absence and presence of atropine, $10^{-7}$M. A binding curve was constructed showing that the specific binding saturated between 15–30 minutes. Assuming that the rate of alkylation is faster than the rate at which the PrBCM-receptor complex dissociates (Gill & Rang, 1966) the rate constant for the formation of the reversible complex is approximately $6 \times 10^5$ $M^{-1}$ $s^{-1}$. The maximal binding capacity of the tissue is 9 pmol/g dry weight. This value is low compared to 150 pmol/g wet weight for longitudinal ileum muscle of the guinea-pig (Young, Hiley & Burgen, unpublished). Since ileum muscle consists of 90% smooth muscle cells (Rang, 1967) compared to approximately 70% for amnions, the latter can have only 1–2% of the receptors in the ileum.

Further evidence that the specific binding of $^3$H-PrBCM was to muscarinic receptors was obtained by incubating membranes with atropine at concentrations ranging from $10^{-10}$ to $10^{-7}$M, after which $^3$H-PrBCM (2·4 nM) was added. The uptake was measured at 12 min, that is before saturation was achieved. The concentration of atropine causing 50% inhibition of uptake under these conditions was about $10^{-9}$M. This value is in good agreement with the $pA_2$ value for atropine of 8·8 for this tissue (Evans & Schild, 1959). The binding of $^3$H-PrBCM was not affected by tubocurarine, $10^{-6}$M.

Pharmacological studies, together with these results suggest the tissue has few spare receptors.

REFERENCES

CUTHBERT, A. W. (1962). *Ph.D. Thesis, University of London.*

EVANS, D. H. L. & SCHILD, H. O. (1959). Quoted by Arunlakshana, O. and Schild, H. O. Some quantitative uses of drug antagonists. *Br. J. Pharmac.*, **14**, 48–58.

GILL, E. W. & RANG, H. P. (1966). An alkylating derivative of benzilylcholine with specific and long-lasting parasympatholytic activity. *Mol. Pharm.*, **2**, 284–297.

RANG, H. P. (1967). The uptake of atropine and related compounds by smooth muscle. *Ann. N.Y. Acad. Sci.*, **144**, 756–767.

## The binding of labelled tetrodotoxin and cobra toxin by the rat diaphragm

D. COLQUHOUN*, H. P. RANG† and J. M. RITCHIE

*Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut, USA*

Denervation of skeletal muscle brings about two important changes: first a spread of acetylcholine (ACh) sensitivity away from the endplate (Miledi, 1960); and secondly, the development of tetrodotoxin (TTX)-resistant action potentials (Redfern & Thesleff, 1971). One way that these two phenomena might be linked would be that the normal TTX-sensitive sodium channels acquire, after denervation, a sensitivity to ACh and lose their sensitivity to TTX. We have examined this possibility by comparing, in rat diaphragm, the number of TTX binding sites in normal muscle with the number of cobra toxin binding sites (presumed ACh receptors (Lee, Tseng & Chiu, 1967; Lester, 1970)) that appear after denervation.

Hemidiaphragms from normal rats were incubated for 4 h at 21° C with $^3$H-TTX (Colquhoun, Henderson & Ritchie, 1972) at concentrations from 0·6 nM to 590 nM, $^{14}$C-inulin was included in the incubation medium so that the extracellular space could be estimated and used to calculate the amount of TTX bound to the muscle.

The curve (128 points) relating the amount of bound TTX to the TTX concentration was analysed with a least squares procedure into the sum of a linear component, and a saturable hyperbolic component, as in Colquhoun *et al.* (1972). The saturable component has an equilibrium dissociation constant of 12·7 nM (95% likelihood interval 7·1 to 22·2 nM), a value roughly consistent with electrophysiological results.

The TTX-binding capacity of the saturable component is 3·9 fmole/mg wet (95% likelihood interval, 2·50 to 6·31 fmole/mg wet). If the fibre diameter is assumed to be 40 $\mu$m and the transverse tubular system is ignored, the membrane area would be

† Present address: Department of Physiology and Biochemistry, University of Southampton, Southampton SO9 5NH.

about 0·7 $cm^2$/mg wet weight, so that the TTX site density would be 33 sites/$\mu m^2$ (cf. 27 sites/$\mu m^2$ in rabbit vagus C fibres (Colquhoun *et al.*, 1972)).

