the absence (○) and in the presence (●) of 10 μM carbamoylcholine.

number of binding sites. A further increase of specific [^{3}H]Ph_3MeP^+ binding in the presence of [^{3}H]Ph_3MeP^+ concentrations higher than about 15 μM was observed; the increase probably reflects the existence of a second, low-affinity, binding site, the nature of which has not yet been investigated.

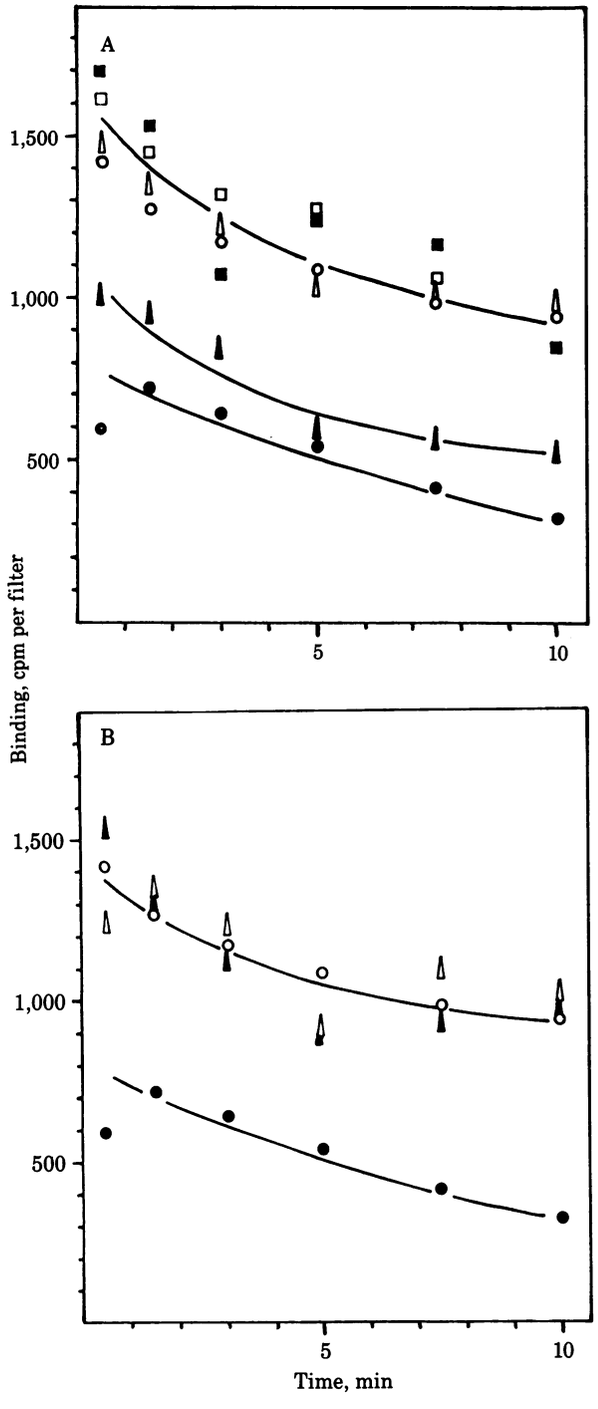

FIG. 5. Effect of Ph_3MeP^+ on ^{22}Na efflux from acetylcholine receptor-rich membrane fragments. Protein concentration was 6 mg/ml; efflux was measured in Na-rich medium. (*A*) No preincubation. Open symbols, leakage; filled symbols, efflux in the presence of 10 μM carbamoylcholine. ○ and ●, control without Ph_3MeP^+; △ and ▲, efflux in the presence of 0.1 mM Ph_3MeP^+ in the dilution medium; □ and ■, efflux in the presence of 1.0 mM Ph_3MeP^+ in the dilution medium. (*B*) Effect of preincubation with Ph_3MeP^+. Open symbols: leakage; filled symbols, efflux in the presence of 10 μM carbamoylcholine. ○ and ●, control without Ph_3MeP^+, same as in *A*. △ and ▲, prior to dilution the membrane suspension was incubated for 10 min at room temperature with 10 μM Ph_3MeP^+; the dilution medium also contained 10 μM Ph_3MeP^+.

