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BETA-ALANINE DISTRIBUTION IN THE LOBSTER, *HOMARUS AMERICANUS*

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Abstract—1. Whereas the distribution in lobster of γ-aminobutyric acid (GABA) was restricted to nerve and muscle tissues, that of beta-alanine (BA) was more widespread and uniform.

2. In abdominal, thoracic and subesophageal nerve cords and in cleaned peripheral motor nerve fibers, the GABA–BA ratio was 2–5 : 1.

3. In walking leg peripheral nerves, the GABA–BA ratio was generally close to 1 or less than 1.

4. Within abdominal nerve cords, the GABA–BA ratio was appreciably greater than 1 only in interganglionic connectives.

INTRODUCTION

BETA-ALANINE (BA) is the amino acid analog possessing one more methylene group than glycine and one less than γ-aminobutyric acid (GABA). It has been reported to be physiologically active, although at reduced relative potency, at mammalian central nervous system and crustacean peripheral neuromuscular synapses presumed to utilize either of these other two compounds as a neutrotransmitter (Kravitz *et al.*, 1963a; Werman *et al.*, 1968; Curtis & Johnston, 1974). In the lobster, it was found to be the only blocking substance other than GABA consistently distributed preferentially in inhibitory as opposed to excitatory nerve fiber extracts (Kravitz *et al.*, 1963b).

The specific accumulation of BA in a patient with a metabolic disorder characterized primarily by neurological dysfunction (Scriver & Perry, 1972) provoked further interest in the relevance of this compound or some aspect of its metabolism to normal neural function. A study was therefore undertaken of the distribution of BA in different tissues and in different regions of the nervous system of the lobster, *Homarus americanus*.

MATERIALS AND METHODS

At present, no reliable, simple method is available for specific quantitation of the tissue levels of BA. The semi-quantitative method employed here involved isolation of the total amino acid fraction of tissue extracts by ion-exchange chromatography followed by a two-dimensional separation of individual components in a paper electrophoresis–chromatography system.

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Tissue extraction

Various tissues were removed rapidly from 0·5 to 2·5 kg lobsters (*Homarus americanus*). They were either stored immediately at −20°C or dissected further for finer analysis. Storage in 10% (w/v) TCA did not alter results. In all cases, samples were rinsed briefly with chilled lobster saline (462 mM NaCl, 16 mM KCl, 26 mM $CaCl_2$ and 8 mM $MgCl_2$), blotted and weighed before freezing. All further dissections were performed in a saline-filled chamber maintained at 8–10°C. Central nerve cord samples were cleansed of the tightly adhering ventral artery and of any other non-neural tissue contaminants. In several experiments, abdominal ganglia were dissected further to distinguish between glial, neuronal and neuropilar components (Otsuka *et al.*, 1967). The ventral connective tissue sheath was removed along with as much of the perineural tissue as possible; this constituted the "perineural" sample. Most of the remaining glial cells then were blown away with a jet of saline. The neuronal cell body layer then was carefully undercut to remove this layer intact ("neuronal" sample) prior to removing the remainder of the ganglion's contents ("neuropile" sample). Nerves used included the abdominal ganglionic first and second roots (short central stumps) and the sensorimotor, sensory, and motor-inhibitory bundles excised from the meropodite segment of the first two pairs of walking legs (Kravitz *et al.*, 1963a). In several instances, the large motor fibers were carefully dissected free of the smaller nerve fibers and connective tissue strands of the motor-inhibitory and sensorimotor bundles and were pooled separate from them (Kravitz *et al.*, 1963b). No attempts were made to separate fibers according to function. However, the functional integrity of these motor nerve fibers was demonstrated crudely at the termination of each dissection when cutting them invariably caused an appropriate transient motor response of the dactyl. Hemolymph samples were obtained either by cranial drainage or by cardiac puncture.

Tissue samples (1–150 mg wet weight) were homogenized in 0·3–1·2 ml of ice-cold 10% (w/v) TCA

containing GABA-2,3-^{3}H (N) (2–10 Ci/mM) and BA-1-^{14}C (4·29 Ci/mM) (New England Nuclear) in quantities which did not contribute significantly to staining intensity. After sitting for 10 min in ice, the suspensions were centrifuged briefly in a Misco Electric Micro Centrifuge. The supernatant was removed and the residue was washed once by repeating the procedure. The supernatants were pooled.

Ion-exchange chromatography

The entire TCA extracts were applied to 0·6 × 5·5 cm column beds of precycled AG50W-X2, 100–200 mesh (Bio-Rad Laboratories) resin. The unbound materials which washed through were combined with the subsequent 15 ml water eluate. This initial fraction should have contained neutral compounds and organic acids. The ampholytes (including GABA and BA) were next removed with 10 ml 0·5 N NH_4OH in fraction 2, after which strong bases were eluted in fraction 3 with 15 ml 2 N NH_4OH. Fraction 2 was dried at 50°C under vacuum and resuspended in 100 μl of water. This procedure typically resulted in recovery in this fraction of at least 80% of the added ^{3}H and ^{14}C.

Two-dimensional separations

Variable aliquots of the second fractions were spotted on 8 × 13 in. Whatman 3MM paper and were subjected to electrophoresis for 2 hr at 400 V in pyridine–glac. acetic acid–water (6 : 55 : 939), pH 3·5. After drying, the sheets were turned 90° and ascending chromatography was carried out for 4 hr in *n*-butanol–glac. acetic acid–water (60 : 15 : 25). The sheets were then air-dried and stained by dipping briefly through a 0·2% (w/v) solution of ninhydrin reagent in acetone containing 2% (v/v) pyridine. Spots were localized by heating the paper for 10 min at 100°C. Semi-quantitative estimates of GABA and BA were made by comparing the staining intensity and size of their presumed spots with a series of standard sheets to which 0·01–0·5 μmole of these compounds had been applied and separated. In this way, it was possible to record, with no greater than a twofold error, the quantities of BA and GABA in the extract aliquots applied and thereby to calculate the concentrations of these substances in the original tissue samples.