Cobra toxin binding capacity was measured by saturating muscles with $^3$H-Naja nigricollis toxin (100 nM for 7 h, followed by 3 h washing in toxin-free solution). In normal muscle the uptake of cobra toxin was $8·7 \pm 1·4$ (S.E. of mean, $n=6$) fmole/mg wet: 7–14 days after denervation it increased to $50·9 \pm 2·8$ ($n=6$) fmole/mg wet. The increase after denervation, 42 fmole/mg wet, is thus about 10 times greater than the TTX binding capacity of the normal muscle. It is thus unlikely that denervation changes can be explained simply by interconversion of channels from one type to another.

This work was supported in part by a grant NS08304 from the U.S.P.H.S. We are grateful to Dr. J.-P. Changeux for supplying the purified α-toxin.

REFERENCES

COLQUHOUN, D., HENDERSON, R. & RITCHIE, J. M. (1972). The binding of labelled tetrodotoxin to non-myelinated nerve fibres. *J. Physiol., Lond.* (in press).

LEE, C. Y., TSENG, L. F. & CHIU, T. H. (1967). Influences of denervation on localization of neurotoxin from elapid venoms in rat diaphragm. *Nature*, **215**, 1177–1178.

LESTER, H. A. (1970). Postsynaptic action of cobra toxin at the myoneural junction. *Nature*, **227**, 727–728.

MILEDI, R. (1960). The acetylcholine sensitivity of frog muscle fibres after complete or partial denervation. *J. Physiol., Lond.*, **151**, 1–23.

REDFERN, P. & THESLEFF, S. (1971). Action potential generation in denervated rat skeletal muscle. *Acta physiol. Scand.*, **82**, 70–78.

## Studies on 5-hydroxytryptamine receptors of neurones from *Hirudo medicinalis*

P. A. SMITH and R. J. WALKER*

*Department of Physiology and Biochemistry, Southampton University, Southampton SO9 5NH*

5-Hydroxytryptamine (5-HT) has been shown to be present in leech Retzius cells (Kerkut, Seddon & Walker, 1967) and to have an inhibitory action on these cells (Kerkut & Walker, 1967) but the ionic mechanism for this inhibition has not been determined. Intracellular recordings were made from Retzius cells in isolated ganglion chain preparations of the leech *Hirudo medicinalis* using glass microelectrodes filled with molar potassium acetate. The potentials were amplified and displayed on a Tektronix 502A oscilloscope and permanent traces obtained using an A.E.I. pen oscillograph. The Ringer used had the following composition: NaCl 115 mM; KCl 4 mM; $CaCl_2$ 2mM; Tris-chloride buffer 10 mM; glucose 10 mM; pH 7·4.

Results from changing either external or internal chloride levels suggested that the major ion involved in 5-HT inhibition was chloride. The 5-HT response was measured in normal and in 50 mM chloride Ringer, chloride being replaced by acetate. From the Nernst equation the internal chloride concentration was calculated to be 9 mM (mean of five experiments), giving a chloride equilibrium potential of about 65 mV. Changing either external sodium or potassium levels or using a Ringer containing 20 mM $MgCl_2$ had no effect on the 5-HT response. Strychnine also had an inhibitory effect on Retzius cells and this inhibition was also mediated through a change in chloride permeability (Prichard, 1971). However, desensitization experiments and the use of a specific 5-HT antagonist, mianserin, suggested that 5-HT and strychnine acted on different receptors.

Equipotent molar ratios were determined for analogues of 5-HT, the results being a mean of five experiments. These studies indicated the importance of the hydroxyl group of 5-HT for maximum 5-HT like activity. Tryptamine was 120 (range 38–200) times less active than 5-HT. Substitution of the hydroxylgroup for either chloro or methoxy reduced the potency 55 (range 9–140) and 77 (range 21–285) times respectively. The addition of a methyl group on the alpha carbon of the side chain of 5-HT or the