EFFECTS OF ETHIDIUM BROMIDE ON CELLS OF THE COCKROACH CENTRAL NERVOUS SYSTEM

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SUMMARY
The effects of ethidium bromide were tested on isolated cockroach interganglionic connectives, either with the perineurium intact or desheathed. Intact connectives showed small, reversible depolarizations in response to 25 mM-ethidium. Desheathed connectives showed much larger, concentration-dependent depolarizations and the membrane potentials repolarized only slowly after washing out ethidium. The ethidium-induced depolarization could be blocked by raising the K concentration in the salines and was also shown to be reduced by rubidium, caesium, ammonium and ouabain. These treatments also reduced the degree of staining of nerve cords by ethidium, which was demonstrated to enter the cells.

INTRODUCTION
Ethidium bromide (EthBr), an intercalating agent of DNA (see Waring, 1981), is used in many areas of cell biological research. The intercalating properties of ethidium allow it to be used in buoyant-density procedures; for example, to separate nicked and closed-circular DNA (Radloff, Bauer & Vinograd, 1967); and several other intercalating agents have been shown to be mutagens of prokaryotic cells. EthBr has been used to monitor permeability lesions of cell membranes, as it is normally impermeant (Gomperts, 1983), and has also been shown to induce demyelination in the vertebrate central nervous system (see Blakemore, 1982) and to disrupt selectively neuroglia in cockroach interganglionic connectives (Smith, Leech & Treherne, 1984).

Experiments on yeast cells have shown that at low concentration (less than 1 mM), ethidium is actively taken up by the transport system for monovalent cations (see Pena & Ramirez, 1975) and that, at higher concentrations, ethidium can cause an all-or-none disruption of the diffusion barrier of these cells to potassium (Theuvenet et al. 1983). In a previous study on the cockroach (Smith, Leech & Treherne, 1984), glial disruption was induced by application of 25 mM-EthBr. If ethidium causes a breakdown of the membrane permeability properties of central nervous system (CNS) cells, then this may be one step involved in glial disruption. Such changes in membrane permeability could be monitored by looking at the electrophysiological properties of the nerve cord. Although it is difficult to record from glial cells, axonal effects can be easily followed. The results described here were obtained using intracellular microelectrodes to look at changes in single axons, and the sucrose-gap method to look at the compound response of the nerve cord.
The mode of entry of EthBr into other types of cell does not appear to have been investigated. Experiments on yeast cell raise the possibility that ethidium may be actively transported into CNS cells of the cockroach via the Na-pump, the only pump that has been demonstrated to be present in this system.

MATERIALS AND METHODS

Experiments were performed on interganglionic connectives of adult male cockroaches (Periplaneta americana) from the laboratory culture. Nerve cords were isolated and mounted in a five-compartment experimental chamber (Treherne, Schofield & Lane, 1973) in which conventional intracellular and sucrose-gap recording techniques could be used. The two left-hand compartments of the chamber contained stimulating electrodes. The central compartment was separated, by a compartment through which isotonic mannitol was flowing, from the right-hand compartment, which contained the cut end of the nerve cord bathed in isotonic KCl. Microelectrode records were obtained by impalement of the cord in the central compartment of the chamber, and sucrose-gap records represent the potential recorded in the right-hand compartment with reference to the central compartment.

Salines were perfused continuously through the central, earthed compartment of the chamber and could be changed using a multi-way, non-return valve (Holder & Sattelle, 1972). Nerve cords with the perineurium either intact or surgically removed (de-sheathed, using fine needles), were used to investigate some effects of the in vitro application of EthBr.

Standard cockroach saline had the following composition: 157 mM-NaCl, 3 mM-KOH, 2 mM-CaCl₂, 2 mM-MgCl₂, 5 mM-trehalose, 86 mM-HEPES buffer (pH 7.2). EthBr (obtained from Sigma) was added (hypertonically) to salines on the day of the experiment. Experiments were performed at room temperature (22–29°C).

All probabilities were calculated from the Mann–Whitney U-test and are single-tailed.