Radioactivity measurements

Radioactivity of column fractions and of chromatogram strips was determined in a scintillation cocktail prepared from 19·00 g PPO and 1·27 g POPOP (New England Nuclear) dissolved in 3·94 l. of toluene containing 150 ml of BBS-3 (Beckman). Counting was performed in a Packard 3380 Tri-Carb Liquid Scintillation Spectrometer under double-label conditions. One-dimensional electropherograms and chromatograms were scanned for radioactivity in a Packard 7201 Radiochromatogram Scanner at lower efficiencies.

RESULTS

Identification of BA and GABA

Several experiments were performed to demonstrate that spots designated as BA and GABA truly contained these compounds free of significant levels of ninhydrin-positive impurities. First, when two standard mixtures of amino acids and amino acid analogs (including arginine, lysine, histidine, aspartate, glutamate, glutamine, taurine, cystine, methionine, glycine, alanine, leucine, tryptophan, tyrosine and serine), only one of which contained BA and GABA, were fractionated by the two-dimensional procedure, these two compounds were clearly separated from the others and from each other. Second, authentic radioisotopically labeled BA and GABA co-migrated, respectively, only to the same locations as stained lobster central nervous tissue spots designated as BA and GABA on the basis of migration behavior. Third, when this region of the paper sheets was eluted with water and the eluates were subjected to uni-dimensional chromatography in a phenol–water solvent system which separates close structural analogs of these compounds (Smith, 1969), the radioactivity again co-migrated with the BA and GABA spots, which were the only ninhydrin-positive substances detected (Fig. 1). Based upon these results, it was concluded that BA and GABA in lobster tissue extracts could be reliably identified merely by stain localization on combined electrophoresis-chromatography runs.

BA and GABA levels in various lobster tissues

Several different lobster tissues were analyzed for their contents of BA and GABA. Some of the results are indicated in Fig. 2. Whereas GABA was restricted to nerve and muscle tissues, the distribution of BA was more widespread. Only in nervous tissue did the level of GABA consistently equal or exceed the quantity of BA. The samples fell into four different categories with respect to the semi-quantitative results: (1) in hemolymph and hepatopancreas, GABA was not detectable while BA was present at 0·2–0·6 μmole/g wet tissue; (2) in central nerve cord and in walking leg closer and abdominal superficial flexor muscles, both GABA and BA were found in the range 0·2–0·7 μmole/g; (3) in walking leg motor-inhibitory nerves, both GABA and BA were present at roughly 16 μmoles/g; and (4) in walking leg sensory and sensorimotor nerves and opener muscle, GABA was found at 0·5 μmole/g while BA was detected at three to six times greater concentrations. The calculated values obtained for GABA in different nerve samples were in reasonably good agreement with those reported by Kravitz *et al.* (1963a). While the values obtained here for BA in nerves are quite different from those reported by McBride *et al.* (1974), the results from other tissues are generally compatible.

BA and GABA levels in different parts of nervous system

The distribution of BA and GABA in various parts of the lobster central and peripheral nervous

systems was studied. Figures 1–3 illustrate the typical difference in pattern produced by extracts of central compared with peripheral nervous tissue. In abdominal, thoracic and subesophageal nerve cords, the total GABA content was about two to five times greater than the BA content. In contrast, in walking leg peripheral nerves (e.g. the sensorimotor bundle and the motor-inhibitory bundle to the carpopodite main flexor), the reverse relationship was generally observed. The motor-inhibitory bundle to the walking leg opener muscle usually had about equal quantities of each (Fig. 3).

Finer dissections were performed to determine the distribution of these two compounds within the central and peripheral nervous tissues. Dissection of abdominal ganglia into first and second nerve roots, perineural tissue layer, neuronal cell body layer and inter-ganglionic connective revealed that only in the connectives was the GABA–BA ratio considerably greater than unity (Fig. 4). In this portion, GABA was in approximate two- to fivefold excess of BA levels. Moreover, when peripheral nerve bundles were separated into motor and small nerve fiber-connective tissue fascicles, GABA tended to predominate over BA in the former, while the reverse was true in the latter (Fig. 5). The approximate ratio of GABA to BA in the cleaned motor fibers was, however, no greater than 2–5 : 1.

DISCUSSION

Ample evidence has been presented to support the candidacy of GABA as a peripheral inhibitory neurotransmitter in the lobster (Kravitz, 1967; Otsuka *et al.*, 1967). In the course of these studies it was noted that BA was the only other blocking compound besides GABA that was preferentially distributed in isolated inhibitory rather than excitatory peripheral nerve fibers (Kravitz *et al.*, 1963b). It was not, however, unique to the former fibers, as was GABA. Furthermore, GABA was found in large excess compared to BA in these fibers and was about 50 times as active as an inhibitory neuromuscular agent.

The studies reported in this paper confirm the specific distribution of GABA in lobster nervous tissue. It was virtually absent from hepatopancreas and hemolymph but was present in all samples of nervous tissue examined. Its presence in muscle extracts can probably be ascribed primarily to the numerous nerve endings and connective tissue which adhere tightly to the isolated tissue (Iversen & Kravitz, 1968; Orkand & Kravitz, 1971).

