

## **Tetraethylammonium contains an impurity which alkalizes cytoplasm and reduce calcium buffering in neurons**

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Commercial tetraethylammonium chloride contains about 5% of the impurity triethylamine. External triethylamine raises cytoplasmic pH, which reduces the intracellular calcium concentration transient following calcium influx during membrane depolarization. The calcium influx is unaffected, so the cell's ability to buffer calcium transients is reduced. Purified tetraethylammonium does not have these effects.

Tetraethylammonium (TEA) is widely used to block voltage-dependent potassium channels in excitable tissue<sup>4</sup>. Neurons treated with TEA respond to membrane depolarization with an enhanced cytoplasmic calcium concentration transient following calcium influx through voltage-dependent channels<sup>11</sup>. A recent study<sup>10</sup> demonstrates that cytoplasmic alkalization enhances intracellular calcium transients without affecting depolarization-induced influx through calcium channels, apparently by reducing the cell's ability to buffer calcium transients. This report shows that commercially prepared TEA also alkalizes cytoplasm and reduces calcium buffering, and that these effects are due entirely to the presence of an impurity, triethylamine. Purified TEA still blocks potassium channels, but does not affect internal pH or calcium buffering.

Experiments were performed on neurons from the abdominal ganglion of the sea hare *Aplysia californica*. Ganglia were desheathed without enzymes and the membrane potentials of identified neurons were controlled by voltage clamp. Cells were bathed in a normal artificial sea water (NASW) or one in which TEA was substituted isosmotically for NaCl (TEA SW). Detailed formulations are given in ref. 11.

Cytoplasmic pH was measured with a pH-sensitive microelectrode, while intracellular calcium activity was monitored photometrically in cells injected either with the photoprotein aequorin or the dye arsenazo III. The procedures for voltage clamping neurons and measuring internal pH and calcium activity are described in detail elsewhere<sup>6–8</sup>.