RESULTS

Fig. 1A,B,C shows a series of records from an intact connective demonstrating the effects of 25 mM-EthBr. A small, reversible positive shift of the sheath potential (A), d.c. sucrose-gap potential (b) and intracellular resting potential (c) can be seen. These voltages are relative to the bath potential. Fig. 1D,E,F shows sucrose-gap d.c. records from desheathed connectives and shows large, concentration-dependent depolarizations, which were accompanied by loss of the action potential. The recovery of the resting potential is very slow and because of this each preparation received only one test pulse of EthBr. Control experiments in which NaBr was added in place of EthBr did not show these effects.

A standard 10-min pulse of EthBr was generally used, but the effect of longer exposure was also tested. The resting potential of desheathed connectives during a 40-min exposure to 5 mM-EthBr did not reach a steady level and very little recovery was observed during a 60-min washout period.

No evidence was found from these experiments on cockroach nerve cord for a threshold concentration above which there was an all-or-none response, in the range 0.5 to 25 mM-EthBr. Intracellular records of the shift in resting potential showed graded responses similar to those seen from sucrose-gap records.

An example of an intracellular record from a giant axon exposed to 5 mM-EthBr (in normal saline) is shown in Fig. 2. In this experiment, small hyperpolarizing current
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Fig. 1. A, B, C. A series of pen-recorder traces from an intact connective. A. A microelectrode recording of the sheath potential (Pichon & Boistel, 1967) that had an initial value of 10 mV. A 25 mM solution of EthBr in normal saline flowing at 3-4 ml/min was introduced for 10 min (marked by broken lines). 
B. A sucrose-gap d.c. record; the resting potential had an initial value of -64 mV. C. An intracellular, axonal resting potential (initial value -74 mV).
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D, E, F. Sucrose-gap records from desheathed nerve cords. Flowing EthBr solutions were introduced (broken vertical lines) at concentrations of 5 mM (D, initial $E_m$ = -70 mV), 10 mM (E, initial $E_m$ = -68 mV) and 25 mM (F, initial $E_m$ = -64 mV). An upward deflection of the traces indicates a positive shift of potential in all records. Bars: 20 mV (vertical), 10 min (horizontal).

Pulses (5 nA, 1 s) were passed through the recording electrode. The voltage displacement resulting from the injection of current was largely offset after the cell was impaled on the electrode, and the effects of application of EthBr on this displacement, which should reflect changes in membrane resistance, were monitored. Eight axons (in different preparations) were investigated in this way and six of these cells showed an apparent increase in resistance while two showed a fall in resistance. All of these axons, however, initially showed a slowing of the falling phase of their action potential during EthBr application, which may arise from a reduction in conductance of $K^+$. If EthBr also reduces the resting $K^+$-permeability of the axon then the membrane resistance should increase and the resting potential may fall. The effects on the action potential are easier to explain if EthBr caused an increase in resting $K^+$-permeability, leading to conduction block, and possibly a reduction of the voltage-dependent delayed $K^+$-conductance, though this makes it difficult to explain the apparent increase of input resistance.

EthBr may produce these effects by interacting with the external surface of the cells or it may have an intracellular action. EthBr fluoresces when in contact with nucleic acids; hence, if it enters cells, highly fluorescent nuclei should be seen in treated nerve cords. If EthBr does not enter the cells, then this eliminates the possibility of an intracellular effect. Examination of both intact and desheathed connectives, treated with EthBr, showed the presence of fluorescent nuclei, indicating that ethidium had
entered the cells (Fig. 3). It is interesting to note that the fat body associated with the nerve cord becomes stained very intensely.

This demonstration of intracellular ethidium now raises the question of how this entry occurs, as EthBr is normally impermeant, at least in mast cells (Gomperts, 1983). Unless the membrane properties of cells within the cockroach CNS are markedly different from those of mast cells, it is difficult to imagine the entry of EthBr being purely passive. This leaves the possibility that, by analogy with yeast cells,

