ELECTRICAL RESPONSES OF FROG PRIMARY AFFERENTS TO NEUTRAL AMINO ACIDS: RECEPTOR MULTIPLICITY AND IONIC REQUIREMENTS

by

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ABSTRACT

The aim of the present study was to examine some characteristics of the responses of frog primary afferents to applied neutral amino acids. A sucrose gap recording technique was used. The preparation consisted of isolated, hemisected spinal cord or dorsal root ganglion (DRG). The following results were obtained:

(i) 6-aminomethyl-3-methyl-4H-1,2,4-benzothiadiazine-1,1-dioxide (AMBD) selectively depresses depolarizing responses of intraspinal terminals of dorsal roots (DRT) and of DRG to taurine, beta-alanine, and kojic amine but not those to GABA or glycine;

(ii) depolarizing responses of primary afferents to GABA, and possibly taurine, are not likely to be mediated substantially by an indirect increase in extracellular potassium concentration ([K]o) since barium enhances these responses on DRG and depresses them on DRT (taurine > GABA) at a time when responses of both sites to increased [K]o are blocked;

(iii) hyperpolarizing responses of DRT to neutral amino acids, as exemplified by baclofen, are sensitive to changes in [K]o and are blocked by barium, suggesting they are due to an increase in K conductance.

All these results are consistent with the existence of multiple populations of receptor-ionophore complexes for neutral amino acids on primary afferents.
CONDENSÉ

Le but de la présente étude a été d'examiner certaines caractéristiques de réponses électriques liées aux acides aminés neutres sur les afférents primaires de grenouilles au moyen de la technique du "sucrose gap" sur des préparations isolées de la moelle épinière ainsi que les ganglions de racine dorsale (DRG). Les résultats suivants furent obtenus:

(i) 6-aminomethyl-3-methyl-4H-1,2,4-benzothiadiazine-1,1-dioxide (AMBD) agit sélectivement sur les termiiaux intraspinaux de racines dorsales (DRT) et DRG en abaissant les réponses dépolarisantes des acides aminés suivants: taurine, beta-alanine et kojic amine, mais non GABA, ni glycine;

(ii) les réponses dépolarisantes des afférents primaires liées à GABA, et possiblement taurine, ne sont probablement pas médiaiser de façon importante par une augmentation indirecte de la concentration extracellulaire du potassium ([K]o), puisque barium rehausse ces réponses sur DRG et les atténue sur DRT (taurine > GABA), à un moment où les réponses liées à une augmentation de [K]o sont bloquées sur les deux sites;

(iii) les réponses hyperpolarisantes de DRT liées aux acides aminés, exemplifiées par baclofen, sont sensibles aux variations de [K]o, et sont bloquées par barium. Ceci suggère une étroite liaison entre l'augmentation de la conductance du potassium et lesdites réponses.

Tout ces résultats sont en accord avec l'existence de populations multiples de complexes récepteur-ionophore pour les acides aminés neutres sur les afférents primaires.
PREFACE

Format of the Thesis

In accordance with the Guidelines Concerning Thesis Preparation of the Faculty of Graduate Studies and with the Thesis Format adopted by the Department of Pharmacology and Therapeutics, McGill University, on March 2, 1973, the results in this thesis are presented in a form suitable for publication in a learned journal.

This thesis is composed of five chapters. Chapter 1 contains an introductory review of literature that is pertinent to work presented in this thesis. Chapters 2, 3 and 4 have been submitted for publication to Brain Research. An overall discussion of the results is presented in Chapter 5. Chapter 6 contains a list of references for chapters 1 and 5.

Most of the text of chapter 2 was written by Dr. A. Padjen. This chapter also includes data (i.e. synaptic potentials and responses of bullfrog DRG) provided by Harout Hassessian. All other text and data presented in this thesis were provided by the author.
ACKNOWLEDGMENTS

The preparation of a thesis is a major endeavor requiring team effort. I would thus like to acknowledge the efforts of the many people who have helped make this thesis possible:

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<td>AMBD</td>
<td>6-aminomethyl-3-methyl-4H-1,2,4-benzothiadiazine-1,1-dioxide (also known as TAG)</td>
</tr>
<tr>
<td>BALA</td>
<td>beta-alanine</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>D</td>
<td>dextrorotatory</td>
</tr>
<tr>
<td>DR-DRP</td>
<td>dorsal root-dorsal root potential</td>
</tr>
<tr>
<td>DR-VRP</td>
<td>dorsal root-ventral root potential</td>
</tr>
<tr>
<td>DRG</td>
<td>dorsal root ganglia</td>
</tr>
<tr>
<td>DRP</td>
<td>dorsal root potential</td>
</tr>
<tr>
<td>DRT</td>
<td>dorsal root terminals (intraspinally)</td>
</tr>
<tr>
<td>DRT9</td>
<td>dorsal root terminals of segment IX</td>
</tr>
<tr>
<td>DRT10</td>
<td>dorsal root terminals of segment X</td>
</tr>
<tr>
<td>EC50</td>
<td>effective concentration for 50% of maximal response</td>
</tr>
<tr>
<td>EPSP</td>
<td>excitatory postsynaptic potential</td>
</tr>
<tr>
<td>4-AP</td>
<td>4-aminopyridine</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
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<tr>
<td>GAD</td>
<td>glutamic acid decarboxylase</td>
</tr>
<tr>
<td>GLY</td>
<td>glutamate</td>
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<tr>
<td>HT</td>
<td>glycine</td>
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<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>K</td>
<td>potassium ion</td>
</tr>
<tr>
<td>KJA</td>
<td>kojic amine</td>
</tr>
<tr>
<td>[K]o</td>
<td>extracellular potassium concentration</td>
</tr>
<tr>
<td>L</td>
<td>milliliters</td>
</tr>
<tr>
<td>ml</td>
<td>millimoles per liter</td>
</tr>
<tr>
<td>mV</td>
<td>millivolts</td>
</tr>
<tr>
<td>n</td>
<td>number of samples</td>
</tr>
<tr>
<td>PAD</td>
<td>primary afferent depolarization</td>
</tr>
<tr>
<td>PAH</td>
<td>primary afferent hyperpolarization</td>
</tr>
<tr>
<td>SE</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>TAU</td>
<td>taurine</td>
</tr>
<tr>
<td>TEA</td>
<td>tetraethylammonium</td>
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<tr>
<td>THIP</td>
<td>4,5,6,7-tetrahydroisoxazolo(5,4-c)pyridin-3-ol</td>
</tr>
<tr>
<td>uM</td>
<td>micromoles per liter</td>
</tr>
<tr>
<td>VR-DRP</td>
<td>ventral root-dorsal root potential</td>
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CHAPTER 1

INTRODUCTION
1.1.1 General introduction

Pharmacology is the field of medical science directed towards the study of the mode of action of chemicals in biological organisms whether or not such chemicals are present naturally therein. During this century, this field has reinforced the concept that many of these chemicals produce their effects on biological tissue via interaction with specific receptors. A main focus in pharmacology is therefore the identification, characterization and elucidation of the role of the receptors in the normal functioning of living tissue, and ultimately, in the behavior of the organism as a whole.

Pharmacological techniques have lead to the realization that a multitude of different receptors exist throughout the body, including the spinal cord. The focus of this thesis is on receptors localized on a population of neurons, known as primary afferents, which mediate sensation. The general issue that will be addressed is whether more than one type of receptor exists on these cells for a group of chemicals known as neutral amino acids.

1.1.2 General organization of primary afferents

The central nervous system (CNS) continuously receives sensory information from its periphery in response to a large variety of stimuli (eg. visual, auditory, mechanical, nociceptive, thermal). One pathway of referral of sensory information which is of particular relevance to this work is the collection of sensory neurons, referred to as primary afferents, whose soma form the dorsal root ganglia (DRG) and whose axons (ie. primary afferent fibres) enter the spinal cord as dorsal roots. Dorsal roots contain no other axons but those originating from DRG soma (Wilhelm & Coggeshall, 1981). After entering the spinal cord, these axons give rise to several stages of progressively finer branches ultimately
ending in boutons (axonal dilatations) that make synaptic contact with other neurons (e.g. motoneurons, interneurons). Each axon often ends in multiple boutons interconnected by thin axon segments (referred to as en passant). This general organization appears to be similar in both amphibians and mammals although quantitative differences exist (Grantyn et al., 1984). Sites of sensory fibre termination occur throughout the spinal cord but predominantly within the dorsal horn and especially in the most superficial layers (Hunt, 1983). The stimulus-induced sensory information is thus received by specialized receptors at the peripheral end of these sensory neurons and is propagated, in the form of action potentials, through dorsal roots to the terminals (DRT) forming the first sensory synapses inside the spinal cord. More recent anatomical studies (Wilhelm & Coggeshall, 1981; Coggeshall, 1985) in both amphibians and mammals have provided compelling data indicating that many primary afferents branch before entering the spinal cord and that some of these fibres also exist in the ventral root, thus deviating from the classical schema outlined above.

1.2 Regulation of sensory input

1.2.1 Primary afferent heterogeneity

Considering the quantity of sensory information arriving from thousands of primary afferents into the spinal cord, it would not be surprising for such input to be coded or regulated. One possible means for coding derives from the inherent anatomical, physiological and pharmacological heterogeneity of primary afferents. For instance, measurements of axon diameter, conduction velocity and degree of myelination fall into multimodal distributions, indicating anatomically discrete populations of sensory fibres (e.g. large-diameter, fast-conducting, myelinated fibres).
Similarly, distinctions occur in physiological roles and pharmacological profile to reinforce the notion that primary afferents are indeed heterogeneous. So far, glutamate, ATP, plus at least five peptides (substance P, somatostatin, vasointestinal polypeptide, gastrin and angiotensin II) are candidates for neurotransmitters released by primary afferents of both amphibia and mammals (Krnjevic, 1974; Hokfelt et al., 1980; Kawagoe et al., 1985; Salter & Henry, 1985; Cuello et al., 1976; Inagaki et al., 1981; Hunt, 1983). Possible relationships between the anatomy, physiology and pharmacology of some primary afferents have also been proposed, particularly for small-diameter, finely myelinated or unmyelinated primary afferents involved in cutaneous nociception and suspected of containing substance P (Buck et al., 1982). These fibres tend to terminate in the more superficial layers of the dorsal horn (e.g. substantia gelatinosa) while larger myelinated fibres involved in muscle reflexes end in deeper layers (e.g. motor nuclei) (Hunt, 1983).

