

THE MECHANISM OF TUBOCURARINE ACTION ON MECHANORECEPTOR CHANNELS IN THE PROTOZOAN *STENTOR COERULEUS*

By DAVID C. WOOD

Psychobiology Program, University of Pittsburgh, Pittsburgh, PA 15260, U.S.A.

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SUMMARY

(+)-Tubocurarine (TC) decreases the probability that the protozoan, *Stentor coeruleus* Ehrenberg, will contract in response to mechanical stimulation, because it selectively depresses mechanoreceptor currents. Resting membrane properties and action potentials are not significantly altered by the drug.

Stentor incubated in media containing radioactively labelled TC (TC*) retain TC* after extensive washing despite a rather high apparent K_D ($19.7 \mu\text{mol l}^{-1}$). The incubation curve for TC* binding exhibits an initial exponential rise followed by a linear increase. Wash-out of bound TC* and elimination of the exponential component of the incubation curve is observed if the TC* incubation is followed by a 5-s exposure to 8% urea; therefore, the exponential component represents a reversible binding process. TC* binding in the exponential component is highly correlated ($r < -0.96$) with the depression in receptor current and response probability when incubation time, drug concentration and drug (gallamine, TC, decamethonium and succinylcholine) are varied. These correlations suggest that the exponential binding is to functional mechanoreceptors.

Mechanoreceptor currents are decreased by hyperpolarization and increased by depolarization, indicating that the mechanoreceptor channel is voltage-dependent. At hyperpolarized potentials the channels are in a form (the U form) which cannot be opened by mechanical stimulation; at depolarized potentials they are in a form (the R form) which can be opened. TC appears to bind to the U form with higher affinity than to the R form, since depolarization reduces the amount of bound TC* and relieves the depression of mechanoreceptor current produced by TC.

INTRODUCTION

Following the work of Jenkinson (1960), (+)-tubocurarine (TC) has been regarded as a 'competitive antagonist' of acetylcholine at the vertebrate neuromuscular junction. However, the reports of Manalis (1977), Katz & Miledi (1978) and Colquhoun, Dreyer & Sheridan (1979) have made it clear that TC affects postsynaptic ionic channels in the neuromuscular junction as well as acetylcholine receptors. TC has also

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been found to depress synaptic transmission in invertebrates both at cholinergic (Marty, Neild & Ascher, 1976; Ascher, Marty & Neild, 1978) and non-cholinergic synapses (Stefani & Gerschenfeld, 1969; Ascher, 1972; Carpenter, Swann & Yarowsky, 1977; Yamamoto & Washio, 1979; Cull-Candy & Miledi, 1983). In these cases, the primary effect of the drug is upon ionic channels rather than upon transmitter receptors.

TC also depresses the probability with which contractile protozoa respond to mechanical stimuli by contracting (Tartar, 1961; Applewhite, 1972; Wood, 1977). Intracellular recordings from one of these protozoa, *Stentor coeruleus*, reveal that its contractile response to mechanical stimulation is mediated by the production of mechanoreceptor and action potentials (Wood, 1970, 1982) similar to the potentials observed in the non-contractile protozoa, *Paramecium* (Eckert, 1972; Eckert, Naitoh & Friedman, 1972) and *Stylonychia* (de Peyer & Machemer, 1977, 1978). The mechanoreceptor and action potentials observed in these protozoa are produced by transient increases in membrane conductance, i.e. the opening of ionic channels. Therefore, TC could be depressing the probability of contraction in protozoa by acting upon one or more of the ionic channels involved in producing the contractions.

Since TC depresses the production of contractions elicited by mechanical stimuli but not those elicited by photic or electrical stimuli (Wood, 1977), the drug appears to act upon the mechanoreceptor mechanism and not upon the channels producing the action potential since these channels are involved when a contraction occurs, whatever stimulus is employed. If TC is acting upon the mechanoreceptor, and more specifically its ionic channel, then its site of action in *Stentor* departs in several respects from its site of action in other preparations. In particular, the mechanoreceptor channel in *Stentor* is selectively permeable to Ca^{2+} ions (Wood, 1982), whereas the ionic channels previously reported to be blocked by TC carry Na^+ , K^+ or Cl^- ions. Secondly, the opening of mechanoreceptor channels is strongly voltage-dependent in the vicinity of the resting potential, whereas such voltage dependence is not evident in the other channels affected by TC. Mechanoreceptor function also does not appear to depend on the presence of a 'neurotransmitter' as for the other channels. Therefore these studies were initiated to determine the mechanism by which TC acts upon *Stentor* for comparison with its action in other preparations.

MATERIALS AND METHODS

Animals

Stentor coeruleus Ehrenberg of the Stella strain were cultured in 1-litre beakers or high-walled Petri dishes in a medium containing (in mmol l^{-1}) CaCl_2 , 2.5; MgSO_4 , 1.0; NaNO_3 , 1.0; KCl , 0.1; and Tris-HCl buffer, 0.75; to maintain a pH of 7.6–7.9. The *Stentor* were daily fed *Tetrahymena thermophila* which had been grown axenically in a 1% proteose peptone medium. The *Tetrahymena* were washed twice by centrifugation and resuspended in culture medium bacterized with *Kiebstella aerogenes*. After 1 day in the bacterized medium the *Tetrahymena* were again washed twice before being used as a food organism. Heat-killed wheat grains were often added

to these cultures to provide nutrient for the support of a bacterial population on which the *Tetrahymena* could feed.

Materials

(+)-Tubocurarine di- (^{14}C) methyl ether iodide (TC*) was obtained from Amersham/Searle Corporation (specific activity: 88–112 mCi mmol $^{-1}$). Unlabelled (+)-tubocurarine chloride (TC) was obtained from Calbiochem, decamethonium bromide (Deca), succinylcholine chloride (Succ), gallamine triethiodide (Gall) from K & K Laboratories and acetylcholine chloride (ACh) from Sigma.

Procedures

Mechanical stimulus sensitivity tests

On the day before mechanical stimulus sensitivity testing, 15–20 *Stentor* were placed together with 1.8–2.0 ml of culture medium in a 5-ml beaker, the bottom of which was covered with ruled filter paper. At least 30 min prior to a test, this beaker was mounted on top of a 674 g steel disc which could be dropped a calibrated distance (0–10 mm). The fall of the disc was abruptly stopped when its lower flat surface came into contact with the flat surface of a second steel disc rigidly mounted on top of a weighing table. All-or-none contractions of sessile *Stentor* occurring as a result of this stimulation were observed through a stereomicroscope. A drop of 4 mm was found to be just sufficient to produce contraction of 90–100 % of undrugged control animals and all the behavioural data reported here employ this stimulus intensity. Data employing other stimulus intensities were also obtained and are in all cases compatible with those reported. These data are omitted solely to simplify description of the results.

During a behavioural test sequence, an initial 4-mm drop test was used to verify that more than 90 % of the *Stentor* responded to the mechanical stimulus. After a 5-min rest, 12.5–200 μl of the drug solution to be studied was injected to raise the final volume in the test beaker to 2 ml. For studies in which drug concentration was manipulated, a second drop test was then performed after a 2-min incubation period and a third test was performed after a 30-min incubation period. For the incubation time studies incubation periods ranged between 1 and 32 min before the second drop test.