When neurons are exposed to TEA SW, the cytoplasmic pH rises from an

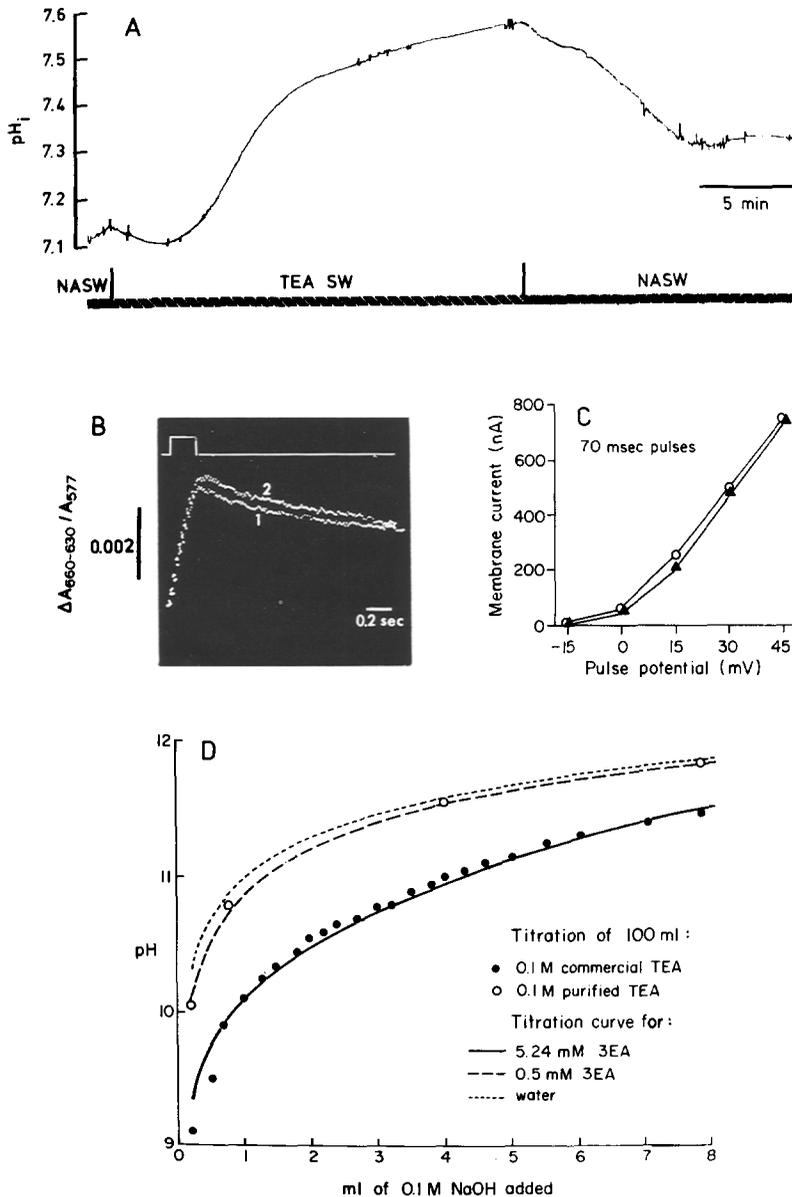


Fig. 1. Effects of commercial TEA on cytoplasmic pH and calcium current, and titration of impurity in TEA. A: internal pH ( $pH_i$ ) monitored in cell L8 with a pH-sensitive microelectrode. Membrane potential clamped to  $-40$  mV throughout experiment. The bathing medium was changed to tetraethylammonium chloride sea water (TEA SW) and back to normal artificial sea water (NASW). Temperature  $15^\circ\text{C}$ . B: differential absorbance change at 660–630 nm, normalized to the absorbance at 577 nm ( $\Delta A_{660-630}/A_{577}$ ), in an unidentified cell near R15 filled with  $250\ \mu\text{M}$  arsenazo III and depolarized to  $+20$  mV for 0.2 sec from a holding potential of  $-40$  mV at the time shown in the top trace: trace 1 in NASW and trace 2 in TEA SW. After correction for the pH-dependence of arsenazo III's calcium sensitivity<sup>10</sup>, the calibration bar corresponds to an average intracellular calcium concentration change of about 277 nM in NASW and 261 nM in TEA SW. Temperature  $15^\circ\text{C}$ . C: magnitude of inward calcium tail currents measured 1 msec after 70 msec depolarizing pulses of different amplitudes in NASW ( $\circ$ ) and TEA SW ( $\triangle$ ). Eight responses were computer averaged at each potential, and capacitance current was automatically subtracted by the procedure described in (ref. 10). Cell R2. Temperature  $7^\circ\text{C}$ . Holding potential  $-40$  mV. D: titration of 0.1 M solutions of commercial TEA-Cl ( $\bullet$ ) and 90% purified TEA-Cl ( $\circ$ ). The lines are theoretical titration curves for 5.24 and 0.5 mM triethylammonium and pure water.