1.2.2 Presynaptic Inhibition

Superimposed upon the sensory inflow along DRT is presynaptic inhibition. This phenomenon is thought to play an important role in controlling the activity of all types of primary afferent fibres in the vertebrate CNS (Eccles, 1964; Schmidt, 1971).

The current concept of presynaptic inhibition is relatively recent and was first documented in the vertebrate spinal cord. Frank & Fuortes (1957) discovered that conditioning muscle afferent volleys depressed monosynaptic excitatory postsynaptic potentials (EPSP's) recorded from soma of motoneurons without detectable changes in motoneuronal membrane properties. Although they did not suggest any possible mechanisms, they termed this inhibition "presynaptic". Frank (1959) subsequently acknowledged technical limitations in recording electrical phenomena of
dendritic origins from the distant soma by changing the terminology to "remote inhibition". But a group led by Eccles (Eccles et al., 1961) provided evidence against the idea of remote postsynaptic inhibition by showing that the falling phase of the monosynaptic EPSP, thought to be a manifestation of remote electrical phenomena, was unaltered during the inhibition. Furthermore, they found that the EPSP depression had the same time course as depolarization of primary afferent fibres (PAD) recorded extracellularly as a negative dorsal root potential (DRP). These findings led to the proposal that PAD is responsible for EPSP depression. Numerous subsequent studies showing parallel alterations of PAD and presynaptic inhibition following physiological and pharmacological manipulations have repeatedly confirmed this causal relationship (Schmidt, 1971).

1.3 Role of PAD in presynaptic inhibition

It may seem at first perplexing that depolarization of presynaptic terminals can lead to a depression of neurotransmitter release since such depolarization has been recognized as normally being a necessary first event in the process of neurotransmission (Zucker & Lando, 1986). There is however no evidence that PAD on its own can induce the release of primary afferent transmitter (Nicoll & Alger, 1979). Presynaptic depolarization can thus conceivably lead to a reduction of transmitter release either through inactivation of voltage-sensitive sodium channels which are necessary for action potential propagation or through an increased conductance for one or more small ion species whose combined equilibrium potential is lower than the threshold potential for transmitter release (or action potential propagation). The latter mechanism would decrease the axonal length constant and cause a shunt of currents (either active or electrotonic) sufficient to hinder or block impulse conduction.

The most conclusive evidence regarding the occurrence and
mechanism of presynaptic inhibition comes from studies utilizing the much simpler invertebrate preparations. In their now classic study in the crayfish neuromuscular junction, Dudel and Ruffler (1961) were the first to demonstrate that presynaptic inhibition causes a decrease in the number of transmitter quanta released from the presynaptic terminal. Shunting appears to be the predominant mechanism for presynaptic inhibition in this as well as many other invertebrate preparations, especially since the phenomenon therein is often associated with presynaptic hyperpolarization (Fuchs, 1977; Kawai & Niwa, 1977; Baxter & Bittner, 1981). A number of possible scenarios (not necessarily mutually exclusive) by which such shunting can lead to depression of transmitter release have been proposed. These include: (i) blocking of action potential penetration of terminals, especially at points of low safety factor for propagation such as axonal branches or dilatations (Atwood, 1976; Dudel, 1965) (ii) reducing the amplitude and duration of spikes reaching the terminals assuming that the transmitter release process is graded in relation to such spike characteristics (Takeuchi & Takeuchi, 1962, 1966; Kennedy et al., 1974; Baxter & Bittner, 1981) (iii) limiting the spread of electrotonic potentials into terminals (if action potentials cannot or do not normally propagate to synapses) thereby lessening transmitter release because of a smaller electrotonic depolarization at the site of release (Atwood et al., 1984).

Recent intrafibre recording studies in frog (Padjen & Hashiguchi, 1983) have provided evidence that PAD is associated with an increase in conductance of DRT, in support of previous work using sucrose gap technique (Padjen et al., 1973). This represents the first experimental evidence that current shunting plays a major role in presynaptic inhibition in the vertebrate nervous system as well, as originally suggested by Eccles (1964).

Based mostly on pharmacological grounds, evidence has accumulated in the past decade for the possibility of primary afferent hyperpolarization contributing to presynaptic
inhibition in vertebrate spinal cord. Such evidence adds more credence to the importance of shunting (versus depolarization) as a mechanism of presynaptic inhibition therein. This topic will be further discussed in section 1.7.2 (see also chapter 4).

1.4 Origin and nature of PAD

The pathway responsible for PAD generation via conditioning afferent volleys appears to be multisynaptic, as suggested by a variety of data including some characteristics of the PAD such as its long latency, slow time course, and patterns of spatial and temporal summation (Burke & Rudomin, 1977). It is therefore likely that interneurons are involved in the genesis of PAD. Three explanations have been proposed for the mechanism of PAD generation by interneurons: electrical, ionic and chemical.

1.4.1 Electrical

PAD was first hypothesized to occur as a result of extracellular current flows generated by active interneurons (Gasser & Graham, 1933). Short-latency electrical interactions among motoneurons or between motoneurons and DRT have been described in frog spinal cord (Grinnell, 1970; Shapovalov et al., 1978; Alvarez-Leeffmans et al., 1979). If similar interactions were to occur between interneurons and DRT, then the latter neurons should behave as passive conductors of the extracellular currents. Such does not seem to be the case in frog spinal cord where PAD can be produced by electrical stimulation of motoneurons (unlike mammalian spinal cord). Chronic deafferentation abolishes the intraspinal field potentials (associated with PAD) evoked by electrical stimulation of ventral roots, as would be expected if the DRT are generating the extracellular PAD-associated...
currents (Glusman & Rudomin, 1974). This data, along with the long latency and long duration characteristic of PAD (Burke & Rudomin, 1977), make this an unlikely mechanism for PAD generation.

1.4.2 Ionic (extracellular K)

It was originally proposed by Barron and Matthews (1938) that DRP (in both amphibian and mammalian spinal cord) could arise as a result of changes in ionic concentrations around DRT due to neuronal activity by a mechanism analogous to that responsible for after-potentials in peripheral nerve (i.e. increases in extracellular K). This theory remained unchallenged until new technology was introduced in the early seventies—the K-sensitive microelectrode. Using this technology in both mammalian and frog spinal cords, it was found that maximal increases in extracellular K concentration ([K]o) are distributed in layers of the dorsal horn where primary afferent terminals seem to be concentrated and where maximal PAD has been localized (Sykova, 1983). The most probable sources for this K accumulation in the dorsal horn after afferent stimulation are unmyelinated terminal arborizations of primary afferents as well as neighboring neurons, especially interneurons. The former source appears to be minor since, even under optimal conditions for K accumulation, blockade of synaptic transmission (e.g. by magnesium) causes measurements of [K]o to decrease to about 10-15% of control values (Nicoll, 1979).

The magnitude of K accumulation seems to increase with increasing frequency, intensity and duration of afferent stimulation (Sykova et al., 1976; Nicoll, 1979; Shefner & Levy, 1981). Thus, Nicoll (1979), using both glial cell and K-selective microelectrode recordings, reported that 10% of the amplitude of DRP set up by a single electrical volley can be attributed to a transient increase in periaxonal [K]o but that during high frequency stimulation this could rise to 80-
90%. Such tetanic activity has been detected following certain nociceptive cutaneous stimuli in frog (Czeh et al., 1981) suggesting that it may not be unphysiological.

From the above discussion, it appears that the measured magnitude of K accumulation during DRP following single or short trains of afferent stimuli is insufficient to account totally for the recorded potential. Other evidence (Nicoll & Alger, 1979) also supports an alternate or additional mechanism for PAD generation: (i) the time course for the peak of detectable increases in [K]o is much slower than that of the DRP (Krnjevic & Morris, 1975; Nicoll, 1979) (ii) low-frequency dorsal root stimulation produces decremental summation of measured [K]o while the size of DRP (evoked by stimulation of adjacent dorsal root) remains constant or decreases slightly (Nicoll, 1979) (iii) in frog spinal cord, ventral root stimulation evokes a DRP but does not cause any increase in [K]o (Nicoll, 1979) (iv) some pharmacological (ie. barbiturates, picrotoxin) or physiological (ie. flexor reflex different stimulation) manipulations in mammalian spinal cord produce opposite effects on DRP and [K]o (Bruggencate et al., 1974) (v) picrotoxin, a known blocker of GABA but not K-evoked responses (Nicoll, 1979; Osorio et al., 1979), depresses (but does not totally block) DRP, particularly the early component, to an extent dependent on concentration and the presence of synaptic transmission. Based on some or all of these observations, a number of groups (Krnjevic & Morris, 1975; Barker et al., 1975b; Nicoll, 1979) have suggested that evoked PAD may have (at least) two components, a fast, sharp phase and a slower, longer-duration phase, operating by different mechanisms. It is proposed that the latter component is mostly mediated by potassium while the former one is a result of activation of axo-axon synaptic, which are probably GABAergic (see section 1.5.2).
1.4.3 Chemical (axo-axonic synapses)

Not only did Eccles and colleagues strongly support the existence of presynaptic inhibition across the first sensory synapse in vertebrate spinal cord (as discussed above) but they also were the first to postulate that such a phenomenon was the result of the release of a specific chemical transmitter from axo-axonic synapses formed by the terminals of interneurons and the terminals of primary afferent fibres (Eccles, 1964). The first of a long list of subsequent supportive evidence (Burke & Rudomin, 1977; Nicoll & Alger, 1979) from other laboratories came from Gray (1962) who discovered the anatomical substrate for such a process in spinal cord. Later studies confirmed the presence of axo-axonic synapses in areas of the spinal cord where primary afferent terminals are thought to be located, such as the substantia gelatinosa (Ralston, 1965) and the motor nuclei (Conradi, 1969). Furthermore, using cobaltous chloride iontophoresis to label dorsal root fibres of frog spinal cord, Szekely and Kosaras (1977) demonstrated unequivocally that the postsynaptic component of some axo-axonic synapses are of dorsal root origin.