Electrophysiological recordings

Microelectrode recordings were made from 10–50 M Ω , 0.5 mol l $^{-1}$ KC1-filled glass microelectrodes. Voltage and current electrodes were simultaneously inserted into the frontal field of *Stentor* cooled to 8.5–10°C as described previously (Wood, 1982). All voltage readings were corrected for the presence of tip potentials. Currents were monitored using a virtual ground circuit. When voltage steps were employed, a third microelectrode was placed in the bath near the penetrated animal and the transmembrane potential was recorded as the difference in potential between the intracellular voltage electrode and this bath electrode. Mechanical stimuli were applied by moving the impaling microelectrodes and hence the impaled animal 25 μm or less through the medium (Wood, 1975, 1982). The speed of this movement was monitored by a linear

velocity transducer in series with the solenoid armature which produced the movement.

In most of the reported studies, animals were penetrated while in culture medium and preliminary measurements of resting potential, membrane resistance and action potential production were made. The cell was then voltage clamped and receptor currents in response to an ascending series of 5–7 mechanical stimuli were measured using a 2-min interstimulus interval. Only animals with resting potentials more negative than -40 mV, input resistances greater than $4\text{ M}\Omega$, action potentials exceeding 60 mV and maximal receptor currents equal to or exceeding 2 nA were retained for further study. For preparations meeting these criteria, $0\text{--}60\text{ }\mu\text{mol l}^{-1}$ TC solutions made up in culture medium were then infused over a 3-min period which was sufficient to exchange the medium in the test chamber eight times. After an additional 3-min period, measurements of resting potential, membrane resistance, action potentials and receptor currents were repeated. Additional measurements of receptor current, following procedures mentioned in the Results section, were then employed. In a few studies initial penetration and TC infusion were carried out in media containing elevated KCl concentrations.

TC binding assay*

Stentor (15–35) were placed in a 5-ml beaker together with $1.8\text{--}1.975$ ml of culture medium at room temperature. In the saturation studies, $12.5\text{--}200\text{ }\mu\text{l}$ of 0.6 mmol l^{-1} TC* was then pipetted into the beaker to produce a final volume of 2 ml. After a 2-min incubation period the animals were collected together with a small amount of the incubation medium into a disposable pipette and transferred into a 10-ml culture medium wash at room temperature. By means of similar manual pipetting the animals were given three additional 1-min washes in 10-ml baths. Longer washing for up to 30 min did not substantially reduce the amount of bound TC* per animal. After being washed, the *Stentor*, while still alive, were individually pipetted onto a filter paper disc which was then oven dried. Eight to fifteen 3.2-mm circles, each centred on the green spot marking an animal's remains, were punched from the filter paper and placed into separate vials containing 3 ml of Scintiverse scintillation fluid for counting. Three control circles were also punched from each filter paper disc and counted to provide a background measurement. Each sample was counted three times for 1 min in a Packard Tri-Carb Liquid Scintillation Spectrometer at 92 % efficiency. Control circles averaged 24–26 c.p.m.

For the incubation time studies, the procedures described above were followed except that the incubation period was varied between 0.5 and 32 min, a constant final TC* concentration of $15\text{ }\mu\text{mol l}^{-1}$ was used and the incubation medium was maintained at $15\text{--}15.5^\circ\text{C}$, since the variability in the c.p.m. of bound TC* is reduced at this lower temperature. For those studies employing a urea wash, the animals were incubated, following the above incubation procedure, but were immediately thereafter pipetted into 10-ml of 8 % urea for 5 s. This wash was terminated by pouring the urea solution into 80 ml of culture medium, collecting the animals by pipette and transferring them through two additional culture medium washes following the usual wash procedure. Additional incubation time studies were performed in which the normal incubation procedure was followed but the four 10-ml washes contained 10 % sucrose in the culture medium.

To determine the degree of inhibition produced by other drugs on TC* binding, 0.95 ml of culture medium containing 15–25 *Stentor* was cooled to 15–15.5°C for 5 min. Culture medium (1 ml) containing the drug was then introduced. Five minutes later, 0.05 ml of 0.6 mmol l⁻¹ TC* was introduced for a 2-min incubation period which was followed by the usual wash procedure.

The assay procedures described above were developed so that the condition of the animals in the TC* binding assay would be very similar to their condition in the mechanical sensitivity or electrophysiological studies. One consequence of this approach, which involved measuring TC* bound to individual living cells, was that only small numbers of counts were measured because of the small size of the cells ($\sim 10 \times 10^{-9}$ l). The data from the urea and sucrose treatment studies indicate that these small numbers of counts are bound to the cell surface where the drug appears to be bound in concentrations at least a 100 times greater than those present in the incubation medium. The small numbers of counts/animal did produce a large proportionate variability in the results due in part to the random sampling nature of the counting process. Variability, as indicated by the standard error of the mean, was kept to useable limits by using large samples containing 25–50 animals for computing means. A small proportion (< 4%) of the animals studied gave very large counts, occasionally as much as 10 times the sample mean. Disruption of the cell surface before or during the TC* incubation has previously been shown to produce such large increases in counts (Wood & See, 1976). To avoid the disproportionate influence such animals would produce on the reported means, data from all animals whose results were more than 2 standard deviations from the sample mean were not used in the final calculations. No animals had measured counts more than 2 standard deviations less than the sample mean and hence no animals were eliminated from the low end of the sample distribution. The resultant means and standard deviations are very close to the sample medians and interquartile ranges.

Correlation between any two of the dependent variables studied – probability of response, receptor current amplitude and c.p.m. of bound TC* – were made by calculating the Pearson product-moment correlation coefficient between the variables using the four or five data points for mean group values as displayed in the relevant figures.

RESULTS

Concentration dependence of the TC-induced depression of mechanoreceptor function

TC and other drugs containing two quaternary amine groups spaced 1.0–1.4 nm apart depress *Stentor*'s probability of contracting in response to mechanical stimuli, whereas drugs of other structures do not have this effect (Wood, 1977). The concentration dependence for this depression in response probability (Fig. 1A) was obtained by mechanically stimulating the animals 2 min after drug introduction; a very similar curve was obtained when stimulation was applied 30 min later.

The physiological basis of this behavioural effect was determined by studying the effect of TC on mechanoreceptor currents, action potentials and resting membrane properties. Undrugged cells which were voltage clamped to their resting potential produced mechanoreceptor currents which increased in amplitude as the velocity of the