Ph_3MeP^+ Blocks ^{22}Na Efflux from Acetylcholine Receptor-Rich Membrane Vesicles. A convenient method for measuring acetylcholine receptor function *in vitro* is monitoring the ^{22}Na efflux after dilution of preloaded acetylcholine receptor-rich membrane vesicles (10). Efflux can be stimulated by cholinergic agonists—e.g., carbamoylcholine. In the experiment shown in Fig. 5*A* efflux stimulated by 10 μM carbamoylcholine was slightly reduced by 0.1 mM Ph_3MeP^+ and was completely blocked by 1.0 mM Ph_3MeP^+ in the dilution medium.

The blocking effect of Ph_3MeP^+ on the ion channel could be potentiated by preincubating the receptor-rich vesicles with the lipophilic cation. In this case, efflux stimulation was fully inhibited by 10 μM Ph_3MeP^+ (Fig. 5*B*), a concentration at which [^{3}H]acetylcholine binding is not yet blocked.

Ph_3MeP^+ Is a Competitive Inhibitor of [^{3}H]PCP Binding. To test further the possibility that Ph_3MeP^+ is an ion channel ligand we investigated the effect of Ph_3MeP^+ on [^{3}H]PCP binding. PCP is a general anesthetic and hallucinogen and was shown to bind to the ion channel of the acetylcholine receptor complex, thereby blocking ion translocation (11, 12). Binding of PCP is also stimulated by cholinergic agonists (12). Specific binding of [^{3}H]PCP either in the absence or in the presence of carbamoylcholine is completely blocked by Ph_3MeP^+ (Fig. 6). Nonspecific binding in this experiment was determined in the presence of 5 mM amantadine, which was previously shown to

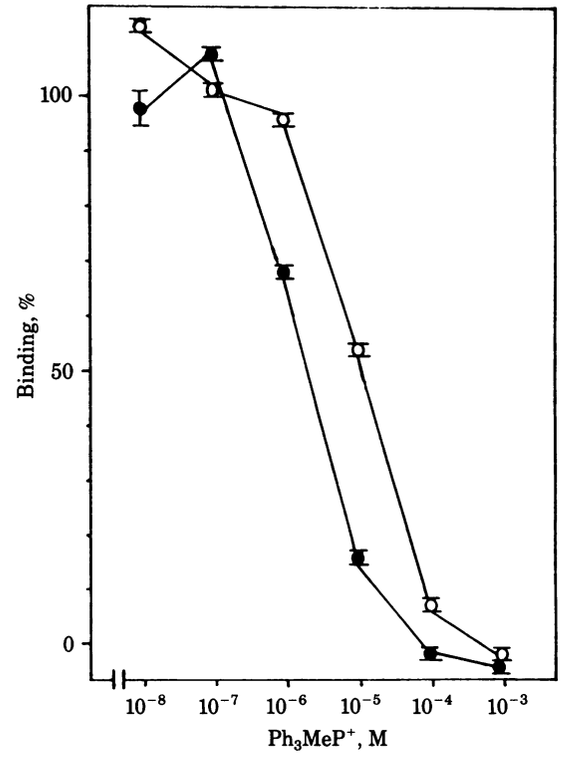

FIG. 6. Displacement of [^{3}H]PCP by Ph_3MeP^+. Protein concentration was 0.65 mg/ml; membranes were equilibrated in Na-rich medium; [^{3}H]PCP was 8.75 nM. Aliquots were filtered 5 and 5.5 min after dilution into Na-rich medium. Bars represent upper and lower values, symbols are mean values. Binding was measured in the absence (○) and in the presence (●) of 10 μM carbamoylcholine. Binding in the absence of Ph_3MeP^+ was set as 100%: 0.34 pmol/mg of protein for ○ and 1.36 pmol/mg of protein for ●. Binding in the presence of 2 mM amantadine was set as 0%: 0.06 pmol/mg protein for ○, 0.13 pmol/mg of protein for ●.

be a channel ligand (13, 14). All three compounds are competitive with respect to binding to the acetylcholine receptor complex; amantadine blocks not only [^{3}H]PCP binding but [^{3}H]Ph_3MeP^+ binding as well (data not shown).