Padden and Hashiguchi (1983) provided additional evidence that is consistent with the presence of axoaxonic synapses and the release of PAD neurotransmitter. As mentioned above, they detected an increase in membrane conductance associated with PAD as well as an extrapolated reversal potential close to but less negative than the resting membrane potential.

One of the more likely candidates for the role of PAD neurotransmitter is presently considered to be the neutral amino acid, GABA (Levy, 1977).
1.5 Neutral amino acids as mediators of PAD

1.5.1 Description of neutral amino acids

Neutral amino acids are polar amino acids which are electrically neutral, and therefore neither acidic nor basic, at physiological pH. Figure 1.1 displays the chemical structures of some neutral amino acids which are relevant to this work.

1.5.2 GABA

The history of neutral amino acid association with CNS function is extremely short. GABA (gamma-aminobutyric acid) was not known to be a constituent of vertebrate CNS until 1950 when this was reported by three laboratories (Roberts & Frankel, 1950; Awapara et al., 1950; Udenfriend, 1950). It was observed that GABA (not being a constituent of protein) occurs in high concentration in such tissue. Although this suggested an important function for GABA in vertebrate CNS, its role as an inhibitory transmitter therein remained in serious doubt for many more years to come. Based on indirect evidence, a group led by Florey and Elliot (Bazemore et al., 1957) had suggested such a possible role by demonstrating that the mysterious factor I from mammalian brain extracts, which Florey had previously found to strongly inhibit crayfish stretch receptor neurons, was GABA. Such a role remained disputed until Krnjevic and Phyllis (1963), working in mammalian cortical neurons, became the first to provide convincing support for the postulate that GABA is a natural inhibitory transmitter in the brain. This was subsequently confirmed by a large number of studies (Krnjevic & Schwartz, 1967; Dreifuss et al., 1969; Krnjevic, 1974) so that this neutral amino acid is presently considered to be an
established inhibitory neurotransmitter with effects on neurons throughout the CNS, including spinal cord.

In vertebrate spinal cord, GABA is thought to be a neurotransmitter responsible (at least partially) for PAD and presynaptic inhibition on the first sensory synapse, as mentioned earlier. A considerable body of evidence is consistent with the existence of GABAergic axoaxonic synapses (Levy, 1977; Nicoll & Alger, 1979) including the following: (i) GABA and its synthesizing enzyme, glutamic acid decarboxylase, are present in significantly higher concentrations in the dorsal than in the ventral horn of the spinal cord, especially in areas associated with PAD (Miyata & Otsuka, 1972) (ii) glutamic acid decarboxylase has been localized, by electron microscopic immunohistochemistry, to axon terminals synapsing onto other axon terminals of primary afferent origin (Hunt, 1983) (iii) a high-affinity, sodium-dependent uptake system for GABA is present in spinal cord, including nerve terminals (Iversen & Johnston, 1971; Davidoff & Adair, 1975) (iv) depletion of endogenous GABA levels in spinal cord, either surgically (leading to interneuronal loss) or pharmacologically, results in PAD depression (Miyata & Otsuka, 1972; Bell & Anderson, 1972) (v) GABA depolarizes DRT and such a response is depressed to a similar extent by the same antagonists which depress presynaptic inhibition and PAD evoked by afferent volleys (Eccles et al., 1963; Davidoff, 1972; Barker et al., 1975a).

1.5.3 Taurine

High concentrations of taurine were first discovered in vertebrate CNS at about the same time as GABA (Roberts et al., 1950) but progress in elucidating a functional role for this neutral amino acid has not proceeded as smoothly as that of its more famous structural relative. Current neurobiological functions for taurine include neurotransmitter, neuromodulator or general stabilizer of excitable membranes. A number of
criteria for the identification of taurine as a neurotransmitter have apparently been met in vertebrate CNS, especially cerebellum (Kontro & Oja, 1983; Wu et al., 1985). These include (i) presence of taurine and its synthesizing enzyme in nerve terminals (ii) stimulated release (possibly calcium-dependent) and high-affinity uptake (iii) direct inhibitory effects on the excitability of neurons through increased chloride conductance (Okamoto et al., 1983) (iv) discovery of a relatively specific antagonist of taurine responses (see section 1.6.1).

In frog spinal cord, taurine (also not a protein constituent) is present in higher concentration than any other free amino acid tested (Collins, 1974) making it likely that it serves an important function therein. Whether this function is that of an inhibitory neurotransmitter is less certain than for GABA. On DRT it produces depolarizing responses with similar potency to GABA but with slower time course. Unlike GABA, there does not appear to be a high-affinity, sodium-dependent taurine uptake process in this preparation (Davidoff & Adair, 1976), thus possibly explaining the potency and time course of taurine responses (Nicoll & Iwamoto, 1978). Even some previous quantitative estimates of taurine content in vertebrate CNS tissues have been disputed as being overestimates using more sensitive methodology (Tachiki & Baxter, 1979). The best evidence so far for taurine being a mediator of PAD is pharmacological (see section 1.6.1).

1.5.4 Glycine

Although this neutral amino acid has been recognized, like GABA, to be an established inhibitory neurotransmitter in the vertebrate CNS (Krnjevic, 1974), its depolarizing effects on vertebrate DRT are poor and cannot be blocked by any known antagonists, including strychnine (Barker et al., 1975a). For these reasons, little further consideration will be given to
1.6 Responses of primary afferents to neutral amino acids: receptor multiplicity

The most important characteristic for classifying a receptor is its pharmacological profile, which includes the potency (especially rank order) of agonists and competitive antagonists that interact directly with it. The latter aspect of such a pharmacological profile is perhaps more revealing, especially since antagonists have led to the realization that many agonists can interact with more than one receptor type, often on the same tissue. Such is the case with neutral amino acid effects on primary afferents of frog spinal cord.

1.6.1 Receptors for GABA versus taurine

Using sucrose gap recording (of DRT), Barker and coworkers (1975) applied a series of neutral amino acids and three antagonists (i.e. bicuculline, picrotoxin and strychnine) to isolated frog spinal cord. Based on antagonist sensitivity of the depolarizing responses, they suggested the presence of at least three distinct populations of neutral amino acid receptors on DRT: a receptor for GABA (sensitive to bicuculline and picrotoxin), a receptor for taurine or beta-alanine (sensitive to all three antagonists tested), and a receptor for glycine (insensitive to all three antagonists tested). These authors also compared quantitatively the action of antagonists on amino acid and synaptic responses and further proposed that GABA mediates the final step in the pathway producing DRP evoked by adjacent dorsal root stimulation (DR-DRP) while taurine (or beta-alanine) mediates the final step of the pathway leading to DRP after ventral
root stimulation (VR-DRP).

Recent studies on isolated toad spinal cord (Yarbrough et al., 1981) provided further support for part of such studies by demonstrating blockade of the depolarizing responses of taurine (and beta-alanine) on DRT, but not those of GABA or glycine, by a novel antagonist, 6-aminomethyl-3-methyl-4H-1,2,4-benzothiadiazine-1,1-dioxide (AMBD).

1.6.2 GABA receptor sub-types (GABA-A versus GABA-B)

The pharmacological profile of the classical GABA-A receptor site in vertebrate CNS has been extensively studied (Böwery et al., 1984). It is antagonized by the alkaloid convulsant, bicuculline, which competes for the GABA recognition site. GABA responses are also antagonized by another convulsant, picrotoxin, but this agent does not appear to bind at the same site as GABA. It apparently interacts in a non-competitive manner with the associated ionophore. Other medically important agents which have been shown to interact allosterically with the GABA-A receptor are the sedative barbiturates and benzodiazepines. These drugs are thought to enhance the binding of GABA to its GABA-A recognition site by associating with their own distinct binding sites. Interactions amongst the various allosteric sites and the GABA-A recognition site seem to be quite complex and will not be further discussed so as not to drift too far away from the main focus of this work.

Agonists that bind directly at the GABA-A recognition site include muscimol, piperidine-4-sulphonic acid, isoguvacine, THIP and delta-aminovaleric acid. Some of these may not be selective only for the GABA-A site (e.g. muscimol). Since taurine (and beta-alanine) depolarizing responses on DRT can also be antagonized by bicuculline, these neutral amino acids may also share binding sites with GABA. The proposed nature of the ionophore coupled to the GABA-A receptor will be discussed later (section 1.7.1).
In 1979, Bowery and Hudson discovered that GABA could reduce the evoked (but not basal) release of radioactive noradrenaline from peripheral tissues in a manner that was not sensitive to bicuculline nor mimicked by many GABA agonists. They thus suggested the presence of a GABA receptor on sympathetic nerve terminals distinct from the well-known bicuculline-sensitive site. At about the same time, Brown & Higgins (1979) found similar results at pre-ganglionic sympathetic neurons. Since then, many other physiological and biochemical studies have supported the existence of a novel presynaptic inhibitory GABA receptor with similar pharmacological characteristics in a variety of peripheral as well as central nervous tissues (Bowery, 1983). This receptor has been classified as GABA-B while the classical site has been designated GABA-A. The GABA-B site is defined (Bowery et al., 1984) as a bicuculline-insensitive GABA receptor which is: (i) selectively activated in a stereospecific manner by baclofen (beta-para-chlorophenyl-GABA) (ii) not activated by recognized GABA agonists (examples above) (iii) not influenced by barbiturates or benzodiazepines (iv) dependent on physiological concentrations of divalent cations (v) and not associated with those ionophores which are linked to the GABA-A receptor complex (see section 1.7.1). As mentioned previously, the existence of a competitive antagonist is probably the key pharmacological characteristic for defining a receptor as well as elucidating a functional role for it. Some laboratories have provided evidence for delta-aminovaleric acid (Muhayaddin et al., 1982) and delta-aminolaevulinic acid (Brennan & Cantrill, 1979) as being possible GABA-B antagonists. Chapter 4 discusses this point further.