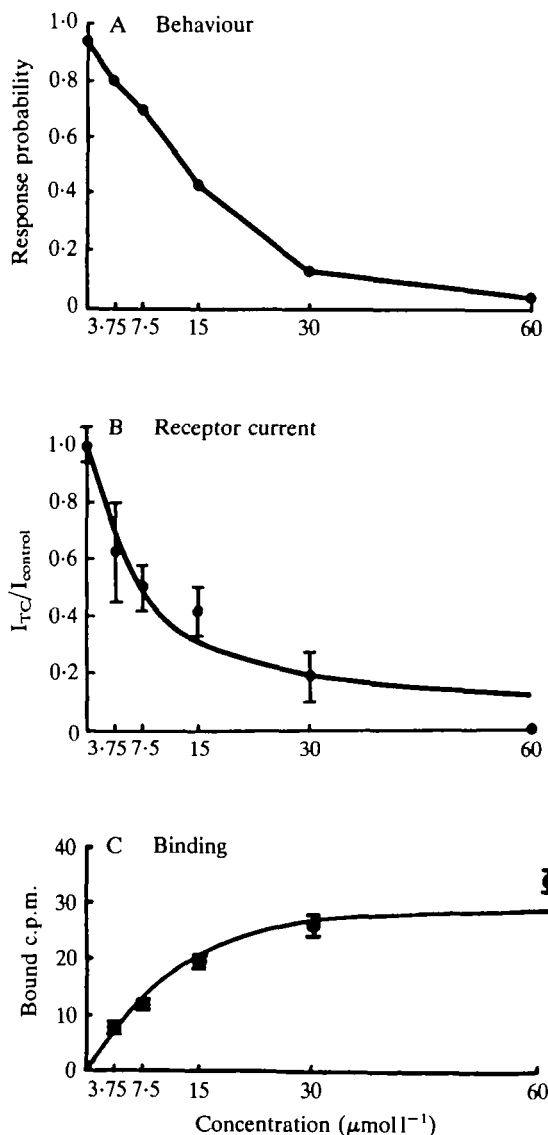


Fig. 1. (A) The concentration dependence of the effect of TC on contractile behaviour measured 2 min after drug introduction. The probability of response to mechanical stimulation produced by a 4-mm drop was between 0.9 and 1.0 before drug introduction. Two minutes after drug introduction the probability of response to this stimulus was markedly depressed in a concentration-dependent fashion. Group sizes were between 20 and 53 at each TC concentration. (B) The concentration dependence of the blockade of mechanoreceptor current produced by TC. Sample records showing inward mechanoreceptor currents in culture medium and in the presence of 30 $\mu\text{mol l}^{-1}$ TC are shown above Figs 7 and 10. This figure shows what proportion of the asymptotic receptor current present prior to TC introduction was present after its introduction ($I_{TC}/I_{\text{control}}$). Five cells were tested at each TC concentration. Means \pm s.e.m. are shown. (C) The concentration dependence of TC* binding. Means \pm s.e.m. of the c.p.m. of TC* bound to individual cells after a 2-min incubation in the TC* concentration shown. Data from 58–74 animals at each point.

Table 1. *Effect of TC on electrophysiological parameters of Stentor*

	Control	30 $\mu\text{mol l}^{-1}$ TC
Resting potential (mV)	-54.7 ± 2.2	-53.0 ± 4.1
Action potential peak (mV)	$+19.0 \pm 4.5$	$+20.4 \pm 2.2$
Action potential threshold (mV)	-20.0 ± 2.6	-17.1 ± 3.3
Input resistance ($\text{M}\Omega$)	17.0 ± 3.6	18.8 ± 1.7

Values are means \pm s.d.

mechanical stimulus was increased, until an asymptotic mechanoreceptor current level was obtained at high stimuli velocities (Wood, 1975; see the top of Figs 7 and 10 for examples). This asymptote was assessed by averaging the inward currents elicited by three or four stimuli producing the largest currents. Following drug addition, mechanical stimuli of increasing velocity again elicited mechanoreceptor currents of increasing amplitude which approached a lower asymptote. The asymptotic mechanoreceptor current of cells in TC relative to their previous control asymptotic current is plotted against drug concentration in Fig. 1B. TC depressed mechanoreceptor current in a concentration-dependent fashion which is well correlated ($r = 0.96$) with the depression of response probability.

This strong correlation suggests that the effect of TC on behaviour is due to its depression of mechanoreceptor current. In behavioural studies this specificity was suggested by the failure of TC to alter *Stentor*'s electrical stimulus threshold or probability of response to photic stimuli (Wood, 1977). In the present studies, TC at concentrations of 30 $\mu\text{mol l}^{-1}$ or less was found to reduce mechanoreceptor current while not altering any of the other electrophysiological parameters measured – resting potential, specific membrane resistance, action potential amplitude and action potential threshold (Table 1; Fig. 2). At concentrations above 60 $\mu\text{mol l}^{-1}$, TC did cause cells to shed pigment granules and thus non-specific effects were present at these higher drug concentrations.

The binding of radioactively-labelled TC* to living *Stentor* at times and under conditions similar to those used in the behavioural and electrophysiological studies was also studied on the assumption that TC acts by binding to and altering the function of specific molecular structures in the cell. For *Stentor* incubated for 2 min in TC* solutions of varying concentration, the amount of TC* bound per cell after

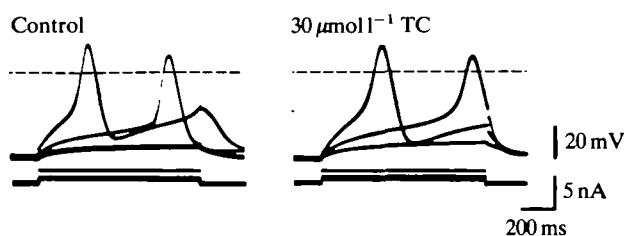


Fig. 2. Outward rectangular current pulses (bottom traces) were used to stimulate action potentials (top traces) before (Control) and after 30 $\mu\text{mol l}^{-1}$ TC introduction. The dashed line represents 0 mV.

a 4-min wash follows a hyperbolic saturation function (Fig. 1C). This curve is well approximated by a Langmuir isotherm ($K_D = 19.7 \mu\text{mol l}^{-1}$). The somewhat elevated amount of bound TC^* at $60 \mu\text{mol l}^{-1}$ may be partially attributable to the shedding of pigment granules which was generally accompanied by excessively large and erratic amounts of bound TC^* .

As the amount of bound TC^* increases the asymptotic mechanoreceptor current and probability of response decrease. The relationship between bound TC^* and mechanoreceptor current is approximately linear and these variables are well correlated ($r = -0.98$). The correlation coefficient between bound TC^* and probability of response is also high ($r = -0.98$).

Analysis of the mechanism of TC binding

The behavioural, electrophysiological and binding data presented above are consistent in showing that TC has a relatively low affinity for its binding sites on *Stentor*; therefore, TC^* would be expected to dissociate from its binding sites during the post-incubation wash procedure and be lost to the wash medium. Since this expected wash-out did not occur, a series of studies was initiated to analyse the kinetics and sites of TC^* binding.

The time course of the behavioural effect of TC was quantified by comparing the probability of response prior to drug injection (0.94) with that observed at various intervals after the addition of $15 \mu\text{mol l}^{-1}$ TC. Thirty-two minutes after drug addition the probability of response had fallen to 0.66, an asymptotic value which is somewhat higher than that observed in other studies. The data obtained after other incubation periods are calculated as a proportion of this total depression in probability of response (0.94–0.66) and plotted in Fig. 3A. TC depressed the probability of response to the asymptotic level in 4 min.

A similar study of the effect of incubation time on mechanoreceptor current was not attempted because 3 min were required to infuse new medium into the electrophysiological test chamber to minimize disturbance of the animal impaled by the microelectrodes. It was evident, however, that the depression of mechanoreceptor current was complete by the time the first stimulus was applied, i.e. 4 min after the start of the infusion.

The incubation curve for the binding of $15 \mu\text{mol l}^{-1}$ TC^* has two components: (1) an exponential component which has approached its maximum value by 4 min of incubation and (2) a linear component which becomes clearly visible after 4 min of incubation (Fig. 3B). Four additional incubation curves generated in subsequent studies also revealed these two components. The curve drawn in Fig. 3B is produced by a kinetic equation of the form:

$$\text{c.p.m.} = M(1 - e^{-mt}) + nt, \quad (1)$$

where $M = 18.1$ c.p.m., $m = 0.018 \text{ s}^{-1}$ and $n = 0.0012 \text{ s}^{-1}$. This equation was derived on the assumption that both a reversible and an irreversible or compartmentalization process occur during TC^* binding.