The values for half-maximal displacement of [^{3}H]PCP binding by Ph_3MeP^+ (2 μM in the presence, 10 μM in the absence of carbamoylcholine) are in good agreement with the K_d values found in the direct binding experiment (compare Fig. 4).

DISCUSSION

The ion-translocating part of the receptor ("ion channel" or "ionophore") so far has been investigated predominantly by using radioactively labeled perhydrohistrionicotoxin (H_{12}-HTX) (15–20). Just recently, PCP was introduced for the same purpose (11, 12, 21). There are striking similarities in the binding characteristics of these "channel ligands" with those of Ph_3MeP^+. Binding of all three compounds is stimulated by cholinergic agonists. This effect is inhibited by the antagonist α-bungarotoxin; antagonists alone (*d*-tubocurarine), however, stimulate binding also but to a lesser degree. Above a certain agonist concentration, stimulation of Ph_3MeP^+ and H_{12}-HTX binding is decreased. Local anesthetics inhibit binding of channel ligands. H_{12}-HTX, PCP, and Ph_3MeP^+ block the ion channel at concentrations at which there is no effect on binding of cholinergic agonists.

However, there is one important difference between Ph_3MeP^+ on the one hand and H_{12}-HTX and PCP on the other: stimulation of binding of the latter two drugs is inhibited by desensitization. In fact, for this reason H_{12}-HTX has been proposed as a tool for monitoring different functional states of the receptor complex; in contrast, desensitization has no effect on Ph_3MeP^+ binding. In addition, there are strong indications suggesting differences in the binding sites of the ion channel for H_{12}-HTX and PCP: affinities obtained for various channel drugs (adiphenine, dibucaine, dimethisoquine, piperocaine, procaine, quinacrine, tetracaine, amantadine) are different when determined by inhibition of PCP or H_{12}-HTX binding (12). Furthermore, affinity of piperocaine for PCP-binding sites is enhanced 10-fold after receptor stimulation, whereas it is reduced by 90% for H_{12}-HTX-binding sites (12, 22).

We conclude from these observations that channel ligands do not necessarily bind to identical sites, although they appear to bind competitively. Thus we would like to introduce Ph_3MeP^+ as a channel ligand, the properties of which can be summarized as follows: Ph_3MeP^+ is a lipophilic cation exhibiting affinity for the ion channel of the acetylcholine receptor complex and blocking it upon binding. Its binding site is coupled with the binding site for the usual cholinergic effectors; binding is stimulated by both receptor activation and binding of antagonists and is not inhibited by desensitization.

At present we cannot explain the observation that not only the channel-opening agonists but also antagonists stimulate Ph_3MeP^+ binding. The cation-binding site of the ionophore appears to be exposed by cholinergic effectors in a step preceding agonist-specific opening of the channel.

Recently a report on the effects of lipophilic cations (tetraphenylarsonium, tetraphenylphosphonium, and Ph_3MeP^+) on motility and other physiological properties of the bacterium *Bacillus subtilis* has been published (23). The authors found major changes in various parameters, including swimming speed and frequency of tumbling. They concluded from their evidence that lipophilic cations perturb the process of conversion of the protonmotive force into work. Whether this fact can be related to a similar channel blocking activity as described here could well be worth further investigation. At any rate, measurements of voltage dependent Ph_3MeP^+ binding to membrane systems should be interpreted with care.

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