The distribution of BA in lobster tissues was, however, considerably more widespread. This compound was found not only in all the samples of nervous tissue examined but also in extracts of hepatopancreas, muscle and hemolymph. It was found in particularly large concentrations in the walking leg nerve and opener muscle samples.

In most animal tissues, BA is found bound primarily in the form of carnosine or anserine, respectively dipeptides of BA and histidine or 1-methylhistidine (Scriver & Perry, 1972). The results presented here are presumed to be uncomplicated by BA originating from these sources for the following three reasons. First, in the two-dimensional separation procedure employed, carnosine and anserine migrate to a region distinct from the one occupied by free BA. Second, when a solution of carnosine is carried through the entire procedure employed for BA recovery from tissues, the product recovered is truly carnosine devoid of noticeable free BA. Third, when 0·1 μmole of carnosine is added to a lobster tissue homogenate and the sample treated immediately by the usual BA recovery procedure, no increase in BA staining is observed.

BA was present in roughly equal or greater concentrations compared to GABA in samples consisting primarily of small nerve fibers, connective tissue or glial cells. More surprising, however, were the similarly large concentrations of BA relative to GABA in the samples consisting primarily of neuronal cell bodies or motor fibers. This result is believed not to be an artifact of unusually low GABA or high BA levels for the following reasons. First, the animals exhibited overtly normal behavior immediately prior to use. Second, the nerves were functionally active upon completion of dissection, as determined by the crude behavioral observations. Third, isolated lobster ganglia in culture readily form and retain radioactive BA from a uracil precursor concurrent with appreciable macromolecular synthesis (Grossfeld, in preparation). Fourth, if one assumes that all of the GABA present in stripped motor-inhibitory bundles is in the approximately 50 μm dia. inhibitory fibers (Kravitz *et al.*, 1963b), then one can estimate (knowing the number and lengths of fibers and the semi-quantitative GABA content) the GABA concentration in these fibers as 50–75 mM. This is in surprisingly good agreement with the 100 mM concentration estimated by enzymatic assay of isolated nerve fibers (Kravitz *et al.*, 1965). Moreover, this implies, if most of the BA in these stripped nerve bundles is also present in the inhibitory fibers (Kravitz *et al.*, 1963b), that the BA concentration in these fibers could be as great as 25–50 mM. It is disturbing that such high levels of BA were found here when Kravitz *et al.* (1963b) reported lower levels and McBride *et al.* (1974) reported its absence from the same isolated nerve fibers. Perhaps this discrepancy reflects the considerable physiological variability often experienced in working with lobster walking leg motor nerves. Since even the most carefully dissected isolated lobster nerve fibers are contaminated by tightly ensheathing glial cells (Kravitz *et al.*, 1963b; de Lorenzo *et al.*, 1968), the true intracellular location of the GABA and BA in these preparations cannot be definitively ascertained

by the methods employed. The results do, however, suggest the need for further studies of the role of BA in normal lobster nerve cell metabolism and physiology.

It was noted in these studies that the amount of BA in comparison to GABA remains high or increases somewhat in extracts prepared from progressively more peripheral samples of lobster nervous tissue. Thus, the BA–GABA ratio in the thoracic ganglia (or abdominal ganglion cell body layer) corresponds roughly to that in the motor-inhibitory nerve bundle innervating the walking leg opener muscle. Although these tissue samples are rather crude for such comparisons (the more finely dissected samples do not, however, differ markedly in general result), the persistence of BA from the central neuron somata to the peripheral nerve endings has possible implications for specificity of inhibitory transmitter (GABA) transport and release in this system. A precedent exists for transport and release of close structural analogs in cat splenic nerve, where octopamine is a co-transmitter with norepinephrine yet possesses only 1/50 the latter's pressor activity (Molinoff & Axelrod, 1972).

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EXCITATORY ACTIONS OF GABA AND OF ACETYLCHOLINE IN SEA URCHIN TUBE FEET*

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Abstract—1. The musculature of isolated tube feet of several species of sea urchins was found to contract in response to GABA and to acetylcholine (ACh). The action of GABA but not that of ACh is blocked by bicuculline and picrotoxin.

2. The action of GABA but not that of ACh requires the presence of Na^+; it is reduced when the external Cl concentration is diminished.

3. The following cholinomimetics were found to be effective: acetyl-β-methylcholine, carbamylcholine, butyrylcholine and nicotine. The contractile effect of these agents, but also that of GABA was prevented by the cholinolytics atropine, banthine, decamethonium, flaxedil, hexamethonium and mytolon.

4. The action of ACh and of acetyl-β-methylcholine, but also that of GABA, was greatly enhanced by anticholinesterases (eserine, neostigmine).

5. Both ACh and GABA were detected in tube foot extracts. ACh is specifically localized in those parts that contain nerve tissue, with concentrations up to 75 μg/g wet wt.

6. It is concluded that GABA causes excitation of cholinergic motoneurones and does not affect the muscle fibers directly. The cholinoreceptors of the muscle fibers have both muscarinic and nicotinic properties.

INTRODUCTION

VARIOUS muscles of echinoderms have been shown to contract in response to acetylcholine (ACh). There is substantial evidence that in these animals neuromuscular transmission, at least in non-visceral muscle, involves a cholinergic mechanism. Substantial quantities of acetylcholine have been detected in the radial nerves. The relevant literature has been exhaustively reviewed by Welsh (1966), Fänge (1969) and by Penthreath & Cobb (1972).

Osborne (1971) reported the occurrence of GABA (0·9–1·1 μmoles/g wet wt) in radial nerves of representative species of echinoid and asteroid echinoderms, but the function of GABA in the central nervous system of these animals has not been determined. GABA was found to have no effect on the much studied lantern retractor muscle of sea urchins (Mendes *et al.*, 1970), but Hill (1971) reported slight inhibition of the spontaneous contractions in the isolated cloacal muscle of a holothurian. The cholinoceptive musculature of the sea urchin esophagus was reported to be unresponsive to GABA (Florey & McLennan, 1959).