The proportion of the total depression in probability of response produced at given incubation times is highly correlated ($r = -0.99$) with the counts of bound TC^* as predicted by the exponential term of the above equation. The linear component of the

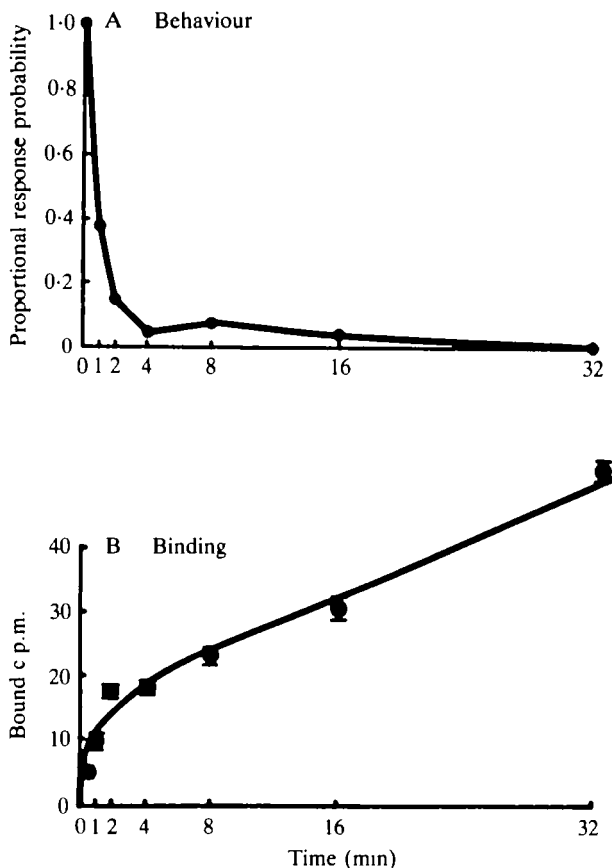


Fig. 3. (A) Time course of the TC-induced depression in response probability. The probability of response to a 4-mm drop was between 0.9 and 1.0 for all tested groups prior to drug introduction. The points show the proportion of the total decrease in probability of response to a second mechanical stimulus applied at varying times after introduction of $15 \mu\text{mol l}^{-1}$ TC. Data from 70–138 animals at each point. (B) Incubation curve for TC* binding. Means \pm s.e.m. of the counts bound to individual animals after selected incubation periods in $15 \mu\text{mol l}^{-1}$ TC*. Data from 42–57 animals at each point. The curve was drawn using the kinetic equation and constants presented in the Results section.

incubation curve has no obvious behavioural correlate. Therefore only the exponential component of TC* binding appears to represent binding to sites functionally important in mechanoreception.

Because of the functional difference between the two components of TC* binding, I attempted to determine if these components represented binding to different cellular sites. Cells were induced to shed specific cellular components prior to TC* incubation: (1) pigment granules were shed in response to immersion in cold water (Tartar, 1961); (2) the arrays of cilia surrounding the frontal field, called the membranellar bands, were shed during brief (5 s) immersion in 8% urea or 4-min immersion in 10% sucrose (Tartar, 1957) and (3) the cytoplasm was washed away after the cell had been ruptured on the surface of a DEAE filter (Wood & See, 1976). Only pretreatment of cells in 8% urea or 10% sucrose produced significant ($P < 0.01$ in both cases; t -test) reductions in the c.p.m. of bound TC* after a 2-min TC* incubation (control,

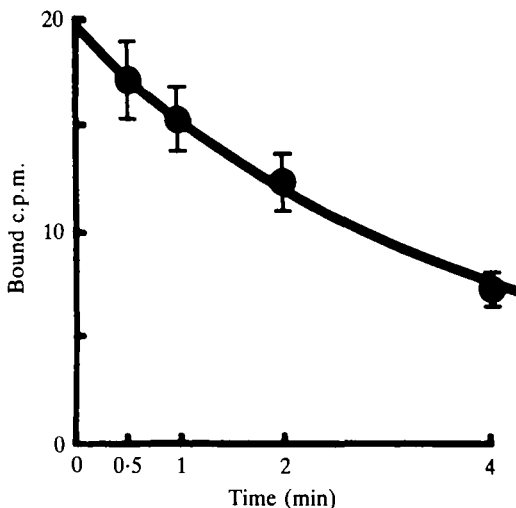


Fig. 4. Wash-out of bound TC* after urea treatment. Animals were incubated for 2 min in $15 \mu\text{mol l}^{-1}$ TC*, exposed to 8% urea for 5 s and then given additional culture medium washes for 0.5, 1, 2 or 4 min. Means \pm s.e.m. ($N = 35\text{--}76$ animals). A simple exponential decay function with an intercept at 19.6 c.p.m. and a rate constant equal to 0.004 s^{-1} was used to fit the data.

19.0 ± 1.6 , $N = 59$; urea-treated, 4.8 ± 3.3 , $N = 66$; sucrose-treated, 4.7 ± 3.4 , $N = 28$; mean \pm s.e.m.).

Although these data initially suggested that approximately 75% of the exponential component of TC* binding is to the membranellar band, subsequent analysis indicated that the urea and sucrose treatments inhibited TC* binding *via* a different mechanism. If the membranellar band were the site of mechanoreceptors which bind

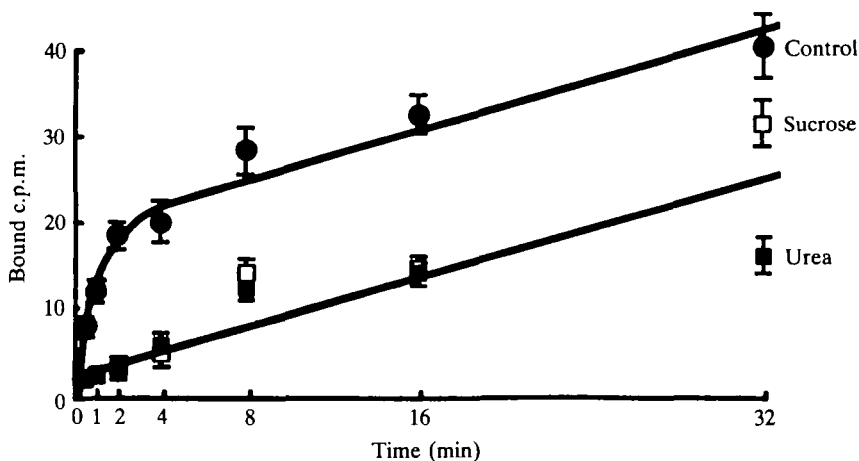
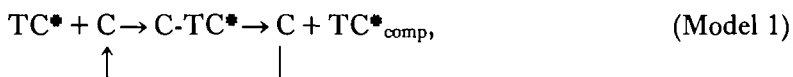


Fig. 5. The effect of 8% urea (■) or 10% sucrose (□) washes on the TC* incubation curve. To generate the control curve (●) animals were incubated for varying periods in $15 \mu\text{mol l}^{-1}$ TC* and then washed in culture medium. For the urea and sucrose data the same procedure was followed, except that the first wash was in 8% urea for 5 s or all the washes were 10% sucrose. Means \pm s.e.m. ($N = 29\text{--}46$ animals). The linear portion of the control curve has a least squares fitted slope of $0.69 \text{ c.p.m. min}^{-1}$, while the line fitted to the combined urea and sucrose data has a slope of $0.67 \text{ c.p.m. min}^{-1}$.