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Fig. 2. Intracellular records of resting and action potentials from an axon in a desheathed preparation showing the effects of EthBr (5 mM) in normal saline. A. An action potential recorded before the introduction of EthBr. B, C, D, E, F were taken 1, 5, 7 and 10 min, respectively, after the introduction of EthBr; and F was taken 10 min after the reintroduction of normal saline. G. A pen-recording of the resting potential from the same axon as above. An upwards deflection represents a positive shift of the resting potential. Hyperpolarizing current pulses (5 nA, 1 s) were passed through the recording electrode, which was connected to a WPI M-701 microprobe. These current pulses were balanced with the electrode in the cell. Bars: A–F, 40 mV (vertical), 2 ms (horizontal); G, 20 mV (vertical), 5 min (horizontal).
Fig. 3. Ethidium bromide fluorescence. This nerve cord, with the perineurium intact, was treated in vitro for 10 min with 50 μM-EthBr in K-free saline. The nerve cord was pre-soaked for 20 min in the K-free saline before application of the EthBr solution. A. The connectives between the 2nd and 3rd abdominal ganglia; B. The junction of the connectives with the 6th ganglion. f, fat body; t, tracheole. ×140.

EthBr is transported into cells, with the sodium-pump being the most obvious mechanism. The uptake of ethidium by yeast cells is inhibited by raising the [K] in the bathing medium. Cockroach nerve cords treated with EthBr in salines of high [K] become stained, but much less intensely than cords treated in normal saline.

If the electrophysiological effects of EthBr are a result of some intracellular event and, if elevation of [K] reduces the amount of ethidium entering the cells, it should be possible to modify these effects on the resting potential of axons by changing the saline [K]. The effect of different [K] on the response of desheathed connectives to ethidium is shown in Fig. 4. [K] was varied by substitution with Na, and EthBr (5 mM) was added to these salines. Fig. 4a shows the response of a preparation that was pre-washed with K-free saline before application of EthBr. The size of the response (at the end of a 10 min pulse) is significantly larger (n = 3, P = 0.028) than in 3 mM-K (n = 4) saline (Fig. 1b). The ethidium-induced depolarization was reduced and eventually abolished by raising the saline [K] (Fig. 4b,c).

Rb also inhibited ethidium uptake by yeast cells but was only about 50% as effective as K, while Cs had very little effect. The response of desheathed connectives to ethidium in K-free salines containing Rb or Cs was also tested. These cations (15 mM) were added by substituting them for Na in K-free saline. Rb (Fig. 4a) and K (Fig. 4b) have equal effects at the same concentration (P = 0.14, n = 4), Cs (Fig. 4c) is less effective but has a significant competitive action (P = 0.028, n = 4) when compared with the effect of ethidium in K-free saline. NH₄ (15 mM) has an effect similar to that of Cs at the same concentration.

The results described above are consistent with the transport of ethidium into axons by the Na-pump in competition with K. In this case, addition of ouabain, which inhibits the axonal Na-pump in cockroach (Pichon & Treherne, 1974), might be expected to reduce or block the effect of ethidium. Perfusion or desheathed connectives with 0.2 mM-ouabain caused a small, rapid, depolarization and addition of 5 mM-EthBr to this saline caused a further depolarization (Fig. 4f), though this shift was
Fig. 4. Sucrose-gap records showing the response of desheathed nerve cords to 5 mM-EthBr in different salines. The experimental protocol (except for A) was as follows: the preparation was equilibrated in normal (3 mM-[K]) saline for 30–40 min and then transferred to the test saline (without EthBr) for 10 min. This was followed by a 10-min pulse of EthBr (in test saline) and finally by washing in normal saline. The preparation shown in A was prewashed for about 30 min in K-free saline. The test salines and initial resting potentials (RP) are as follows: A, K-free, RP = -79 mV; B, 15 mM-[K], RP = -71 mV; C, 30 mM-[K], RP = -67 mV; D, 15 mM-[Rb], RP = -79 mV; E, 15 mM-[Cs], RP = -70 mV; F, 0.2 mM-ouabain, RP = -71 mV; G, 0.2 mM ethacrynic acid, RP = -77 mV. Bars, 20 mV (vertical), 10 min (horizontal).

significantly smaller ($P = 0.028$, $n = 3$) than that induced by ethidium in the absence of ouabain. The effect of ethacrynic acid (0.2 mM), which inhibits glial Na-pumps (Pichon & Treherne, 1974), was also tested. Ethacrynic acid has no effect on the resting potential of desheathed connectives (Pichon & Treherne, 1974) but addition of 5 mM-EthBr to this saline caused a depolarization, which was consistently larger ($P = 0.028$, $n = 3$, Fig. 4G) than in the absence of ethacrynic acid. Tetraethylammonium ions (TEA, 50 mM, added by substitution for Na) also appeared to cause a slight, but not significant ($P = 0.20$, $n = 3$), reduction in the amplitude of the ethidium response. Such a reduction might be expected, as TEA can block the Na-pump (Sachs & Conrad, 1968).