GABA-B receptors have been found (often with regional distribution distinct from that of GABA-A receptors) throughout the vertebrate CNS (Bowery et al., 1984). An abundance of binding (Hill & Bowery, 1981; Price et al., 1984) and electrophysiological (Davidoff & Sears, 1974; Bourne & Padjen, 1980; Henry, 1982; Capek & Esplin, 1982; Desarmenien et al., 1984; Schlichter et al., 1984) evidence points to
their location on DRT and therefore a possible role in presynaptic inhibition. Neonatal administration of capsaicin, a neurotoxin selective for small unmyelinated or finely myelinated DRT, has been shown to reduce these GABA-B receptors to a greater extent than GABA-A (muscimol binding) sites (Singer & Placheta, 1980). This, along with electrophysiological data showing the absence of GABA-B sites on large myelinated DRT (Padjen & Hashiguchi, 1982) and their presence on primary afferents of thinner diameter (Desarmenien et al., 1984), suggests that the GABA-B receptor may be located exclusively on small-diameter DRT.

1.7 Responses of primary afferents to neutral amino acids: ionic mechanisms

The ionic mechanism of neutral amino acid responses on DRT remains unresolved largely because of technical limitations owing to the size of DRT relative to intracellular microelectrode tips. The primary afferents can only be impaled at pre-bifurcated axonal sites (usually near where the dorsal root enters the spinal cord) of only large-diameter fibres or in soma (DRG), where neutral amino acids also produce depolarizing responses (DeGroat, 1972). The determination of the reversal potential of these responses, which would provide the best evidence for identifying the ion(s) mediating observed conductance changes, can therefore only be arrived at indirectly at sites electrotonically distant from the synaptic events. An additional problem in frog spinal cord is the strong rectifying property of axonal membranes allowing only an estimation of the reversal potential through extrapolation (see Padjen & Hashiguchi, 1983). Despite these difficulties, results obtained using currently available extracellular and intracellular recording techniques have provided good evidence for understanding the ionic events responsible for such responses.
1.7.1 GABA-A

On DRT, GABA-A receptor activation leads to a depolarizing response. There are four possible ionic mechanisms (not mutually exclusive) that can produce such an electrical event: decreased K conductance or an increased conductance to calcium, sodium or chloride. The first mechanism is unlikely because of the observed increase in membrane conductance upon application of GABA (Padjen & Hashiguchi, 1982). From extracellular studies (Padjen & Smith, 1981), it appears that part of the GABA depolarizing response may be calcium-dependent but this would not explain presynaptic inhibition. Extracellular sodium manipulations (Barker & Nicoll, 1973; Padjen et al., 1973; Nishi et al., 1974) have lead to controversial interpretations because of the complex electrical effects seen upon sodium removal. Unless extracellular sodium concentration is reduced to less than 10% of control, sodium reduction does not lead to depression of the GABA response on DRT (Nicoll & Padjen, unpublished observations) thus making sodium unlikely to be the major ion species mediating this response. The strongest ionic candidate appears to be chloride, as at other GABA-A sites throughout the vertebrate nervous system (Krnjevic, 1974). Evidence includes intracellular recording from DRG where changes in extracellular chloride concentration shift the reversal potential of the GABA depolarizing response in the expected direction (Nishi et al., 1974; Gallagher et al., 1978; Hattori et al., 1983; Akaike et al., 1985). The implication of chloride being the major ionic species responsible for GABA-A responses is that the chloride equilibrium potential would have to be in the depolarizing direction relative to the resting membrane potential, as determined in some studies on DRG (Hattori et al., 1983; Ishizuka et al., 1984; Akaike et al., 1985) where both GABA and chloride equilibrium potentials were found to be.
identical. It is assumed that GABA-A responses on DRT are mediated through a similar ionic mechanism as those on DRG even though some studies in which extracellular chloride concentration was varied have produced results not fully consistent with such a mechanism (Barker & Nicoll, 1973; Nishi et al., 1974; Nicoll & Padjen, unpublished observations). Preliminary intracellular studies on extraspinal dorsal root axons (Hashiguchi & Padjen, 1981) have however shown expected changes in extrapolated reversal potential of PAD with use of chloride-filled microelectrodes, thus making it more probable that PAD occurring at DRT is chloride-mediated.

1.7.2 GABA-B

On frog DRT, baclofen produces a pure hyperpolarizing response (Davidoff & Sears, 1974; Fukuda et al., 1977; Bourne & Padjen, 1980), in contrast to GABA-A responses. While it is difficult to expose pharmacologically a hyperpolarizing component of GABA responses, such opposing response polarities indicate distinct ionic mechanisms and, consequently, GABA receptors. Since GABA-A responses seem to be due to chloride efflux, the baclofen-induced hyperpolarizing responses should involve other ionic events.

At about the same time as Bowery and colleagues were discovering bicuculline-insensitive GABA effects in the peripheral nervous system, Dunlap & Fishbach (1978, 1981) demonstrated similar results on cultured embryonic chick DRG. They provided evidence that GABA could decrease action potential duration by reducing voltage-dependent calcium currents at concentrations that did not alter resting membrane potential or conductance, thus revealing a novel way of producing presynaptic inhibition. Some of these results were later supported by studies on rat small-diameter DRG (Desarmenien et al., 1984; Schlichter et al., 1984) but the data obtained did not exclude the possibility that the effects on calcium conductance are secondary events. In hippocampus,
stronger evidence was provided for another ionic explanation of GABA-B responses (Newberry & Nicoll, 1985; Inoue et al., 1985c; Gahwiler & Brown, 1985). Here, GABA-B responses on both pre- and postsynaptic sites of CA3-CA1 synapses appear to be predominantly due to K efflux since: (i) reversal potentials for both GABA-B responses and post-burst hyperpolarization are identical. (ii) changes in concentration of extracellular K, but not chloride, affect GABA-B responses and shift their equilibrium potential in the expected direction (iii) GABA-B responses are associated with an increase in membrane conductance and are blocked by potassium conductance blockers such as cesium, barium and 4-aminopyridine.

1.7.3 Taurine

Very little work has been done on the ionic mechanism of taurine responses in vertebrate CNS. On primary afferents, indications are that such responses, like those of GABA, are mediated by chloride ionophores (Nishi et al., 1974; Akaike et al., 1985), as has been shown in cerebellum (Okamoto et al., 1983). Some pharmacological evidence however suggests that the two neutral amino acid responses involve distinct ionic components. In hippocampus, Zeise (1985) demonstrated that 4-aminopyridine has a differential effect on the two responses: taurine-evoked responses are depressed while those of GABA are increased.

Additional pharmacological experiments on DRT using bicuculline and picrotoxin (Bourne & Paden, 1980) have shown that the taurine response may also consist of a bicuculline-resistant, hyperpolarizing (GABA-B?) component. This component is much more easily exposed than that of GABA responses. In fact, even under normal conditions taurine responses occasionally exhibit biphasic responses on DRT (Nicoll & Iwamoto, 1978; Paden, unpublished observations).
1.8 Objectives

In light of the issues that have been introduced above, the specific objectives of this project were to try and answer the following questions:

(i) Do the neutral amino acids, GABA and taurine, interact at different receptors on DRT?

(ii) Are such receptor(s) really located directly on membranes of primary afferents or do neutral amino acids exert their effects on primary afferents indirectly through an increase in [K]o?

(iii) What are some characteristics of electrical responses of DRT to baclofen, in view of its possible interaction at a novel (additional) GABA receptor on primary afferents?
Figure 1.1 Chemical structures of some neutral amino acids.
GABA

BETA-ALANINE

TAURINE

HYPOTAURINE

BACLOFEN

KOJIC AMINE

3-AMINOPROPANE-SULPHONIC ACID
CHAPTER 2

FURTHER EVIDENCE IN SUPPORT OF TAURINE AS A MEDIATOR OF
SYNAPTIC TRANSMISSION IN THE FROG SPINAL CORD

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Abstract

It was reported that 6-aminomethyl-3-methyl-4H,1,2,4-benzothiadiazine-1,1-dioxide (AMBD, TAG) is a specific blocker of taurine and beta-alanine responses in central nervous system (Yarbrough et al., 1981). We have re-examined the effect of AMBD on amino acid and synaptically evoked responses recorded from isolated hemisectioned frog spinal cord by means of sucrose gap technique. When indirect responses were blocked by adding tetrodotoxin (0.2 uM) or manganese (2 mM), AMBD (conc 0.01 - 0.5 mM) selectively antagonized taurine, beta-alanine, hypotaurine and kojic amine evoked depolarizations of intramedullary part of primary afferents (DRT) and dorsal root ganglia (DRG) without affecting responses to glutamate, glycine (on DRT) or GABA (on DRT and DRG). Depolarizing responses to taurine and beta-alanine (1 mM) were depressed by 50% at 0.1 mM AMBD and often completely antagonized by 0.25 mM AMBD. In normal Ringer solution, AMBD selectively antagonized dorsal root potential evoked by ventral root stimulation (VR-DRP, threshold at 0.02 mM, 90% block at 0.25 mM); other synaptic potentials increased in duration and/or amplitude due to strong convulsant activity caused by higher concentrations of AMBD. These results are in agreement with the proposal that taurine or a taurine-like substance is the mediator of VR-DRP in amphibian spinal cord (Barker et al., 1975b).

Key words: taurine, transmitter, frog spinal cord, dorsal root potential, 6-aminomethyl-3-methyl-4H,1,2,4-benzothiadiazine-1,1-dioxide (AMBD, TAG), taurine antagonist, beta-alanine, hypotaurine, kojic amine.
Introduction

The great abundance and uneven distribution of taurine in the nervous system suggests its possible important role therein. Many steps in the life cycle of taurine have been identified, such as synthesis, release and uptake (cf. Oja and Kontro, 1983), that are compatible with a neurotransmitter role. Its action, an increase in chloride conductance (Okamoto et al., 1983a), however, has been hard to distinguish from other transmitter candidates because conventional antagonists, picrotoxin, bicuculline and strychnine did not distinguish the inhibitory action of taurine, GABA, beta-alanine and glycine in most of the sites examined (cf Knjevic, 1974). On the other hand, the primary afferents of amphibian spinal cord lack strychnine sensitive glycine sites and the taurine (and beta-alanine) effect has been separated from that of GABA by its sensitivity to strychnine and bicuculline/picrotoxin antagonism. On the basis of the same pharmacological profile, ventral root evoked dorsal root potential has been proposed to be mediated by a taurine or taurine-like compound (Barker et al., 1975).