TC, then the loss of this band should have rendered the treated animals less responsive to mechanical stimuli. In fact, cells treated with urea or sucrose were more responsive to mechanical stimuli than were the controls. Urea-treated animals also produced normal mechanoreceptor currents. Furthermore, their behavioural responses were blocked by the addition of TC in the usual concentration range, hence mechanoreceptors which bind TC are still present on cells without membranelles. Moreover, when cells were incubated for 2 min in TC*, immersed for 5 s in 8 % urea and then washed for varying periods of time, the counts of bound TC* were found to decrease as the wash time progressed (Fig. 4). The zero time intercept of this wash-out curve is 19.6 c.p.m./cell, which agrees well with the 19.0 ± 1.6 c.p.m./cell found in control cells. Therefore urea-treated cells, despite shedding their membranelles immediately after the TC* incubation, retained all the TC* normally bound to cells. Apparently the urea treatment allows TC* to dissociate from its initial binding sites and to be carried into the wash medium, whereas a similar wash-out does not occur in control cells. The rate constant for this wash-out (0.004 s^{-1}) and the 'on' rate constant of $923 \text{ mol}^{-1} \text{ s}^{-1}$ derived from fitting equation 1 to the incubation curve yield a $K_D = 4.3 \text{ } \mu\text{mol l}^{-1}$. This value is somewhat smaller than the $19.7 \text{ } \mu\text{mol l}^{-1}$ value derived from the saturation curve, but is sufficiently close to it to suggest that TC binds to and is dissociated from the mechanoreceptor channels with similar constants in urea-treated and control cells. However, in control cells the TC*, after dissociating from the mechanoreceptor channels, appears to become compartmentalized or irreversibly bound within the cell structure, whereas in cells treated with urea or sucrose this TC* is released into the medium. Electron micrographs have not revealed a structural disruption produced by urea or sucrose treatments which might suggest the site of this compartmentalization or irreversible binding (Shigenaka, Yamaoka, Ito & Kameda, 1979).

A simple chemical model based on this analysis is presented below:



where C represents the TC* binding site and $\text{TC}^*_{\text{comp}}$ the compartmentalized or irreversibly bound TC*.

This model is supported by data from studies in which varying periods of TC* incubation were followed by a 5-s, 8 % urea immersion and 4 min of culture medium wash or by 4 min of 10 % sucrose wash. As a result of these treatments the initial exponential portion of the incubation curve was lost but the linear component was retained (Fig. 5). Therefore the linear component of the incubation curve represents a tightly bound or compartmentalized form of bound TC* which cannot be released by 8 % urea or 10 % sucrose treatments.

This model is also supported by data showing that *Stentor* which have been incubated for 2 min in TC recover their ability to contract in response to mechanical stimuli after culture medium washes.

The efficacy of other bisquaternary amine containing drugs

Gall, Deca, Succ and TC depress *Stentor*'s probability of response in different concentration ranges (Fig. 6A). The approximate ED_{50} values obtained from these

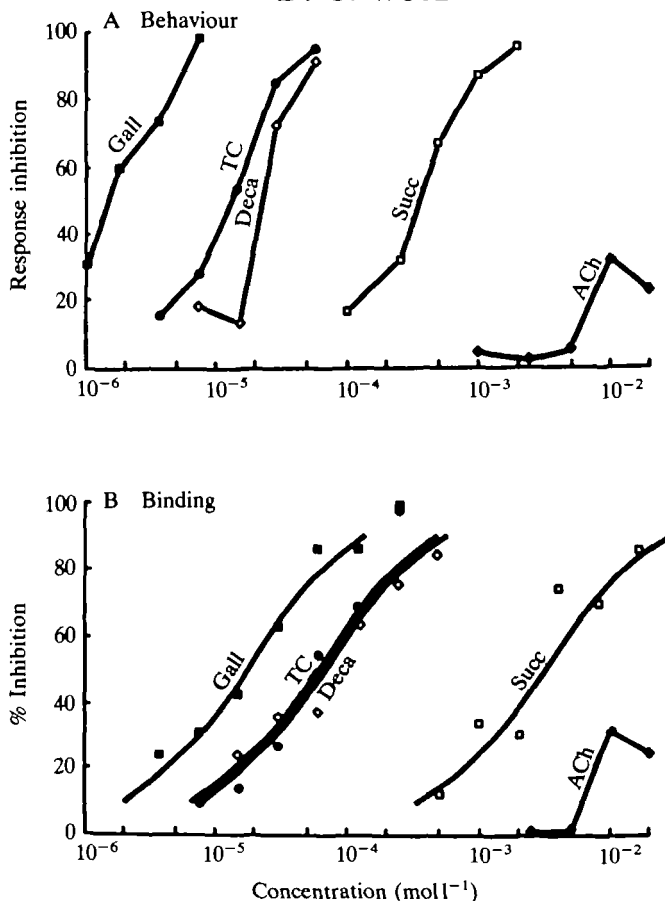


Fig. 6. (A) Response inhibition as a function of drug concentration for the five drugs studied. Response inhibition is defined as the difference between the probability of response before drug introduction and the probability of response 7 min after drug introduction divided by the probability of response before drug introduction and multiplied by 100 % ($N = 32-62$). (B) Percentage inhibition of TC* binding as a function of drug concentration for each of the five drugs studied. Percentage inhibition is defined as the difference between the counts of TC* bound to control animals after a 2-min incubation in $15 \mu\text{mol l}^{-1}$ TC* and the counts of TC* bound when cells were incubated similarly but were in the presence of the indicated drug concentration divided by the control group's counts and multiplied by 100 %. The curves drawn are least squares fits assuming the Langmuir isotherm with unimolecular reactions and competitive inhibition between the drug studied and TC*. Curve fitting was not attempted for the ACh data because the degree of inhibition obtained was too low ($N = 24-57$).

curves are: Gall, $1.4 \mu\text{mol l}^{-1}$; TC, $14 \mu\text{mol l}^{-1}$; Deca, $23 \mu\text{mol l}^{-1}$ and Succ, $360 \mu\text{mol l}^{-1}$. ACh produced no marked depression in probability of response until concentrations of $10-20 \mu\text{mol l}^{-1}$ were attained. These ACh concentrations also produced some cell shrinkage due to osmotic effects.

The affinity of these same drugs for binding sites on *Stentor* was assessed by determining their ability to inhibit the exponential component of TC* binding; direct measurement of their binding was not attempted since these drugs are not commercially available in radioactively-labelled form. For each of the drugs studied, the data points displaying the degree of inhibition of TC* binding produced by the drug were fitted with sigmoid curves of the type expected as a result of competitive inhibition

(Fig. 6B). The K_i values used to generate these curves are: Gall, $9.5 \mu\text{mol l}^{-1}$; TC, $32 \mu\text{mol l}^{-1}$; Deca, $36 \mu\text{mol l}^{-1}$ and Succ, $1.68 \mu\text{mol l}^{-1}$. ACh produced only limited inhibition of TC* binding and no attempt was made to determine its K_i . The order in which these K_i values fall – Gall < TC < Deca < Succ – is also the order in which the ED_{50} values fell in the behavioural measure. The correlation coefficient between $\log K_i$ and $\log \text{ED}_{50}$ is 0.96. This correlation suggests that the different drugs have different affinities for the binding sites on *Stentor* and that this results in their different efficacy in depressing the probability of response.