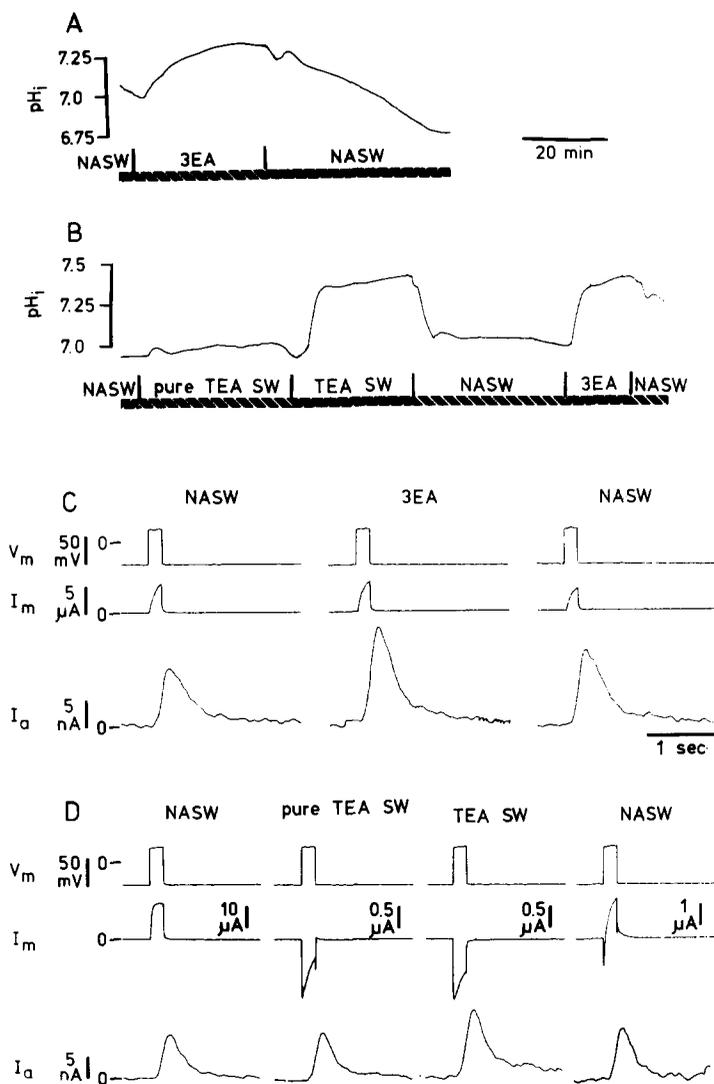


Fig. 2. Effects of triethylamine, commercial TEA SW and purified TEA SW on cytoplasmic pH and calcium transients. A: intracellular pH in cell  $RD_6$  exposed to 25 mM triethylammonium (3EA) in NASW, and then returned to NASW. B: pH monitored in cell L2 when exposed to NASW, 90% purified TEA SW, commercial TEA SW, NASW, 25 mM triethylammonium in NASW, and finally NASW. C: membrane potential ( $V_m$ ), membrane current ( $I_m$ ) and photomultiplier anode current ( $I_a$ ) registering intracellular calcium-indicating photo-emissions from cell L11 injected with aequorin, when depolarized to +25 mV for 0.2 sec and exposed to NASW, 25 mM triethylammonium in NASW, and NASW. D, similar records from cell R2 filled with aequorin, depolarized to +30 mV for 0.2 sec, and exposed to NASW, 90% purified TEA SW, commercial TEA SW, and NASW. Temperature 15 °C in A–C and 18 °C in D. Holding potential –40 mV in A–D.

average level of 7.18 (range 7.10–7.24, 5 cells) to 7.46 (range of pH changes 0.20–0.40 pH units). The effect is reversible (Figs. 1A and 2B).

It was shown previously<sup>11</sup> that TEA enhances the aequorin photoresponses to depolarizing pulses. The non-linear calcium-dependence of aequorin photoemissions<sup>2</sup>

complicates the interpretation of this result. Increased aequorin photoemissions may indicate either a larger average cytoplasmic calcium transient, an increased resting level of intracellular calcium, or a spatially more restricted calcium concentration transient involving the same number of free ions<sup>7,11</sup>. Arsenazo III is a metallochromic indicator linearly sensitive to calcium, so an enhancement of its response, which is a color shift toward blue, indicates an increase in the total number of free calcium ions available in cytoplasm to react with arsenazo III. Five cells were filled with about 250  $\mu$ M arsenazo III. In 3 cells, the absorbance was monitored at 660 nm (the peak of the calcium difference spectrum of arsenazo III) and 577 nm (the isosbestic point), and the normalized absorbance change  $\Delta A_{660}/A_{577}$  accompanying a 0.2–0.3 sec depolarization to +20 mV increased an average of 28% (range 20–33%) after 30 min in TEA SW. In two other cells, the normalized differential absorbance at 660 and 630 nm was monitored, and  $\Delta A_{660-630}/A_{577}$  during a depolarization increased 13% and 33% in these cells in TEA SW. Records from one of these cells are shown in Fig. 1B. Arsenazo III's calcium-sensitivity is pH-dependent, so correction must be made for the expected increase in cytoplasmic pH in TEA SW. After appropriate correction, as described in ref. 10, the results suggest a transient calcium concentration change that is about 27% larger in TEA in these 5 experiments (range 7–40%).

The calcium influx through the membrane may be estimated by recording calcium tail currents following depolarizing pulses<sup>3</sup>. These inward tail currents, identified as calcium currents by their characteristic kinetics, voltage-dependence, and ionic and pharmacological sensitivities<sup>1,7</sup> reflect the magnitude of calcium influx at the end of a depolarizing pulse. In 9 cells, the calcium current was not significantly affected by TEA (average change  $-5\% \pm 6\%$ , mean S.E.; see Fig. 1C). These results suggest that TEA reduces the cell's ability to buffer calcium influx during electrical activity, due to its effect on internal pH, much the same as when internal pH is raised by ammonia treatment as described elsewhere<sup>10</sup>. Intracellular calcium concentration transients are regulated by a variety of processes, including cytoplasmic calcium binding to high affinity ligands, extrusion of calcium by an energy-utilizing pump at the surface membrane, and sequestration by several types of organelles. It is not yet clear which of these processes is most affected by internal pH.