The occurrence of catecholamines in echinoderm nervous tissue (Cottrell, 1967; Cottrell & Penthreath, 1970; Welsh, 1971) is matched by the finding that such amines cause relaxation of various echinoderm muscles (Boltt & Ewer, 1963; Hill, 1971; Penthreath & Cobb, 1972).

Several authors (e.g. Boltt & Ewer, 1963; Mendes *et al.*, 1970; Penthreath & Cobb, 1972) favor the hypothesis of peripheral inhibitory neurones, but direct evidence for this is lacking.

In view of the many uncertainties of echinoderm neuropharmacology it seemed to us interesting to attempt another analysis of neuromuscular pharmacology of an echinoderm preparation. After preliminary investigations we decided to use the tube feet of echinoids. These appendages have received considerable attention by morphologists, and their general structure is now well established (for a review of the literature see Penthreath & Cobb, 1972).

The overall features of the tube foot structure are represented in Fig. 1. In the regular sea urchins, the suckered tube feet consist of a tubular, hollow muscle composed of longitudinally oriented fibers of smooth muscle. On the luminal side this is bordered by a very thin, ciliated coelomic epithelium. On its outer surface the muscle layer is surrounded by a tubular sheath of dense connective tissue. Each tube foot contains a conspicuous nerve (also known

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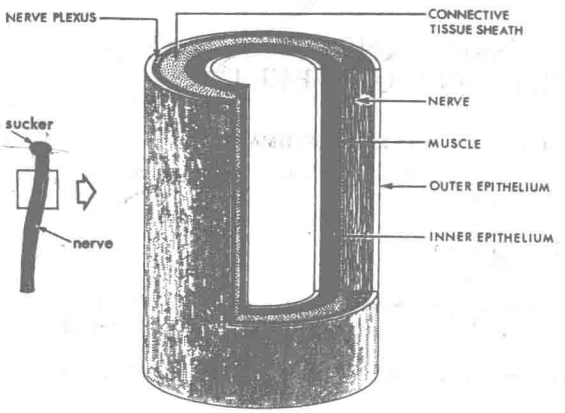

Fig. 1. Idealized diagram of the anatomical features of a suckered tube foot of a typical regular sea urchin (*E. esculentus*).

as the "seam") which runs outside the connective tissue layer from the base to the tip where it spreads out into an apical nerve ring. Continuous with this nerve is a thin nerve plexus that surrounds the entire tube of connective tissue. The tube foot is covered by a ciliated epithelium that is considered by many authors to be a sensory epithelium since most—if not all—cells send processes into the nerve plexus. As yet nothing is known about the mode of innervation of the muscle fibers, nor of their pharmacological properties. We therefore have embarked on an investigation of both the pharmacology and the ultra-structure of sea urchin tube feet.

In this report we will describe and discuss the initial results of our pharmacological studies. They indicate that the muscle fibers are cholinoceptive and that they also contract in response to applied γ-aminobutyric acid (GABA). The latter response appears to be mediated by a cholinergic mechanism. Catecholamines, 5-hydroxytryptamine and glycine were found to be without effect on the muscle responses.

MATERIALS AND METHODS

Experiments were conducted at the Friday Harbor Laboratories of the University of Washington, using *Strongylocentrotus franciscanus*, at the Stazione Zoologica at Naples, Italy, using *Arbacia lixula* and at the University of Konstanz, using *Echinus esculentus* shipped from the Biologische Anstalt Helgoland. In order to obtain maximally extended tube feet, the animals were placed upside down into a large dish filled with sea water. Fully extended tube feet were ligated at their base with aid of surgical thread. A loop was then tied and the tip of the tube foot was ligated also, leaving one end of the thread long for attachment to an isotonic mechano-electric transducer. The tube foot was then mounted in a cylindrical muscle bath. Rectilinear recording (with the aid of chart recorders) was used throughout. Most tube feet were slit open prior to mounting in the muscle bath.

The bathing medium had the following composition in mM: in the case of the experiments on *Strongylocentrotus* and *Arbacia*, NaCl 490, KCl 8, $MgCl_2$ 12 and $CaCl_2$ 10; in the case of *Echinus*, NaCl 433, KCl 8, $MgCl_2$ 50 and $CaCl_2$ 12. The addition of buffers (Tris, TES) was tried but found unnecessary. The following drugs were used: acetylcholine chloride (Merck), γ-aminobutyric acid (Serva), atropinsulfate (Eli & Lilly, Co.), Banthine (G. D. Searle, Co.), benzoylcholine chloride (Hoffmann–LaRoche), bicuculline (K & K Laboratories), butyrylcholine iodide (Serva), carbamylcholine (K & K Laboratories), decamethonium iodide (Fluka), dopamine (Serva, Nutritional Biochemical Corp.), D-tubocurarine chloride (Nutritional Biochemical Corp., Schuchardt), eserine (Burroughs Wellcome Co.), Flaxedil (American Cyanamid), glycine (Merck), glutamic acid (Serva), β-guanidinopropionic acid (Calbiochem), guanidinoacetic acid (Nutritional Biochemical Corp.), hexamethonium chloride (Chemo Puro), 5-hydroxytryptamine creatinine sulfate (Serva, Calbiochem), Mytolon (Sterlin–Wintrhop Research Institute), Na-glutamate (Merck), neostigmine bromide (Mann Research Laboratories), nicotine (Serva), noradrenaline (Fluka), picrotoxin (Schuchardt) and strychnine sulfate (Merck).