The voltage dependence of the mechanoreceptor channel

Mechanical stimulation of cells which were voltage clamped at their resting potential (-50 to -55 mV) resulted in a transient inward current (Wood, 1982; control traces at the top of Figs 7, 10). Hyperpolarizing the cell prior to the mechanical stimulus resulted in a smaller receptor current, whereas depolarizing it to transmembrane potentials between -50 and -20 mV produced receptor currents of increasing amplitude (control curve in Fig. 7). Between -20 and $+20$ mV the

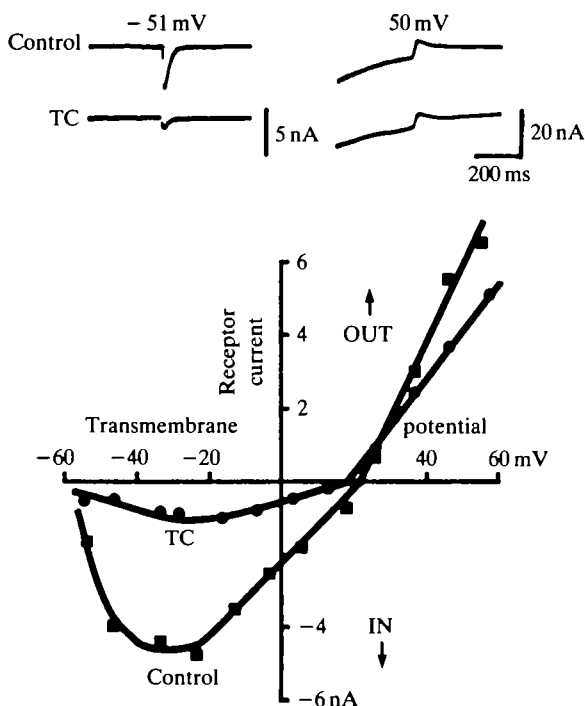


Fig. 7. I-V plots for receptor currents of control cells and cells in $30 \mu\text{mol l}^{-1}$ TC. Sample records at the top of the figure show that introduction of TC markedly depressed receptor currents in a cell voltage-clamped to its resting potential (-51 mV) but only minimally affected outward receptor currents produced at $+50$ mV. The receptor currents produced at $+50$ mV are superimposed upon the delayed outward currents triggered by the $+101$ mV voltage step. TC rather uniformly depressed inward receptor currents elicited from cells voltage-clamped to potentials more negative than the reversal potential ($+21$ mV). At potentials above this, the receptor current depression was much less marked. A control I-V curve was obtained from each of five cells prior to TC introduction and then a second I-V plot was obtained after drug infusion.

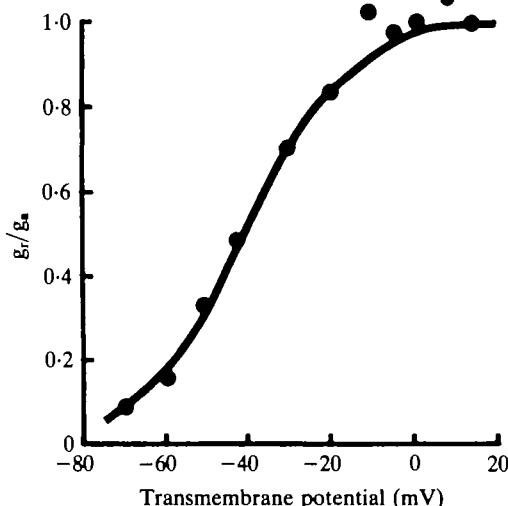


Fig. 8. Voltage dependence of the mechanoreceptor conductance. Data from I-V plots such as that shown in Fig. 7 were first analysed to determine the slope of the relation between -20 and $+20$ mV and this slope was then taken as the maximal conductance, g_s . The ratio of g_r , the conductance at a given transmembrane potential, to g_s is equal to the ratio I_r/I_s , where I_r is the receptor current at that potential and $I_s = g_s(V_m - V_{rev})$.

amplitude of the receptor current decreased linearly to a reversal potential near $+21$ mV. Above $+21$ mV, outward receptor currents were elicited and the conductance change produced by the stimulus was larger than for inward currents. Since the mechanoreceptor current reversal potential is near $+21$ mV, the increase in receptor current observed between -70 and -20 mV cannot be explained by a change in driving force but rather must result from a voltage-dependent increase in the conductance change produced by the mechanical stimulus. This conductance change, $g_r = I_r/(V_m - V_{rev})$, normalized by the conductance change, g_s , observed between -20 and $+20$ mV follows a sigmoid curve which can be well fitted using a Boltzmann distribution function (Fig. 8). An optimal fit is obtained if an e-fold change in receptor conductance is produced by a 12.6 -mV change in transmembrane potential.

These voltage-dependent shifts in mechanoreceptor current are not instantaneous. When the transmembrane potential was stepped from -50 to -70 or -90 mV and followed at varying times by a mechanical stimulus, the amplitude of the receptor current decayed exponentially ($\tau = 100$ ms) from the onset of the voltage step in both cases. This time dependence indicates that the voltage step does not influence the stimulus transduction process directly but rather acts by initiating a conversion of the channel from one form to another. The increase in receptor current produced by depolarizing voltage steps was more rapid (< 50 ms) but harder to quantify because these steps also elicited inward currents through the voltage-dependent channels responsible for producing action potentials.

The voltage dependence of TC binding and receptor current depression

Mechanical stimuli applied to cells bathed in $30 \mu\text{mol l}^{-1}$ TC produced markedly smaller receptor currents than when applied to cells in culture medium (Fig. 1B; traces at the top of Figs 7 and 10). If, 600 ms prior to the mechanical stimulus, the

transmembrane potential was stepped to a value between -50 and $+10$ mV, receptor currents were found to be only 10–30 % as large as those observed at the same potential prior to TC introduction (Fig. 7). However, when the transmembrane potential was stepped to values more positive than $+21$ mV, the outward receptor currents elicited by the mechanical stimuli were 66–82 % as large as those obtained prior to TC introduction. The reversal potential, as evaluated from either the inward or outward receptor currents, was not altered by the presence of $30 \mu\text{mol l}^{-1}$ TC (Fig. 7). Therefore, the driving force term for receptor current was the same for control and drug-treated animals and the TC-induced reduction of mechanoreceptor current observed at transmembrane potentials between -70 and $+20$ mV must have been due to a reduction in the conductance increase produced by the mechanical stimulus. At transmembrane potentials above $+20$ mV, this depression of mechanoreceptor function was largely relieved.

Since this relief from TC depression occurred only for outward currents produced at transmembrane potentials above $+21$ mV, it may have occurred because TC does not affect outward currents or because large transmembrane potential steps reduce the TC depression. To distinguish between these possibilities an experiment was performed in which a 1-s voltage step was applied to cells bathed in $30 \mu\text{mol l}^{-1}$ TC, the membrane potential was clamped again to resting potential and the mechanical stimulus was applied 15 s later. After steps to voltages between -40 and 0 mV, the inward receptor currents elicited from these cells remained small and unaltered

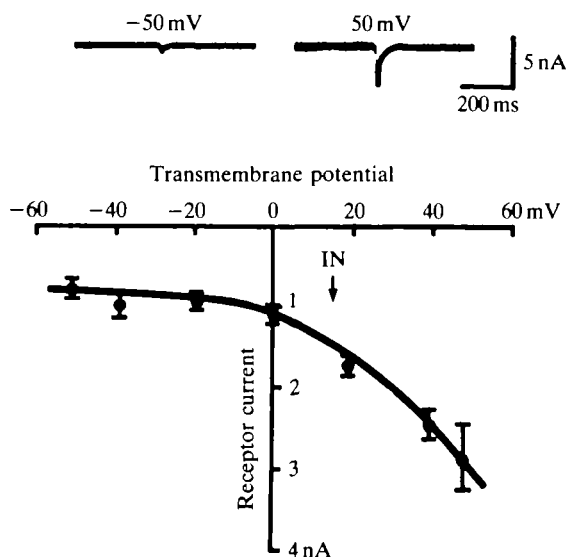


Fig. 9. The effect of voltage steps on the TC blockade of inward receptor current. Cells were voltage clamped for 1 s to the transmembrane potential indicated along the abscissa and then clamped back to their resting potential for 15 s prior to mechanical stimulation and the recording of their receptor current. Sample records at the top of the figure show that when the cell was clamped to its resting potential throughout and $30 \mu\text{mol l}^{-1}$ TC was present, only small receptor currents were observed. When a 1-s step to $+50$ mV was applied to this cell, markedly increased inward receptor currents were recorded. Data collected from four cells show that this increased inward current was observed only after voltage steps exceeding 0 mV. This increase was significant ($P < 0.01$ for voltage steps to $+20$, $+40$ and $+50$ mV; t -test for matched samples).

relative to the currents seen in the absence of such voltage steps (Fig. 9). Inward receptor currents produced after voltage steps to +10 mV or more were significantly larger than currents produced in the absence of the voltage step. Receptor currents observed after voltage steps to +50 mV were larger than those observed after steps to +10 mV or +30 mV, suggesting that even larger or longer voltage steps might produce even more pronounced reductions in the TC depression. Therefore, large voltage steps rather than outward receptor currents reduce the TC-induced depression of mechanoreceptor function.