Recently, it has been reported that 6-aminomethyl-3-methyl-4H,1,2,4-benzothiadiazine-1,1-dioxide (AMBD, TAG, Girard et al., 1982) shows a selective action against taurine (Yarbrough et al., 1981). We sought to address two principal questions. First, does AMBD show specificity towards taurine as claimed in the initial report (Yarbrough et al., 1981). Second, is the pathway, VR-DRP, proposed to be mediated by taurine (Barker et al., 1975) affected by AMBD. Some of the preliminary results have been reported (Padjen et al., 1983; 1984).
**Methods**

The membrane potential of dorsal roots (primary afferents) of isolated hemisectioned Rana pipiens spinal cords and isolated Rana catesbeiana dorsal root ganglia was recorded by means of the sucrose-gap technique as previously described (Barker et al. 1975; Padjen & Smith, 1980). Drugs were applied by superfusion in the compartment bathing the spinal cord via a set of stopcocks. Indirect responses were blocked by the inclusion of tetrodotoxin (TTX; 0.2 uM) in the Ringer solution (referred to as TTX-Ringer solution) or in some experiments by adding 2 mM manganese (Mn-Ringer). Normal Ringer solution used for superfusion (1-3 ml./min; bath volume 0.15 ml) contained the following (in millimoles/liter): NaCl, 115; KCl, 2; CaCl₂, 2; HEPES (pH 7.3), 5; D-glucose, 10. Temperature (12-14 degrees celsius) was maintained within +/- 0.1 degrees celsius using a Peltier device. Permanent records were obtained from a rectilinear pen recorder (Brush model 240b). All drugs were purchased from Sigma, St. Louis, MO., U.S.A., except kojic amine, AMBD (both gifts from Merck Frosst Laboratories, Kirkland, QC) and glycine (Fisher, Fair Lawn, N.J.).

Interpretation of the recorded potentials. Potential difference is measured between the electrode in the central compartment containing spinal cord or dorsal root ganglion and the electrodes in the compartments surrounding distal ends of dorsal roots (cf. Barker et al., 1975). The observed potential changes represent an attenuated version of changes in membrane potential of the intramedullary part of a whole population of primary afferents, in the case of recordings from dorsal roots, or of dorsal root ganglia. Under our recording conditions, an upward deflection of the pen represents depolarization.
Results

Antagonism of neutral amino acid responses by AMBD

In the first part of this study we have re-examined the specificity of AMBD in antagonizing responses of primary afferents to amino acids. These responses were measured in TTX or Mn-Ringer to minimize indirect responses (see Methods). Under these conditions AMBD had no effect on the resting membrane potential of dorsal roots, except for an occasional small (<1 mV) depolarization with the highest concentration of AMBD tested (1 mM).

As previously shown taurine, beta-alanine, hypotaurine and kojic amine responses had characteristically slower time courses than those of GABA or glutamate (cf Barker et al., 1975; Bourne and Padjen, 1980). Though not very potent, AMBD was selective in its blockade of amino acid responses on DRT. Concentration of AMBD (0.1 - 0.25 mM) which markedly reduced responses to taurine had no appreciable effect on responses to GABA. Figure 2-1 illustrates a typical experiment. In this experiment depolarizing responses to taurine (1 mM), hypotaurine (2 mM) and kojic amine (.25 mM) were depressed by AMBD (0.25 mM) to 18, 12 and 48% of control, respectively, with the GABA (.5 mM) response remaining unchanged. In some experiments taurine-evoked depolarization was not only completely antagonized by AMBD but a hyperpolarizing response appeared, in a manner recalling observations previously reported with kojic amine and GABA responses under bicuculline/picrotoxin blockade (Bourne and Padjen, 1980). AMBD antagonized to varying degrees the responses to other amino acids (taurine > beta-alanine > muscimol = aminopropane sulfonic acid), while not affecting responses to glycine (sometimes increased), glutamate nor potassium (not illustrated). All the effects of AMBD were reversed upon its removal. Our results, therefore, essentially confirm the original findings of Yarbrough et al. (1981) about the
selectivity of AMBD in the amphibian spinal cord.

The antagonistic effect of AMBD on amino acid responses was concentration-dependent. Results of these studies are summarized in Figure 2-2 showing that the maximal antagonism of GABA responses by AMBD was 25% (at 1 mM AMBD) at a time when beta-alanine and taurine responses were almost abolished (i.e., 10% of control).

Depolarizing responses to increasing concentrations of taurine did not show saturation until some 10-20 mM and similar behavior was observed with concentration-response curves of GABA and hypotaurine (Figures 2-3 to 2-5). AMBD shifted concentration-response curve of taurine to the right in an apparently non-parallel manner: dose-ratio of AMBD and taurine decreased with increasing taurine concentrations (Figure 2-3). This result appears at variance with the report of Yarbrough et al. 1981. As was obvious from Figure 2-1, hypotaurine, a precursor of taurine, was much less potent in depolarizing DRT than taurine. This is even more clear when comparing hypotaurine and taurine concentration-response curves (Figures 2-4 vs. Figure 2-3).

It is well known that GABA depolarizes somata of primary afferents, i.e. dorsal root ganglia (DRG) (cf. Nishi et al., 1974; Akaike et al., 1985). In view of the fact that the accessibility of DRG cells makes it a model system to study pharmacology of DRT we have examined the action of AMBD on amino acid responses on DRG of bullfrogs. A sample of responses to GABA (0.5 mM), beta-alanine (0.5 mM), hypotaurine (0.5 mM) and taurine (1 mM) is shown in Figure 2-6. The order of potency of these agonists was GABA > beta-alanine > hypotaurine >> taurine, glycine. There was considerable variation in responsiveness of DRG to hypotaurine and taurine, although the hypotaurine responses were at least twice as large as those of taurine. AMBD showed similar selectivity in antagonizing beta-alanine and hypotaurine responses without affecting GABA. Comparison with the responses to the same amino acids on DRT reveals a different rank order of potencies between taurine and hypotaurine (see Figure 2-1). DRG did not
respond to kojic amine, glycine or glutamate in concentrations up to 10 mM.

Effect of AMBD on synaptic potentials.

Three well defined synaptic potentials were studied: dorsal root evoked ventral root potential (DR-VRP), mediated in part by acidic amino acids via N-methyl-D-aspartate preferred receptor (Padjen and Smith, 1980); dorsal root evoked dorsal root potential (DR-DRP), mediated by GABA (Barker et al., 1975) and ventral root evoked dorsal root potential proposed to be mediated by taurine or a taurine like ligand (Barker et al., 1975). A typical experiment is illustrated in Figure 2-9. Increasing concentrations of AMBD caused an increasing depression of VR-DRP. At the same time DR-VRP increased in amplitude and duration. Except for an increase in duration with the highest concentration of AMBD tested, DR-DRP was unchanged. All the changes of synaptic potentials are reversible although with high concentrations of AMBD recovery of VR-DRP may not be complete even after several hours of wash out. These results are also summarized in Figure 2-10.

Addition of AMBD in normal Ringer often caused an increase in spontaneous activity which sometimes resulted in a small depolarization of DRT. Such activity is not recorded on DRG. With high concentrations of AMBD (>0.25 mM), which resulted in great prolongation of DR-VRP and DR-DRP, spontaneous activity tended to decrease.

The changes in synaptic potentials and spontaneous activity observed in AMBD are regularly associated with the action of convulsants. In this respect, AMBD's action differs from other convulsants tested (strychnine, picrotoxin, bicuculline, cf. Barker et al., 1975) by its singular action in increasing DR-VRP.
Discussion

There are two major findings of the present investigation. The first is that AMBD distinguishes depolarizing responses of primary afferents to taurine from those to GABA, thus confirming the original report of Yarbrough et al. (1981). Our results are also in good agreement with selectivity of AMBD observed in cerebellar slices (Okamoto et al., 1983) except for beta-alanine which appears not to be affected by AMBD in cerebellum. These discrepancies point out possible differences in amino acid receptors in different CNS regions. In this regard, it is important to note that the usually very small glycine depolarization of amphibian primary afferents is not sensitive to either AMBD or strychnine (cf. Barker et al., 1975a). This is not the case in other sites, such as spinal interneurons, where AMBD appears not to differentiate between responses to glycine and taurine (and other neutral amino acids, Curtis et al., 1982). In another series of experiments not reported here (cf. Hassessian and Padjen, 1984) we have found that hyperpolarizing responses of amphibian motoneurons to taurine and glycine are equally sensitive to AMBD and strychnine. Additional evidence of the specificity of AMBD was recently provided by studies of GABA- and taurine-mediated increases in [3H]diazepam binding in cerebellar membranes: AMBD (0.25 mM) completely abolished the taurine effect, but only reduced GABA's effect by 30% (Willo & Padjen, 1986).

It is of interest to note that kojic amine, a structural analogue of GABA, was also depressed by AMBD. Together with muscimol, 3-aminopropane sulphonic acid and piperidine-4-sulphonic acid, kojic amine appears to belong to a group of compounds previously shown to be GABA agonists (cf. Yarbrough et al., 1979; Bourne and Padjen, 1980) that are by their sensitivity to AMBD set apart from other GABA agonists. Though beta-alanine produces similarly sized responses on both DRG and DRG, it is very interesting that the rank potencies of
taurine and hypotaurine at these sites are distinctly opposite to each other indicating the possible existence of different sites of action for each amino acid and/or difference of access to the sites of action. The observation that DRG responds to GABA without responding to kojic amine further supports the possibility that the two drugs act at different sites. This notion is further confirmed by our findings, using intracellular recording from large myelinated primary afferents, "that only some 30% of these axons are sensitive to kojic amine although all of them are depolarized by GABA (Padjen & Hashiguchi, 1982).