Acetylcholine determinations were carried out by bioassay, using the isolated ventricle of cockles (*Tapes spec.*) according to the assay procedure described by Florey (1967). The tissue was gently blotted and weighed on a piece of preweighed aluminum foil. It was then dropped, together with the foil, into boiling distilled water and heated for 1 min. The extract was decanted and its volume adjusted to a convenient value, usually 100 times the weight of the tissue. This aqueous extract was then further diluted for bioassay, using unbuffered saline. The final dilutions tested had tissue concentrations of less than 100 μg/ml. The assay was capable of detecting as little as 0·1 ng of ACh/ml.

The GABA content of isolated tube feet was measured using aqueous extracts prepared as described above. Prior to analysis the extracts (about 150 mg tissue in 1 ml water) were dialyzed through cellophane against 2 × 900 ml water at 2°C. The dialyzate was dried *in vacuo* and taken up in 10 ml water. This was acidified with HCl to pH 2–3 and applied to a column (1·5 × 11 cm) of Dowex 50W X2 (previously recycled with 1 N NH_4OH and 1 N HCl). The column was first eluted with water until the effluent was Cl free. It was then eluted with 1 N NH_4OH and the eluate was dried *in vacuo* at 40°C. The residue was taken up in 2 ml of water and applied to a small (0·5 × 3 cm) column of Dowex 1 X2 (in acetate form after recycling with 1 N HCl and 2 N acetic acid). The column was eluted with water. The volume of the eluate was reduced *in vacuo* (40°C) and adjusted to 0·2 ml. This procedure eliminated the acidic amino acids and the fluorescent material that would otherwise interfere with the fluorimetric determination. The enzymatic determination of GABA was carried out according to the technique described by Graham & Aprison (1966), using free GABAase of Worthington Biochemical Corp., Freehold, N.J., U.S.A.*

For the determination of Na^+, K^+, Mg^{++} and Ca^{++} concentrations we collected samples of visceral coelomic fluid from freshly opened sea urchins. Ambulacral coelomic

* The results were corrected for quenching. We are grateful to Dr. Bernd Koidl for carrying out the enzymatic analyses for us.

fluid was obtained by puncture of the exposed ampullae of the tube feet, using Pasteur pipettes drawn out to a fine point of only a few micron diameter. After measuring their volumes the fluid samples were diluted with double distilled water and subjected to analysis by atomic absorption flame-spectrophotometry, using a Zeiss M4QIII flame-spectrophotometer.

RESULTS

General observations

As is shown in Figs. 2–4, isolated tube feet contract in response to ACh as well as to GABA. The effective concentrations were sometimes as low as 10^{-7} g/ml, but in many preparations 100-fold greater concentrations had to be used for an effect to be observed. While some preparations were more responsive to ACh than to GABA, in others the threshold concentration for GABA was one order of magnitude lower than that for ACh.

With *Arbacia* these responses were obtained when the preparations were bathed in the saline medium described under *Materials and Methods*. When this medium was replaced by filtered sea water, the response declined and disappeared within minutes. Preparations kept in sea water generally showed no responses to applied drugs. The reason for this became clear when we investigated the effects of Mg^{2+} and Ca^{2+} concentrations: the addition of Ca^{2+} to natural sea water made the preparations responsive, provided the Ca^{2+} concentration was at least 40 mM. With the artificial saline medium increasing the Mg^{2+} concentration from 12 to 50 mM caused the drug effects to disappear. If the Ca^{2+} concentration was also increased (to 40 mM), however, the contractile responses reappeared. Clearly, then, there is a Mg–Ca antagonism.

In *Strongylocentrotus* preparations this antagonism was noted also but it was less conspicuous. Tube feet of *Echinus* responded to ACh, GABA and the other effective drugs even when the saline medium contained 50 mM Mg^{2+}. Lowering the Mg concentration caused an increase in spontaneous contractions.

When GABA or ACh was left in prolonged contact with the preparations, the induced contraction decayed within 1–2 min. Preparations desensitized in this manner to GABA became refractory to further applications of GABA; they still responded to ACh (Fig. 2). Preparations desensitized to ACh gave no responses to further applications of ACh, they also failed to respond to GABA, as shown in Fig. 2.

Cholinomimetics

Besides ACh, acetyl-β-methylcholine, carbamylcholine (carbachol) and butyrylcholine were found to cause contraction. Carbamylcholine usually was more effective than ACh, butyrylcholine much less. No effect was seen with benzoylcholine. Nicotine was usually much more effective than ACh in causing contraction.

Cholinolytics

In most experiments we found D-tubocurarine (even in concentrations as high as 10^{-4} g/ml) completely ineffective in blocking the contractile responses to ACh. In only one experiment (tube foot of *Arbacia*) did D-tubocurarine (10^{-4} g/ml) cause a complete block of ACh (10^{-5} g/ml). Flaxedil, Banthine, hexamethonium, decamethonium and mytolon were found to antagonize the effects of both GABA and ACh. Atropin antagonized the action of ACh in *Strongylocentrotus* and *Arbacia*, but failed to do so in *Echinus*. Decamethonium was more effective than hexamethonium. The concentrations required to antagonize ACh were generally

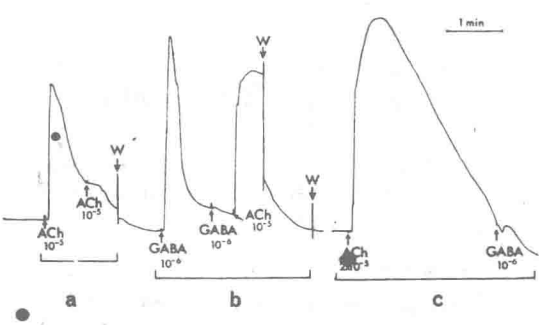

Fig. 2. Responses of an isolated tube foot of *Arbacia* to ACh and to GABA. (a) Desensitization to ACh, (b) desensitization to GABA does not affect contractile response to ACH but (c) desensitization to ACh also prevents reponse to GABA. Concentrations are given in g/ml. W, Washing with saline.