This effect was completely reversible. Four to 6 min after the voltage step, the receptor current was again reduced to the low levels observed before the voltage step.

Since brief depolarizations relieved the TC depression, a sustained depolarization before TC introduction should more effectively eliminate TC depression. Accordingly, animals were placed in depolarizing KCl solutions (Wood, 1982), voltage clamped to their depolarized resting potential, and mechanically stimulated to record the elicited receptor current. TC ($30 \mu\text{mol l}^{-1}$) dissolved in the same KCl solution was then introduced and the mechanical stimulus tests repeated. As previously observed, introducing $30 \mu\text{mol l}^{-1}$ TC in culture medium (0.1 mmol l^{-1} KCl) reduced receptor currents to 25–30% of control values. The TC-produced reduction in receptor

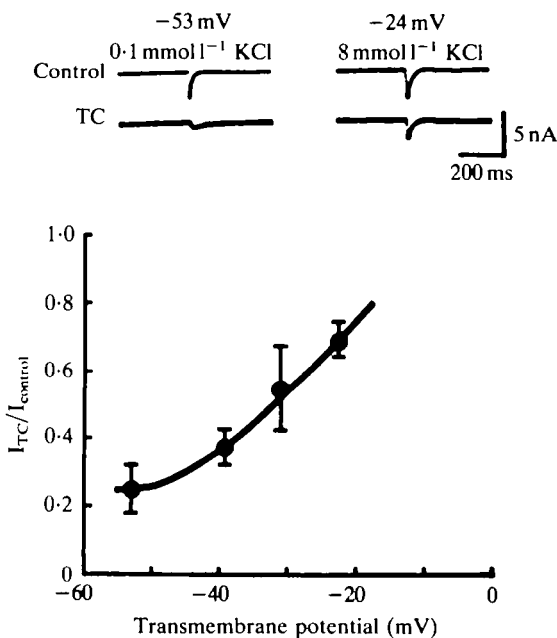


Fig. 10. TC-induced depression of receptor current in depolarized cells. Cells were placed in 0.1, 2, 4 or 8 mmol l⁻¹ KCl solutions and their resting potential and asymptotic receptor current was measured before and after introduction of $30 \mu\text{mol l}^{-1}$ TC. The effect of TC on receptor current recorded from a cell in 0.1 mmol l^{-1} KCl (-53 mV resting potential) is shown in the sample records at the upper left of the figure. At the upper right of the figure sample records taken from a cell in a 8 mmol l^{-1} KCl (resting potential -24 mV) underwent less depression when TC was infused. Data collected from five cells at each KCl concentration (lower part of figure) show that there was significantly less ($P < 0.05$ *t*-test) TC-induced depression of receptor current when cells were in 8 mmol l^{-1} KCl than when they were in 0.1 mmol l^{-1} KCl (means \pm s.e.m.).

current was significantly less marked in 4 and 8 mmol l⁻¹ KCl solutions (Fig. 10). This reduction of the TC effect by long-term depolarization with KCl solutions occurred at lower levels of measured depolarization than was required in the voltage step experiments. For example, KCl solutions which depolarized the cells to -22 mV resulted in a doubling of the receptor current seen after TC introduction, whereas a 1-s voltage step to +18 mV was required to attain a similar doubling. This result suggests that the process involved in the relief of the TC depression was only partially completed during the 1-s voltage step, whereas the steady state was more closely approached in the KCl solutions. The data do not eliminate the possibility that K⁺ ions rather than the depolarized transmembrane potential were the effective agent reducing the TC depression.

The ability of KCl solutions, or of the depolarization produced by them, to reduce the TC depression can be attributed to a reduction in the amount of TC bound to *Stentor* or to a change in the efficacy of TC once it was bound. The possibility that TC binding was reduced by these KCl solutions was tested by measuring the binding of TC*. *Stentor* were pre-treated for 5 min in the depolarizing KCl solutions, incubated for 2 min in 15 μ mol l⁻¹ TC* dissolved in the same KCl solution and then washed in culture medium before being counted. Progressively less TC* was bound to cells as the concentration of KCl was increased (Fig. 11). Addition of similar concentrations of CaCl₂ to the incubation medium did not produce a similar reduction in bound TC*; in fact a slight increase in TC* binding was observed. This suggests that the effect of KCl on TC* binding was not a non-specific ionic effect. Instead it appears likely that the depolarization produced by the KCl reduced the amount of bound TC*.

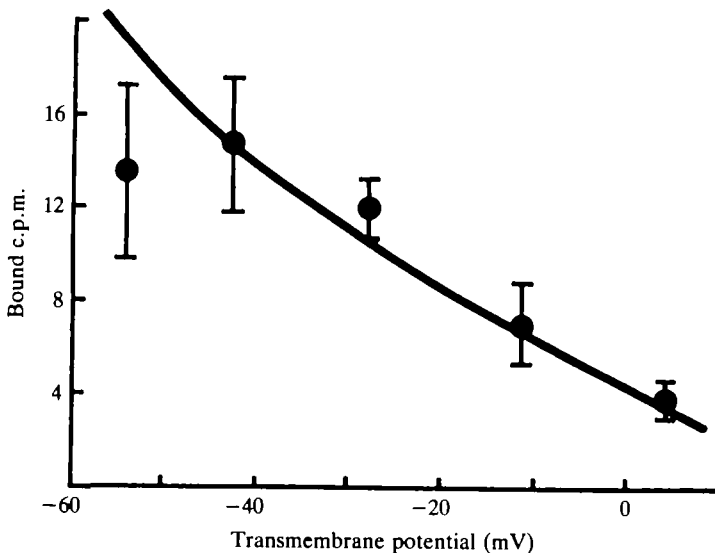


Fig. 11. Depression of bound TC* as a function of transmembrane potential. Cells were placed in 0.1, 2, 4, 8 or 16 mmol l⁻¹ KCl solutions for 4 min prior to and during the 2-min incubation in 15 μ mol l⁻¹ TC*. The transmembrane potential of these cells was assumed to be equal to the values previously observed (Wood, 1982). The presence of 8 mmol l⁻¹ (-12 mV resting potential) or 16 mmol l⁻¹ (+4 mV resting potential) KCl significantly ($P < 0.005$ and $P < 0.001$, respectively; *t*-test) reduced the amount of TC* bound to *Stentor*. Means \pm S.E.M. ($N = 21-58$ animals).

DISCUSSION

TC ($15\text{--}60\ \mu\text{mol l}^{-1}$) markedly reduces the probability that *Stentor* will contract in response to a mechanical stimulus (Tartar, 1961; Wood, 1977; Fig. 1). TC similarly reduces the probability of response of the contractile protozoan, *Spirostomum* (Applewhite, 1972). This effect of TC and other bisquaternary amine drugs appears to be specific to mechanoreception since responses to electrical and photic stimuli are not altered. The specificity of TC action is also indicated by its ability to reduce mechanoreceptor currents while not affecting the other electrophysiological properties measured: resting potential, action potential peak, action potential threshold and specific membrane resistance. Because of this specificity, TC does not appear to alter surface charge density across the entire surface of the membrane. At concentrations above $60\ \mu\text{mol l}^{-1}$, non-specific effects of TC are observed as previously mentioned.