The second finding is that AMBD selectively depresses VR-DRP. This potential has been already shown to be sensitive to antagonism by strychnine and picrotoxin/bicuculline as were the taurine and beta-alanine responses. On the basis of these pharmacological similarities it has been proposed that VR-DRP might be mediated by taurine or a taurine like compound (Barker et al. 1975b). Present results offer additional support for this role of taurine.

One should consider some additional points that may interfere with the above conclusions: 1) VR-DRP pathway in amphibian spinal cord also contains a cholinergic synapse (Kiraly and Phillis, 1961). It has not been possible to examine directly the effect of AMBD on this cholinergic site, but there is no evidence of AMBD interfering with cholinergic transmission (G.G. Yarbrough, personal communication). 2) AMBD causes convulsive activity which may indirectly affect VR-DRP. This seems unlikely because some convulsants, e.g. 4-aminopyridine, do not depress VR-DRP (Collier et al., 1981). 3) There is no evidence of taurine uptake in slices of frog spinal cord (Davidoff and Adair, 1976). It is difficult to accept this argument as exclusive of transmitter role for taurine since another mechanism may be involved in termination of transmitter action (e.g. diffusion).

In conclusion, our results indicate that the transmitter mediating VR-DRP is pharmacologically indistinguishable from taurine. In addition to the previously found correlation,
namely its sensitivity to strychnine, bicuculline and picrotoxin, on which the original proposal for taurine role was made, we have found that AMBD antagonises taurine-evoked depolarizing responses on DRT and VR-DRP in the same concentration range. However strong this argument might be, lack of precise identification of cellular elements containing taurine and demonstrations of its release on specific stimulation prevents us from making a final statement regarding a transmitter role for taurine.
References


Okamoto, K., Kimura, H. and Sakai, Y. Evidence for taurine as an inhibitory neurotransmitter in cerebellar stellate interneurones: selective antagonism by TAG (6-aminomethyl-3-methyl-4H,1,2,4-benzothiadiazine-1,1-dioxide). Brain Research, 265 (1983b) 163-168.


Figure 2-1. Effect of AMBD on the depolarizing responses of the intramedullary part of primary afferents (DRT, for dorsal root terminals) to amino acids. This figure and all others are sucrose-gap recordings on a rectilinear chart recorder whereby upward deflections signify depolarization; drug applications are marked by horizontal bars; concentration of drugs in mM. Abbreviations: GABA - gamma-aminobutyric acid; KJA - kojic amine; TAU - taurine; HYPOTAU - hypotaurine. Note the difference between TAU and HYPOTAU responsiveness of DRT (different gains) and the insensitivity of the GABA response to 0.25 mM AMBD. Antagonism of other responses by AMBD is completely reversible.
Figure 2-2. Summary of the results showing selective effect of AMBD on amino acid responses of DRT expressed as percent of control response (mean ± SE, n=2 - 12). Note that the responses to taurine and beta-alanine are more affected (80 and 70 %, respectively at .25 mM AMBD) than GABA (15%). Glutamate, glycine and responses to 8k (not shown) are unchanged.
EFFECT OF AMBD ON AMINO ACID RESPONSES

on DRT (Rana pipiens)

Percent of control response

Concentration of AMBD (mM)

□ 0.5mM GABA

○ 1mM TAURINE

△ 1mM BETA-ALANINE

× 1mM GLUTAMATE

▼ 3mM GLYCINE

0.010 0.016 0.025 0.040 0.063 0.100 0.158 0.251 0.398 0.631 1.000

2-17
Figure 2-3. Concentration-response curve of AMBD antagonism of taurine responses on DRT. Mean +/- SE, n = 2-9.
EFFECT OF AMBD ON TAURINE RESPONSES

on DRT (Rana pipiens)

Concentration (mM)

TAURINE

TAURINE + 0.25 mM AMBD
Figure 2-4. Concentration-response curve of AMDP antagonism of hypotaurine responses on DRT. Mean +/- SE, n = 2-4.
EFFECT OF AMBD ON HYPOTAURINE RESPONSES

on DRT (Rana pipiens)
Figure 2-5. Concentration-response curve of AMBD antagonism of GABA responses on DRT. Mean +/- SE, n = 2-6.
EFFECT OF AMBD ON GABA RESPONSES

on DE (Rana pipiens)

Ery depolarizing response vs Concentration (mM)

- GABA
- GABA + 0.25 mM AMBD
Figure 2-6. Effect of AMBD on responses of the isolated dorsal root ganglion (DRG) to amino acids. AMBD selectively blocks the responses to beta-alanine (BALA), hypotaurine and taurine without affecting those to GABA. DRG is not responsive to kojic amine, glycine or glutamate (not shown). Note the difference between taurine, hypotaurine, and beta-alanine responsiveness (all at different trace gains) in comparison with DRT (Figure 2-1). AMBD antagonism is completely reversible.
Figure 2-7. Concentration-response curve of AMBD antagonism of hypotaurine responses on DRG. Mean +/- SE, n = 2-4.
EFFECT OF AMBD ON HYPOTAUURINE RESPONSES
on DRG (Rana catesbeiana)

Concentration (mM)

- HYPOTAU
- HYPOTAU+0.25mM AMBD
Figure 2-8. Concentration-response curve of AMBD antagonism of taurine responses on DRG. Note small size of responses, particularly when compared with DRT. Mean +/- SE, n = 2-4.
EFFECT OF AMBD ON TAURINE RESPONSES
on DRG (Rana catesbeiana)

Concentration (mM)

- Taurine
- Taurine + 0.25 mM AMBD
Figure 2-9. Effect of AMBD on synaptic potentials: Dorsal root evoked ventral root potentials (DR-VRP); dorsal root evoked dorsal root potentials (DR-DRP) and ventral root evoked dorsal root potentials (VR-DRP). Sample records taken at chart speed marked by calibration bars (2 min); addition of AMBD is marked by arrows in slow-speed record traces in which the synaptic potentials (large regular vertical traces) are evoked every 1 min; irregular low level potentials represent spontaneous activity. Note a concentration dependent enlargement and prolongation of DR-VRP and DR-DRP and blockade of VR-DRP. Calibration bars in mV and minutes.
Figure 2-10. Summary of the effects of AMBD on synaptic potentials.
EFFECT OF AMBD ON SYNAPTIC POTENTIALS
in frog spinal cord (Rana pipiens)

Percent of control response

Concentration of AMBD (mM)

<table>
<thead>
<tr>
<th>DR-DRP</th>
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0.010 0.016 0.025 0.040 0.063 0.100 0.158 0.251 0.398 0.631 1.000
CHAPTER 3

DIFFERENTIAL EFFECT OF BARIUM ON RESPONSES OF FROG PRIMARY AFFERENTS TO NEUTRAL AMINO ACIDS

G.M. Mitsoglou and A.L. Padjen

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Abstract

The neutral amino acids, GABA and taurine, are implicated as mediators of primary afferent depolarization (PAD), the electrophysiological manifestation of presynaptic inhibition in vertebrate spinal cord. When applied onto the intramedullary terminals of dorsal roots (DRT) of isolated spinal cord or onto isolated dorsal root ganglia (DRG) of frog, they evoke prominent depolarizing responses. These responses could be produced as a result of activation of subsynaptic receptors directly on primary afferents or through an indirect increase in \([K]^o\) from neighboring cellular elements. The present studies were undertaken to examine the latter possibility using barium, a known blocker of \(K\) channels. The action of barium appeared to be consistent with this notion since it depolarized the resting membrane potential and blocked or reversed responses evoked by increased \([K]^o\) on both DRT and DRG. But at the same time barium enhanced responses to neutral amino acids on DRG and only depressed those on DRT. Taurine-evoked responses on DRT were more depressed than those evoked by GABA and regularly displayed partial reversal (i.e. hyperpolarizing components). These results therefore suggest that responses of DRT to GABA are not mediated substantially by indirect increases in \([K]^o\), although the same may not be true for taurine-evoked depolarizing responses.
Presynaptic inhibition in the vertebrate CNS was first discovered and is still best understood in the spinal cord (Frank & Fuortes, 1957; Eccles, 1964) but its precise mechanism remains unresolved. The electrophysiological manifestation of this regulatory phenomenon for synaptic transmission at the level of the first sensory synapse is presently believed to be depolarization of primary afferent fibres (PAD). Application of the neutral amino acids, GABA and taurine, leads to depolarization of the intraspinal terminals of primary afferent fibres entering the spinal cord as dorsal roots (DRT). Such responses are often considered as a model for this phenomenon (Levy, 1977).

Because of the abundance and close proximity of chemical synapses in the vicinity of DRT, depolarization of DRT by neutral amino acids can be achieved theoretically either directly or indirectly.

Direct effects could be mediated via interaction of these neutral amino acids with subsynaptic (or extrasynaptic) receptors localized on DRT. Although understandably inconclusive, a good body of evidence does support a role for GABA (Eccles et al., 1963; Levy, 1977; Nicoll & Alger, 1979) and, to some extent, taurine (Barker et al., 1975a,b; see chapter 2), as neurotransmitters mediating PAD. Such evidence includes immunohistochemical studies demonstrating GABAergic axoaxonic synapses (Hunt, 1983) but such evidence does not presently exist for taurinergic axoaxonic synapses.

Indirect effects could be mediated via diffusion of a by-product (i.e., K) of amino acid interactions with receptors or uptake sites on neighboring neuronal (e.g., interneurons, motoneurons) or non-neuronal (e.g., glial) cells to the surface of DRT. It is known that DRT are sensitive to changes in extracellular K concentration ([K]o) such that increases produce depolarization. The possibility that K is the major mediator of PAD, originally proposed by Barron & Matthews
(1938), gained renewed interest in recent years with the application of K-selective microelectrodes. Using this technology, most studies showed maximal increases in [K]₀ in those layers of the dorsal horn where PAD has been localized and primary afferent terminals concentrated (for review, see Sykova, 1983). In addition, an increase in [K]₀ (though apparently small) was detected upon application of GABA to isolated frog spinal cord, including dorsal horn (Kudo & Fukuda, 1976).

If GABA or taurine were to depolarize DRT indirectly as a result of diffusion of K near DRT, then it is assumed that a blocker of K conductance should be as effective at blocking such responses as those to applied K.