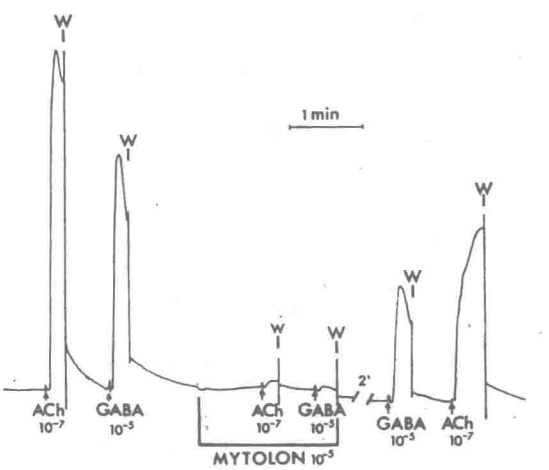

Fig. 3. Isolated tube foot of *Arbacia:* Mytolon blocks the responses to both ACh and GABA. Concentrations in g/ml. W, Washing with saline.

high, usually 10^{-5} or even 10^{-4} g/ml, and it was sometimes necessary to permit the drugs to stay in contact with the preparations for one or several minutes before the blocking action became apparent. The blocking action of Mytolon is exemplified in Fig. 3. Nicotine and the choline esters were effectively blocked by Flaxedil, Banthine, decamethonium and Mytolon.

Anticholinesterases

Eserine was found to greatly enhance the responses to ACh. It also enhanced the contractile responses to applied GABA, as is shown in Fig. 4. In *Arbacia*, the potentiating effect of eserine (even if the drug was applied in concentrations not higher than 10^{-6} g/ml) was transient; the preparations became unresponsive to cholinergic agents or to GABA within several minutes. Neostigmine was similarly effective in potentiating the effects of ACh and of GABA. In *Arbacia* it did not have the adverse effects seen with application of eserine. After a few minutes sensitization with neostigmine, the threshold concentrations of ACh and of GABA were generally reduced by two orders of magnitude. In *Echinus*, neostigmine was more "toxic" than eserine. The preparations showed strong potentiation of ACh effects even after prolonged exposure to eserine but they progressively deteriorated when left in contact with neostigmine. Eserine and neostigmine also enhanced the action of acetyl-β-methylcholine and of butyrylcholine, but they did not change the response to carbamylcholine or nicotine. Benzoylcholine showed no effect in the presence of the cholinesterase inhibitors.

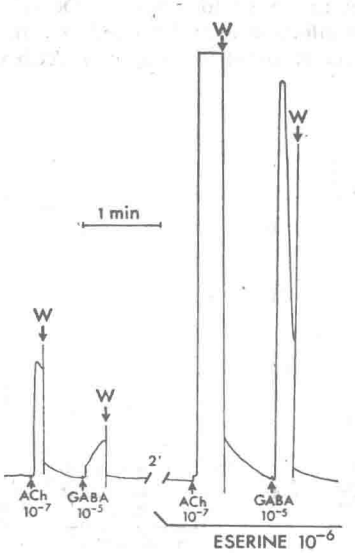

Fig. 4. Isolated tube foot of *Arbacia*: eserine potentiates the effects of ACh and of GABA. Concentrations in g/ml. W, Washing with saline.

GABA analogues and related compounds

Guanidino acetic acid was about one-tenth as effective, whereas β-guanidinopropionic acid was about half as effective as GABA in causing contraction. No response was seen to the application of γ-guanidinobutyric acid. Glycine and L-aspartate were ineffective, even at concentrations of 10^{-4} g/ml.

GABA antagonists

Strychnine, even in concentrations of 10^{-4} g/ml, reduced but did not abolish the contractile response to GABA. In concentrations between 10^{-4} and 10^{-5} g/ml picrotoxin greatly reduced the effect of GABA, but as with strychnine the action was often irreversible, particularly with tube foot preparations of *Echinus*. Even the responses to ACh were sometimes diminished by this treatment and non-specific effects must be assumed to contribute to the action of these alkaloids. In several preparations, however, picrotoxin abolished the responses to GABA without reducing the action of ACh.

Bicuculline (10^{-5} g/ml) was found to effectively antagonize GABA without affecting the response to ACh (see Fig. 5). Experiments with *Echinus* tube feet were not always as clear-cut since in these preparations higher concentrations had to be applied and these caused irreversible non-specific deterioration. In a few preparations of *Echinus* tube feet, bicuculline (10^{-5} g/ml) blocked the action of GABA without reducing the action of ACh.

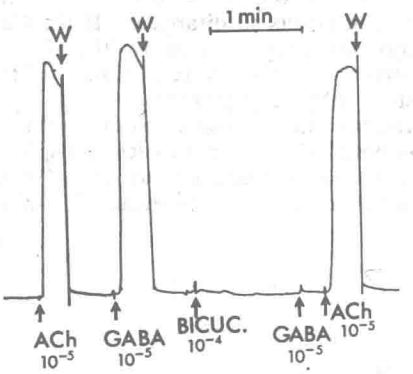

Fig. 5. Isolated tube foot of *Arbacia*. Bicuculline blocks the effect of GABA but does not antagonize ACh.