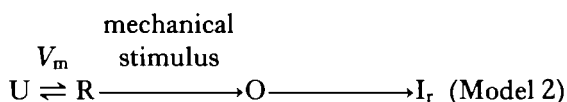
Based on the concentration dependence of the TC-induced depression of contractile behaviour and mechanoreceptor current, the K_D for TC binding appears to be rather large – in the range $5\text{--}15\ \mu\text{mol l}^{-1}$ (Fig. 1A,B). Because of this large K_D , cells incubated in TC* would be expected to lose all bound label to the wash medium during the post-incubation wash procedure. However, such a loss was not observed when the post-incubation washes were with culture medium. On the other hand, if a 2-min TC* incubation was immediately followed by a 5-s immersion in 8 % urea and then culture medium washes, the expected wash-out of bound TC* was observed (Fig. 4). This urea-induced wash-out occurs with a rate constant compatible with that calculated from the K_D and 'on' rate constant. These data suggest that urea treatment blocks the cellular mechanism which normally prevents the wash-out of TC*. Urea treatment does not block TC binding to mechanoreceptors since urea-treated cells retain bound TC* for brief periods of time and since their probability of contracting in response to a mechanical stimulus is still depressed by TC. As the TC* incubation period increases beyond 2 min, the ability of urea treatment to remove bound TC* decreases and the linear component of TC* binding, which appears to be unaffected by urea treatment, is revealed (Fig. 5). Four minutes of washing in 10 % sucrose (Fig. 4) or in an excess ($1\ \text{mmol l}^{-1}$) of Deca (data not shown) similarly eliminated the exponential component of the incubation curve while leaving the linear component. The previously introduced chemical model (1) provides an explanation of these observations. In this model, TC binds initially to a site on the mechanoreceptor channel. When it dissociates from this site it is compartmentalized or irreversibly bound within the cell structure. TC* which has been compartmentalized or irreversibly bound in this way is not removed by culture medium washes and hence is counted in the assay. Treatment of the cells with 8 % urea or 10 % sucrose prevents this compartmentalization or irreversible binding process and thereby results in the loss of the dissociated TC*.

The depression in response probability produced by TC is essentially complete within 4 min after drug injection and is highly correlated with the exponential component of the incubation curve (Fig. 3). Likewise, the depression of mechanoreceptor current is complete 4 min after drug injection. These correlations suggest that the exponential component of the TC* incubation curve represents binding to a functionally necessary component of the mechanoreceptor and could therefore serve as a measure of this component.

This possibility was explored in two additional sets of data which allowed correlations between behavioural or electrophysiological data on the one hand and binding data on the other. In all cases, a 2-min incubation time was used in the TC* binding assays since at this time the exponential component is near its steady state (within 90 % of its final value), and only a small proportion of the measured counts is due to already compartmentalized or irreversibly bound TC*. In the first of these sets of data, the concentration dependence of response probability, receptor current and TC* binding were measured (Fig. 1). The correlation coefficients between response probability and TC* binding ($r = -0.98$) and between receptor current and TC* binding ($r = -0.98$) are high, though the depressions of response probability and receptor current suggest lower dissociation constants ($K_D = 7.5$ and $15 \mu\text{mol l}^{-1}$ respectively) than does the binding curve ($K_D = 19.7 \mu\text{mol l}^{-1}$). In the second set of data, different bisquaternary amine drugs were studied for their ability to depress response probability and to inhibit TC* binding (Fig. 6). The order of efficacy, Gall > TC > Deca > Succ, for depression of response probability is the same as that for inhibition of TC* binding and the log ED₅₀ values for the behavioural measure are highly correlated ($r = 0.96$) with the log K_i values of the various drugs (as calculated on the assumption of competitive inhibition). These additional correlations reinforce the idea that TC* binding provides a measure of functional mechanoreceptor channels.

It is important to consider the possibility that TC* may be binding to sites other than mechanoreceptors with a K_D similar to that with which it binds to mechanoreceptors themselves; i.e. there is non-specific binding. Both mechanoreceptor function (Fig. 7) and TC* binding (Fig. 11) are voltage dependent. This correlation can be explained by assuming that after the 2-min incubation most of the measured TC* was bound to or near the voltage-dependent element in the mechanoreceptor (see below). It seems much less likely that significant amounts of TC* were binding to a second site with both a K_D and a voltage dependence similar to that of the mechanoreceptor. It will therefore be assumed throughout the remainder of the Discussion that the TC* measured after a 2-min incubation was initially bound to mechanoreceptors.

As shown in Fig. 8, the mechanoreceptor conductance is voltage dependent, increasing as the membrane is depolarized. However, depolarization is not sufficient to open the mechanoreceptor channels; a mechanical stimulus is also required. Permissive voltage dependence of this type is also somewhat evident in the function of *Stylonychia* mechanoreceptors (Deitmer, 1981) and clearly evident in molluscan serotonergic receptors (Pellmar & Carpenter, 1980). This voltage dependence can be explained by assuming that the mechanoreceptor channels can exist in three distinct forms: (1) the open or O form, (2) the responsive or R form, which is closed but opens upon mechanical stimulation, and (3) the unresponsive or U form, which is closed and cannot be opened. R can be rapidly (< 400 ms) converted to U or U to R by changes in transmembrane potential. These assumptions result in the following model:



Since g_r is proportional to the number of channels in the O form, it also reflects the proportion of mechanoreceptor channels in the R form. Therefore, the voltage dependence of g_r is a result of the voltage dependence of the R to U conversion and can be described by a Boltzmann distribution function, where the free energy difference between the R and U forms is directly proportional to V_m . This equation best fits the data (Fig. 8) when a value of 12.6 mV per e-fold change is used. Steric, electrostatic and other factors appear to stabilize the R form so that the midpoint of the voltage-dependence curve is at -41 mV. This model is essentially that of Hodgkin & Huxley (1952) and describes the distribution of a mobile charged body for which there are only two stable positions within the uniform field of a parallel plate capacitor, the membrane.

Three sets of data indicate that TC binding interacts with the voltage-dependent shift in the mechanoreceptor: (1) depolarizing voltage steps relieve the depression of mechanoreceptor current produced by previously applied TC (Fig. 9); (2) depolarization of the membrane by increasing K^+ in the bathing medium prior to and during TC* incubation reduces the binding of TC* (Fig. 11); and (3) prior depolarization also reduces the depression in mechanoreceptor current produced by TC (Fig. 10). In each of these cases, less TC appears to bind to the mechanoreceptor at depolarized potentials than at the usual resting potential. This voltage-dependent character of TC binding suggests that the drug is binding to or near the ionic channel component of the mechanoreceptor, since the ionic channel is likely to be the voltage-dependent element in the mechanoreceptor. In this case, the action of TC on *Stentor* provides an additional example of TC action against ionic channels, although previously reported examples (Manalis, 1977; Carpenter *et al.* 1977) have been of Na^+ , K^+ or Cl^- channels whereas the *Stentor* mechanoreceptor channel carries mainly Ca^{2+} ions.

Since TC binds more effectively at resting potential, where the U form of the mechanoreceptor channel predominates, and less effectively at depolarized potentials, where the R form predominates, it appears that TC has a higher affinity for the U than for the R form. As a result a greater proportion of mechanoreceptors will be in the U or U-TC forms in the presence of TC than would be in the U form in the absence of the drug. Since U and U-TC forms do not open when mechanically stimulated, a decrease in mechanoreceptor current and a depression in the animal's probability of contracting to a mechanical stimulus will result.

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