Neutral amino acids are also able to depolarize dorsal root ganglia (DRG) containing the primary afferent soma (De Groat, 1972; Nishi et al., 1974). Because of the inaccessibility of DRT, such responses are considered as a model for PAD occurring at DRT (Deschenes & Feltz, 1976; Akaike et al., 1985). If responses of DRG to the same neutral amino acids are mediated through similar ionic mechanisms, then a blocker of K conductance should have similar effects on such responses as those on DRT.

The purpose of this study was to examine the effect of a known K conductance blocker on the responses of different segments of primary afferents to neutral amino acids, particularly GABA and taurine, and to compare such effects with those on responses to increased [K]₀. Barium was chosen because of its ability to block responses to increased [K]₀, unlike other known K channel blockers such as TEA and 4-aminopyridine (Padjen & Smith, unpublished observations).

Preliminary results have been communicated (Mitsoglou & Padjen, 1985).
METHODS

Experiments were done on isolated hemisected spinal cord or dorsal root ganglia from leopard frog, Rana pipiens (both northern and southern varieties), kept in aquaria at 4 degrees celsius for periods of 1 week to several months (see Barker et al., 1975a; Padjen & Smith, 1983). Frogs were cooled to an anesthetic state by placing in crushed ice and decapitated before dorsal or ventral laminectomy was performed.

The spinal cord (or ganglia) with attached roots was carefully removed to a dissecting dish. Sagittally hemisected spinal cord or partially desheathed ganglia with attached 9th or 10th dorsal roots was then placed in the central compartment (bath volume 0.15 ml) of a sucrose gap chamber. Dorsal roots were led out by way of slits through the sucrose compartment into two separate pools of normal Ringer solution. All slits, as well as a closely fitted cover, were coated with Vaseline (USP) to provide a leak-proof separation of compartments.

The central and sucrose compartments were continuously perfused with unoxygenated Ringer (flow rate 1.5-2.0 ml/min) and 244 mM sucrose (0.5-1.0 ml/min) respectively. Temperature (10-12 degrees celsius) was controlled by means of a Peltier device. Normal Ringer solution (pH 7.2) contained the following (in mM): sodium chloride, 115; potassium chloride, 2; calcium chloride, 2; HEPES (pH 7.3), 5; D-glucose, 10. Tetrodotoxin (0.2 uM) was present in all solutions superfusing the spinal cord to minimize indirect drug responses. Drugs were applied by immediate interchange of solutions superfusing the central compartment via a set of stopcocks. Drug solutions were made by diluting appropriate amounts of concentrated stock solution (in water) in normal Ringer solution. All drugs were purchased from Sigma, St. Louis, MO., U.S.A., except for kojic amine which was a gift from Merck Frosst Laboratories, Kirkland, QC., Canada.
Electrical activity was recorded by monitoring the potential difference between two silver-silver chloride pellet electrodes making contact with solution in the central (ground) and root compartments respectively via salt bridges (4% agar Ringer). Permanent records were obtained from a rectilinear pen recorder (Brush model 2400). Signals were filtered (1 second time constant on amplifier and 5 Hz low-pass filter on polygraph) without distortion of drug responses.

Observed potential changes represent an attenuated version of the average changes in membrane potential of intraspinal axons or soma of a population of primary afferents. Under our recording conditions an upward deflection of the pen represents depolarization. Results are expressed as mean +/- standard error of the mean wherever possible.
RESULTS

Effect of barium on responses of DRT

Figure 3.1 illustrates a single experiment examining the effect of barium (5 mM) on responses of DRT to a variety of amino acids as well as potassium. It is evident that this divalent cation has a marked effect on the resting membrane potential, in this case causing a depolarization of 11 mV. At the same time, it depresses all amino acid-evoked responses but to varying degrees. At this concentration, it blocks and reverses the K-evoked response.

The effect of barium on resting membrane potential takes approximately 30 minutes to reach a steady state after which time further membrane depolarization is minimal. The effect of barium on responses to amino acids and potassium (applied after 30-minute barium incubation) also changes little (ie. within 10%) while in barium. All of these effects are slowly but completely reversible on removal of barium so that they cannot be explained in terms of acute toxicity.

Each of the effects of barium on DRT described above qualitatively was studied in a more quantitative manner, as described below.

Barium as a blocker of K conductance

Barium is a well-known blocker of some (maybe all) K currents in excitable tissues including axons (Krnjevic et al., 1971; Standen & Stanfield, 1978; Arhem, 1980). This characteristic is consistent with our results in two ways:

(1) Barium depolarizes the resting membrane potential of DRT in a concentration-dependent manner, as illustrated in figure 3.2. However, such an effect is quite variable between experiments: at 5 mM, the membrane response to barium ranges from 7-12 mV with mean of 9.54 +/- 0.45 mV; n=18).
(ii) barium depresses, also in a concentration-dependent manner, the electrical responses of DRT to short-duration changes in the K concentration of Ringer solution bathing the spinal cord. This is clear from figure 3.3 showing data from a typical experiment in which the effect of increasing barium concentration was tested against the response of DRT to a 4 mM increase in the Ringer K concentration (i.e., application of 6 mM K).

Since the blocking effect of barium may be dependent on the magnitude of [K]₀ (Standen & Stanfield, 1978; Arhem, 1980) or membrane potential (Standen & Stanfield, 1978), it was important to see the extent of blockade by barium on different concentrations of Ringer K. This point is examined in figure 3.3 showing data from a composite of experiments. It is evident that barium depresses the depolarizing responses of DRT evoked by application of 2.5-8 mM K (up to 9.5 mV depolarization), again in a concentration-dependent manner. In terms of percent of control response amplitude, the effectiveness of barium (0.5-5.0 mM) to block K-evoked responses changes very little (less than 10%) for concentrations of [K]₀ between 4 and 8 mM (3.5 to 9.5 mV depolarization). A concentration of 5 mM is sufficient for total blockade of the K-evoked responses (figures 3.2 and 3.3): in fact, barium consistently reverses the depolarization evoked by increases in [K]₀ (4-8 mM) into hyperpolarization. It also reverses the hyperpolarization induced by K-free Ringer into depolarization.

Effect of barium on responses of DRT to neutral amino acids

It is assumed that if neutral amino acids produce their electrical effects on DRT through indirect increases in [K]₀, then such ionic changes should be equally affected by barium as are experimentally induced increases in [K]₀ (see above).

Because the effect of barium on both K and amino acid-evoked responses of DRT was qualitatively the same (i.e.
depression), it was necessary to compare the interaction between barium and these responses on a more quantitative level. In addition, it was also of interest to look at the effect of barium on responses caused by various concentrations of amino acids. For these quantitative studies, only the responses to two proposed chemical mediators of PAD, GABA and taurine, were analysed.

The same concentration of barium (ie. 5 mM) which totally blocked and reversed responses of DRT to a range of K concentrations, depressed (but never fully blocked) the responses to a range of concentrations (0.1-30 mM) of GABA and taurine (figure 3.4). This effect, as with the K-evoked responses, was concentration-dependent such that less barium (ie. 0.5 mM) produced less depression of amino acid-evoked responses (not illustrated). Barium reduced the maximal response of DRT to GABA and taurine without much change in their EC50. However, the responses of DRT to taurine showed greater depression in the presence of 5 mM barium than those to GABA. In fact, these responses often became multiphasic in barium with the emergence of hyperpolarizing components, as shown in the records below the graph. The appearance of a hyperpolarizing component at higher barium concentrations, although regularly seen with taurine-evoked responses was only occasionally seen with GABA-evoked responses (ie. about 10% of responses).

The range of amplitudes of responses to neutral amino acid application (up to 10.5 mV depolarization) was also similar to that tested with K-evoked responses (cf. figure 3.3). The effectiveness of barium (5 mM) depression, as with the K-evoked responses, changed little (less than 10% for taurine-evoked responses and less than 20% for GABA-evoked responses) with neutral amino acid concentrations greater than 1 mM.

The effect of increasing barium concentration on single responses, of similar amplitude, to increased [K]0 and neutral amino acids proved to be more useful in quantitating differences. Figure 3.5 shows clearly the order of
sensitivity of responses to depression by barium (5 mM) to be: K > taurine > GABA.

Effect of barium on responses of DRG.

Since neutral amino acids and increased [K]o also depolarize DRG, where there appear to be no known synapses or other neuronal elements except somata of primary afferents, it was of interest to compare the effect of barium on responses on this site with its effect on responses of DRT to the same agents. A typical experiment examining the effect of barium on responses of DRG to a variety of neutral amino acids as well as increased [K]o is presented in figure 3.6.

Qualitatively, the effect of barium on DRG was consistent with that of a K conductance blocker, as it was on DRT. But quantitatively, the degree to which barium depolarized the resting membrane potential was much less pronounced on DRG (cf. figures 3.1 and 3.6). On DRG, barium (5 mM) depolarized the membrane potential by 2 mV (+/- 0.15 mV, n=6). With higher barium concentrations (upto 10 mM), no further membrane depolarization was observed (data not shown). As on DRT, the K-evoked response was depressed on DRG, but not nearly to the same degree. On DRG, this response could never be fully blocked with concentrations of barium up to 10 mM.

What is most surprising from figure 3.6 is that in contrast to the results obtained on DRT, the responses of DRG to the same neutral amino acids were markedly increased in the presence of 5 mM barium.

A typical experiment illustrating quantitative differences between the effect of increasing concentrations of barium on single DRG responses to 3 depolarizing agents (GABA, taurine, K) is presented in figure 3.7 (cf. figure 3.5). It is again evident that responses of DRG to neutral amino acids are increased by barium in a concentration-dependent manner, unlike the response to 6 mM K, which is reduced; in this case, to 17% of control (mean 24% +/- 3%,
In summary, neutral amino acid-evoked responses on DRG are consistently increased in the presence of barium while K-evoked responses are consistently depressed (6 experiments, 12 ganglia).

Comparison of sensitivity of primary afferents to GABA and taurine at two separate sites.

If the sensitivity of primary afferents to neutral amino acids were to be limited predominantly to intramedullary segments, then it would be more reason to suspect that such sensitivity is due predominantly to indirect action on primary afferents. A comparison of the responsiveness of primary afferents to GABA and taurine on DRT (figure 3.4A) and DRG (figure 3.8) shows quantitative differences in response amplitudes.