Since TEA is synthesized from the weak base triethylamine<sup>9</sup>, these effects of TEA solutions could be due to some triethylamine being present in commercially prepared TEA, acting in exactly the same fashion as ammonia to alkalize cytoplasm and reduce calcium buffering. I analyzed solutions of tetraethylammonium chloride (Eastman and Aldrich) for triethylamine by two independent methods: pH titration (Fig. 2D) and extraction in ether. In the latter procedure, triethylammonium was converted to triethylamine by bringing 100 ml of 1 M TEA-Cl to pH 13 by addition of 10 N NaOH. This was mixed with 50 ml diethyl ether and shaken vigorously. The ionized TEA dissolves in the water, while the polar triethylamine dissolves in the ether. The aqueous phase was removed with a separatory funnel and neutralized to pH 7 with concentrated HCl. The 5% ether dissolved in water was evaporated under suction, leaving a solution of 1 M TEA-Cl in 0.1 M NaCl, plus 10% of the original concentration of triethylamine, due to the ether which remained in the aqueous phase. (The TEA

could be further purified by using repeated ether extractions of the triethylamine.) The NaCl was removed by evaporating the water, dissolving the precipitate in acetone, filtering out the NaCl, and evaporating the acetone. The purified TEA must not be heated, or more triethylamine will be formed by the Hoffman reaction<sup>5</sup>. The ether phase containing 90% of the triethylamine was acidified with HCl gas, and the triethylammonium chloride precipitate was filtered out, weighed, and analyzed by mass spectroscopy. Both titration and ether extraction procedures yielded an estimate of 5% mol fraction triethylamine in freshly mixed as well as months-old TEA solutions.

At this level of impurity, TEA SW would contain about 25 mM triethylammonium ion. Since this conjugate acid has a  $pK_a$  of 10.8, at pH 7.5, 11  $\mu$ M would be in the form of the membrane-permeant organic base triethylamine. From the measured pH buffer capacity of *Aplysia* cytoplasm<sup>10</sup>, this concentration of triethylamine should raise cytoplasmic pH from 7.1 to about 7.4. Fig. 2A shows that 25 mM triethylammonium has this effect on cytoplasmic pH. Moreover, TEA SW made from commercial TEA has a similar effect on cytoplasmic pH, while TEA SW made from purified TEA, containing only 0.5% triethylamine, has almost no effect on pH (Fig. 2B). Similar results were obtained from 6 cells. Finally, Fig. 2C, D shows that triethylamine enhances calcium concentration transients accompanying membrane depolarization, just as does commercial TEA SW, but purified TEA SW has very little effect. Both pure and commercial TEA block potassium current, which is unaffected by 25 mM triethylamine.

Commercial samples of TEA bromide (Eastman) were found to be free of triethylamine. TEA bromide should therefore be used in applications where the anion does not need to be chloride. Tetramethylammonium (TMA) chloride was also found to be free of trimethylamine. The reported small and variable effect of TMA on aequorin photoemissions<sup>11</sup> was probably a reflection of TMA's reduction of a series resistance artefact in voltage clamp<sup>7</sup>, due to its reduction of outward current. Since both pure and commercial TEA block outward current, but only commercial TEA significantly and consistently enhances aequorin signals, the TEA effect on calcium transients cannot be due solely to its reduction of series resistance error. In support of this conclusion, triethylamine has no effect on outward current or series resistance error, yet still enhances calcium transients.

In conclusion, commercially prepared TEA-Cl alkalizes cytoplasm and reduces neuronal calcium buffering, so that calcium influx accompanying electrical activity results in larger internal calcium transients. These effects are due entirely to the presence of the weak base triethylamine, and may be prevented by its removal. It would seem prudent in the future to check TEA solutions for this impurity by titration, and to purify the substance as needed, as alteration of cellular pH and calcium buffering is likely to have widespread effects, modifying many essential biochemical and physiological processes.

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