**Discussion**

EthBr can enter cells of the cockroach CNS since nerve cords bathed in a 50 μM solution of EthBr had fluorescent nuclei, indicating that ethidium was in contact with
nuclear DNA. This contrasts with the situation in mast cells in which ethidium is impermeant (Gomperts, 1983).

Application of EthBr to cockroach connectives results in a concentration-dependent depolarization of axons. However, the mechanism by which ethidium produces this effect is not clear. The axonal depolarization may result from the disruption of regulation of normal ion gradients. Such disruption would lead to a loss of K from the cells (and an increase in intracellular Na), resulting in depolarization of the cell membrane as electrochemical gradients fall. Inhibition of the Na-pump, which is involved in the regulation of cell resting potentials in cockroach central connectives (Pichon & Treherne, 1974), could be important in such an effect. In crayfish giant axons, ouabain (at a concentration (0.5 mM) that caused 90–100% inhibition of the Na-pump) was shown to cause a 45% reduction in $R_m$ (Lieberman & Lane, 1976) through an increase in the chloride conductance (Lieberman & Nosek, 1976). However, in the cockroach, EthBr did not appear to cause such a decrease in $R_m$, and, if ethidium was acting through inhibition of the Na-pump, then ethidium and ouabain might be expected to have additive effects rather than the competitive action observed. Also, the time-courses of the depolarization resulting from EthBr and ouabain (0.2 mM) were quite different (Fig. 4). This difference makes it seem unlikely that ethidium exerts its effect purely by inhibiting the Na-pump.

Potassium ions appear to compete with ethidium. When the [K] in the saline was raised the depolarization resulting from application of ethidium was reduced and eventually abolished. It is interesting to note that the degree of recovery also increases with [K] (Fig. 4A,B,C). Corresponding differences in the extent of recovery were observed after EthBr application in Rb and Cs-containing salines, the degree of recovery with Rb saline, which offered a greater protective effect, was more complete than with Cs.

The results obtained in high-[K] salines (also the effects of Rb, Cs), might arise from competition for a binding site, which then leads to: (1) inhibition of the Na-pump by ethidium; or (2) reduced transport of ethidium into the cells. In other cell types, ouabain binding has been shown to be sensitive to external K+ (Baker & Willis, 1972). The effects of high [K] could be explained if EthBr caused inhibition of the Na-pump by binding to the same site as ouabain. Alternatively, ethidium and potassium could compete for the same site at which to be transported into the cells.

Ethidium is actively transported into yeast cells where its concentration can reach over 100 times that in the bathing medium (Pena & Ramirez, 1975). The experiments described here provide circumstantial evidence that ethidium may be transported into cockroach CNS cells by the Na-pump, but without detailed examination of the uptake of ethidium its mode of entry remains uncertain.

Theuvenet et al. (1983) described, in yeast cells, an efflux of K as a result of an all-or-none disruption of the diffusion barrier to K by high concentrations of ethidium. The similarity of intracellular records with sucrose-gap d.c. records argues against an all-or-none response, in the concentration range investigated, of cockroach central connectives. The possibility of a concentration-dependent breakdown of membrane permeability properties remains and such an effect may explain the extremely slow
recovery following ethidium treatment. The degenerative response of cells may then be due to elevation of intracellular calcium concentration (see Aviv, Hochner & Spira, 1982) resulting from an influx of calcium through the damaged membrane. If this was the case then EthBr might be expected to have toxic effects on both glia and neurones. However, in cockroach central nervous connectives, application of EthBr leads to selective disruption of the glia (Smith et al. 1984). This result would be expected if ethidium exerts its toxic effect through intercalation of nucleic acids, as neuronal cell bodies are not found within the connectives.

REFERENCES


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