Cl substitution

When the NaCl of the saline medium was replaced by Na_2SO_4 (+ glucose to make up for the osmotic deficit) or by Na-propionate, the contractile response to GABA declined. Potentiation should occur if GABA causes an increase in Cl^- permeability. Evidently this is not the mechanism by which GABA excites the tube foot preparation.

Table 1. ACh content of sea urchin tube feet, as determined by bioassay

Species	Experiment No.	Part of tube foot	No. of tube feet in sample	Weight of tissue (mg)	ACh content wet wt (μg)
A. pustulosa	1	Distal portion		58	17·0
	2	Distal portion		110	14·5
	2	Proximal portion		324	18·0
E. esculentus	3	Strips with nerve	15	4·4	14·0
	4	Strips with nerve	15	7·4	7·0
	5	Strips with nerve	35	9·1	13·0
	3	Strips without nerve	15	9·8	9·0
	4	Strips without nerve	15	8·5	8·0
	5	Strips without nerve	35	29·1	8·5
	6	Inner layers*	10	5·2	1·3
	7	Inner layers*	25	8·8	0·9
	8	Inner layers*	15	4·0	1·7
	6	Outer layers†	10	2·2	30·0
	8	Outer layers†	15	3·2	75·0

* Inner epithelium, muscle, connective tissue sheath.
† Nerve plus nerve plexus, outer epithelium.

TTX and Na-free medium

Even when applied for 1 hr at a concentration of 10^{-6} g/ml, TTX did not diminish the contractile responses to ACh or to GABA. When the Na^+ in the bathing saline was replaced by Li^+, the response to GABA disappeared (usually within 1 min) while the effect of ACh remained. Upon readmittance of Na^+, GABA again caused contraction. This has been observed in tube feet of *Strongylocentrotus* and of *Arbacia*.

ACh content

The ACh content of tube feet (*Echinus*, *Arbacia*), as determined by bioassay on the isolated clam ventricle (Florey, 1967), is surprisingly high (Table 1). A comparison of proximal and distal halves of tube feet (*Arbacia*) showed no significant difference, but when the tube feet (*Echinus*) were divided into parts that contained the predominant amount of nerve tissue and parts that contained little nerve tissue, conspicuous differences became evident.

In a first series of experiments tube feet of *Echinus* were split lengthwise. The strips containing the nerve were extracted separately from the remaining portion. The strips containing the nerve had a mean of 11·3 μg of ACh/g wet wt, while the strips without nerve showed a mean of 8·5 μg/g. From light micrographs of cross-sections, we estimate the volume of the nerve to amount to 10–30 per cent of the volume of the strips that contained the nerve. If all the ACh found were contained in the nerve tissue, this tissue would contain 3·3–5 times 11·3, or 37·4–56·5 μg/g. The part of the tube foot that does not contain the nerve nevertheless contains nerve tissue in the form of a thin nerve plexus (see Fig. 1). It is very likely that it is this plexus that contains the ACh found in the "nerveless" portion of the tube feet, because when tube feet were stripped of their outer two layers (outer epithelium plus nerve and nerve plexus) the remaining muscle, connective

Table 2. Ionic composition (in m-equiv/l.) of perivisceral and water-vascular (= ambulacral) coelomic fluid of one specimen each of *E. esculentus* and *A. pustulosa*, taken from the holding tanks in our laboratory

	E. esculentus		*A. pustulosa*		
	Perivisceral coelom	Ambulacral coelom	Perivisceral coelom	Ambulacral coelom	SW*
Na	455	455	388	394	386
K	10·2	11·2	10·1	12·8	9·3
Mg	50·2	50·6	43·6	43·6	43·7
Cl	12·0	11·8	11·7	11·3	10·6

In the case of *Arbacia* the sea water in the tank (SW*) had a rather low salinity; the attendant had wiped off the salt spray and replaced evaporated water with distilled rather than with sea water. The *Arbacia* had come from Naples, Italy, and had been in this water for 5 days prior to analysis.

tissue and inner epithelium contained (on the average) less than 2·5 per cent of the ACh found in the stripped-off outer layers: the average ACh contents were 1·3 and 52·5 μg/g respectively.

GABA content

Two determinations of GABA were made with two batches of entire tube feet of *Echinus*. The amounts found were 0·9 and 1·2 μg/g wet wt.

Ionic composition of coelomic fluid

In view of the different behavior of tube foot preparations bathed in saline media of different Mg^{2+} concentrations, and in view of the reported elevation of the K^+ concentration in the fluid of the water-vascular system of *E. esculentus* (Berger, 1931) and *Asterias* (Binyon, 1962) we determined the concentrations of Na^+, K^+, Ca^{2+} and Mg^{2+} in samples of the perivisceral and the water-vascular coelom of specimens of *E. esculentus* and *A. lixula*. The data are presented in Table 2. No difference was found between the composition of the fluids of the two coelomic compartments; both kinds of fluid had an ionic composition similar to sea water. This is particularly true for the Mg^{2+} concentration.

DISCUSSION

In arthropods and vertebrates, GABA has actions characteristic of a transmitter substance of inhibitory neurones. Even in the case of the striated muscle of the *vas deferens* of crayfish in which GABA has been reported to cause muscle *contraction* (Murdock, 1971), the ionic mechanism of GABA action is the same as that reported for inhibitory synapses: The contractile effect is due to a permeability increase to Cl^- and the resulting depolarization (Florey & Murdock, 1974).

In the case of the contractile response of the echinoid tube foot, the situation is fundamentally different. A permeability increase to Cl^- can be excluded because reduction of external Cl^- diminishes rather than enhances the response to GABA. Instead the effect of GABA is Na^+-dependent.

In our laboratory, Kleinsteuber (1974) has recently found that GABA can depolarize stretch receptor neurones of crayfish when applied in concentrations below those which cause the usual conductance increase for Cl^-. This depolarizing action is Na-dependent and is accompanied by a decrease in membrane conductance. A Na^+-dependent depolarization by GABA of nerve terminals has also been reported by Barker & Nicoll (1973), who studied the effect of GABA on primary afferent terminals in the spinal cord of frogs. This depolarization by GABA of primary afferents is blocked by picrotoxin and bicuculline (Davidoff, 1972; also reported by Curtis *et al.*, 1972, for the spinal cord of the cat).

Picrotoxin and bicuculline are known as antagonists of the inhibitory, Cl^--dependent actions of GABA in vertebrates and arthropods. Evidently these alkaloids can also prevent the Na^+-dependent actions of GABA. The fact that, at least in the sea urchin tube foot preparation, the excitatory action of GABA can be mimicked by certain GABA analogues like guanidino acetic acid and β-aminopropionic acid, further indicates that the receptor structures responding to GABA in the cases of its excitatory, Na^+-dependent actions are structurally related to the GABA receptors giving rise to the more common Cl^- activation in inhibitory subsynaptic membrane.

The Na^+-dependent excitatory action of GABA on the tube feet of echinoids might be the result of a permeability increase to Na^+ or of a decrease of K^+- or Cl^--permeability. In both cases GABA would cause a depolarization that disappears when Na^+ is removed from the external medium. It cannot be decided at this stage of the investigation whether this excitatory action of GABA represents a transmitter-like action.

The occurrence of GABA in the echinoderm central nervous system (Osborne, 1971) and within echinoid tube feet does not necessarily imply a transmitter role of this compound, since a purely metabolic role cannot be excluded and since it is not yet certain whether the compound actually occurs in *nerve* cells and not in other types of cells.

The finding of excitatory actions of GABA in our tube foot preparation may represent an action on an excitable membrane not normally activated by this substance. On the other hand, GABA may indeed be an excitatory transmitter in echinoderms. Future research will have to resolve this question.

Several lines of evidence indicate that GABA does not act directly on the muscle elements of the tube foot but that its effect is mediated by a cholinergic system: (1) desensitization to ACh prevents responses to GABA, (2) cholinergic blocking agents prevent and (3) anticholinesterases potentiate the action of GABA. The most simple model would call for an excitatory action of GABA on cholinergic motoneurones. The presence of cholinergic motoneurones is indicated by the results of our ACh determinations: The concentration of ACh detected in the tube feet is surprisingly high; the distribution (see p. 9) makes it very likely that this ACh occurs in the nerve tissue.

A cholinergic motor innervation of echinoderm muscle can also be assumed on the basis of several pharmacological studies by other authors who have employed isolated muscles (Boltt & Ewer, 1963; Mendes *et al.*, 1970; Hill, 1971, and others). In all cases the muscles contract in response to low concentrations of applied ACh. The fact that in every echinoderm muscle preparation studied, the action

of ACh is potentiated by anticholinesterases argues for the presence of cholinesterase in the muscle tissue. An electronmicroscopic study of the tube foot musculature (Florey & Cahill, unpublished) indicates that the cholinesterase is localized in the surface membrane of the muscle fibers. The mode of transmission from (cholinergic) nerve fibres to muscle is currently under investigation in our laboratory.

The cholinoreceptors responsible for the action of ACh and the other cholinergic drugs on the sea urchin tube foot cannot be classified as either muscarinic or nicotinic. They respond to the muscarinic agonist acetyl-β-methylcholine and carbamylcholine as well as to nicotine. Both muscarinic blocking agents (atropine, banthine) and nicotinic blocking agents (decamethonium, Mytolon, Flaxedil and, in one case, D-tubocurarine) are effective antagonists. These results confirm with those obtained by Mendes *et al.* (1970) with the latern muscles of sea urchins.

In mammals, in particular in the sympathetic ganglia, carbamylcholine (carbachol) stimulates presynaptic cholinergic terminals to release ACh (McKinstry *et al.*, 1963). The resultant postsynaptic excitation is, therefore, potentiated by inhibitors of cholinesterase—even though carbamylcholine itself is not hydrolyzed by the enzyme. In our echinoderm preparation the action of carbamylcholine appears to be predominantly, if not exclusively, *post*-synaptic: anticholinesterases do not enhance it.

The fact that anticholinesterases enhance the action of ACh as well as that of acetyl-β-methylcholine is evidence that the cholinesterase of the tube foot musculature is a specific acetylcholinesterase.

It is of interest that TTX does not interfere with the excitatory effects of ACh and of GABA. This implies that action potentials involving conventional Na^+ activation are not involved in the generation of the resulting contraction. The failure of Na^+-free saline to abolish the contractile response of tube feet to ACh also supports this contention. At this time it cannot be excluded, however, that the persistence of responses to ACh is due to lack of penetration of TTX or of the Na^+-free medium to the responsive sites. It is also possible that Li^+ can replace Na^+ in the case of the action of ACh.

The ionic composition of the fluid collected from tube foot ampullae is close to that of sea water. The greater responsiveness of tube foot preparations in saline media of low Mg^{2+} concentration obviously is not due to normally low Mg^{2+} concentration of the fluids bathing the cells. Possibly Mg^{2+} ions interfere with the permeability of the epithelia that cover muscle and nerve–tissue (see Fig. 1). In contrast to earlier reports on an elevated K^+ concentration in the ambulacral coelom, we find the K^+ concentration similar to that of sea water. The reported high concentrations which were near twice that found in sea water may be the result of leakage of K^+ from cells injured in the collection procedure.

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