Although of similar potency to GABA on DRT (E.C. 50 of 3 mM), taurine is about 500 times less potent than GABA on DRG (EC50 of 10 mM). This rank order of potency is in agreement with previous observations in bullfrog DRG (Nishi et al., 1974; Paden et al., 1984). The figure also demonstrates that the maximal response of both neutral amino acids is lower on DRG than on DRT with the difference being more dramatic for taurine.

Thus, the sensitivity of primary afferents to taurine is far more limited to intraspinal segments than is that to GABA.
DISCUSSION

We have shown that barium has a differential effect on neutral amino acid-evoked responses at two sites on primary afferents, DRG and DRT, even though it depresses or blocks depolarizing responses to externally applied K at both sites. Although the responses of DRG to neutral amino acids are augmented in the presence of barium, responses on DRT to the same agonists are depressed, but always significantly less than K-evoked responses. Because responses of DRT to taurine are more sensitive to barium depression than are those to GABA, it is suggested that a greater component of the taurine-evoked response may be mediated indirectly through an increase in [K]o near DRT. The following is a detailed discussion of several points necessary for interpreting these results.

Neutral amino acid responses on DRT versus DRG: differential effect of barium.

The differential effect of barium on neutral amino acid-evoked responses on DRG and DRT suggests that the responses at these two primary afferent sites involve different ionic components. From the abundance of evidence currently available, it appears that the responses on DRG are mediated predominantly through an increase in chloride conductance (Nishi et al., 1974; Gallagher et al., 1978; Hattori et al., 1984; Akaike et al., 1985). Because of technical limitations due to the size and inaccessibility of DRT, the ionic mechanism of neutral amino acid effects at this site remains unresolved. Even though some studies using sucrose gap technique have suggested that these responses are dependent on extracellular sodium (Padjen et al., 1973; Barker & Nicoll, 1973) and possibly, in part, on calcium (Padjen & Smith, 1981), it is generally believed that they are mediated predominantly by chloride efflux (Krnjevic, 1974), as on DRG.
It therefore seems more likely that the same receptor-ionophore complexes exist at both primary afferent sites, but that the DRT responses involve indirect (ionic) components that may complement any possible direct depolarizing effects of neutral amino acids mediated via axoaxonic synapses.

Both the barium and sodium sensitivity of DRT responses to neutral amino acids can therefore be explained if it is assumed that the indirect ionic component were due to K released as a result of uptake processes for neutral amino acids (Krnjevic, 1974), which are known to exist in glial (Gottesfeld et al., 1973; Brown, 1979) and neuronal (Iversen & Johnston, 1971) membranes of the vertebrate central nervous system, including frog spinal cord (Davidoff & Adair, 1975). Greater uptake of GABA inside the spinal cord would also explain the lower EC50 of GABA effects on DRT compared to DRG. Site specific (i.e., glial versus neuronal) blockers of neutral amino acid uptake that do not produce electrical effects of their own on DRT would be very useful in further testing this hypothesis but none so far are known to meet these specifications.

Pharmacological studies however, have provided data inconsistent with such a possibility. It was shown (Barker et al., 1975a) that taurine-evoked (1mM) responses on DRT could be completely blocked by picrotoxin, bicuculline or strychnine even though all three antagonists seem to be relatively ineffective blockers of the amino acid uptake process (Iversen & Johnston, 1971; Collins, 1974) at concentrations that depress such responses. However, the depolarizing response evoked by GABA (1mM) was antagonized by up to 30% of control in picrotoxin (and even less so in bicuculline) so that up to 30% of this response may be indirect. A blocking effect of these three convulsants on k conductance is unlikely at the concentrations used to block amino acid-evoked responses (Barker et al., 1975b; Osorio et al., 1979; Nicoll, 1979; Shefner & Levy, 1981).

Such pharmacological data does not exclude indirect effects of neutral amino acids on DRT as a consequence of
interactions with receptors on interneurons (Curtis, 1979) or motoneurons (Nicoll et al., 1976; Shapovalov et al., 1983). In addition to K efflux associated with spread of chloride-mediated synaptic effects on such neighboring neurons, another possible source of increased [K]o from these structures could come from interaction with the novel GABA-B receptors which may be activated by both GABA and taurine (see chapter 4) and which may exist in higher concentrations there. Such receptors are presently thought to be linked to activation of K conductance (Newberry & Nicoll, 1985; Inoue et al., 1985; see chapter 4).

Quantitative differences between the effect of barium on GABA and taurine responses

Barker and co-workers (1975a,b) observed that from 40% to 70% of PAD recorded at the level of the dorsal root (i.e. DRP), after electrical stimulation of an adjacent dorsal root (DR-DRP), was picrotoxin-resistant, as was up to 30% of DRP evoked by GABA (1 mM) application (as mentioned above). These percentages are quantitatively similar to the maximal reduction of the GABA-induced depolarization to 40% of control in the presence of barium (see figure 3.5). All these data, as well as the receptor distribution studies (figures 3.4A & 3.8), suggest that the response of DRT to GABA, although possibly involving a minor-K component, is not mediated substantially through an indirect increase in [K]o. This is consistent with the hypothesis that GABA is a neurotransmitter released from axoaxonic synapses, as originally proposed by Eccles (1964).

Unlike GABA, taurine is much less potent in depolarizing DRG than DRT. Such data, in addition to other pharmacological evidence (see chapter 2), suggest that the two neutral amino acids act at separate receptors, so that their responses should be assessed separately. The possibility therefore exists that densities of receptors and/or coupled ionophores
may be higher for taurine (as for GABA) on DRT than on DRG (cf. maximal responses, figures 3.4A & 3.8). Although both GABA and taurine responses on DRT are depressed by barium, our quantitative studies show that taurine is much more so, despite observations that its receptor appears to be also linked to chloride ionophores (Nishi et al., 1974; Akaike et al., 1985). This suggests that a greater component of its electrical effects on DRT may be indirect. Further evidence may be taken as consistent with such a hypothesis: (i) responses of both DRG and DRT to taurine show similar time course and appearance to responses of the same tissues to increased \([K]_o\) (see figures 3.1 and 3.6) (ii) responses of DRT to taurine often exhibit hyperpolarizing components in the presence of barium (unlike GABA-evoked responses), as is seen with responses to increased \([K]_o\) (iii) taurine-evoked responses are antagonized by 4-aminopyridine, a blocker of voltage-dependent \(K\) conductance (Dubois, 1983; see chapter 4). All these data are consistent with the possibility that responses of DRT to taurine are mediated substantially through indirect increases in \([K]_o\). Use of \(K\)-selective microelectrodes would be of further help in substantiating this claim.

**Effect of barium on \(K\) responses**

Depolarizing responses of neurons to extracellularly applied \(K\) are thought to be mediated, at least initially, by influx of \(K\) ions through channels open at the resting membrane potential. According to Dubois (1981), slow \(K\) leakage and resting sodium currents are open at rest. Since 5 mM TEA (which blocks most \(K\) currents (Hille, 1967) including slow \(K\) conductance (Dubois, 1983) in frog axons) does little to our \(K\)-induced depolarizing responses, it can be assumed that these responses are mediated, at least initially, through leakage channels. Furthermore, Hille (1967) states that the leakage current accounts for most of the conductance of the resting
frog nerve. Such leakage conductance is mostly carried by K ions (Hille, 1973; Arhem, 1980) and is very difficult to block pharmacologically. Although barium has been shown to depress other K currents, it is also capable of depressing the leakage conductance when externally-applied onto amphibian axons (Arhem, 1980).

Unlike its differential effects on the responses of DRT and DRG to neutral amino acids, barium had qualitatively the same (i.e. depressing) effect on the depolarizing responses of the two sites to increased \([K]_o\). However, there were quantitative differences. Such responses on DRT could not only be blocked but even reversed by barium but on DRG they were greatly depressed but never reversed. It is then possible that there is either a lower density of barium-sensitive ionophores on DRG (and hence less resting K conductance) or that the resting K ionophores on DRG are less sensitive to blockade by externally applied barium. The fact that K-evoked responses are less potent on DRG relative to DRT (cf. figures 3.3 & 3.6) favors the former possibility (see below). The alternate possibility that barium is less accessible to the neuronal membrane of DRG than of DRT is unlikely since GABA, which would be expected to have greater diffusional barriers because of its greater size and inactivation pathways (e.g. uptake), is more potent on DRG than on DRT.

The observation of a potassium response polarity reversal in the presence of higher concentrations of barium could be explained by competition of the two ions for a common binding site (Standen & Stanfield, 1978; Armstrong & Taylor, 1980; Arhem, 1980). Displacement of barium by a transient increase in \([K]_o\) (i.e. during the K-evoked response) could reverse channel blockade and therefore the depolarization of the membrane potential. This effect may not be quantitative since responses evoked by increasing \([K]_o\) do not cause greater polarity reversal in the presence of 5 mM barium (c.f. figure 3.3).
Additional factors affecting responses

More precise quantification of the exact contribution of direct and indirect components of neutral amino acid effects on primary afferents is not possible because of factors (eg. changes in membrane polarization level and resistance; electrical synapses) discussed in detail below.

I. Effect of barium on resting membrane potential

Resting membrane currents are thought to be mostly responsible for the magnitude of the resting membrane potential of a particular neuron. As mentioned above, the predominantly K-mediated leakage current accounts for most of the resting conductance of frog nerve. A blocker of resting K conductance (through leakage or slow K channels) should theoretically depolarize the membrane potential of a neuron to an extent dependent on resting sodium permeability relative to that of K.

The fact that the depolarizing effect of barium on resting membrane potential was five times smaller on DRG than on DRT could mean that there is a differential distribution of resting sodium and K channels between DRG and DRT (as suggested above) - ie. since barium produces greater depolarization of DRT, this site may have a greater density of resting sodium channels relative to resting K channels. Such differential channel distribution however would mean that different membrane segments of the same neuron would normally have different resting membrane potentials. This would seem unlikely were it not for the length of sensory neurons and the significant distances between soma and terminals. In fact, intracellular studies have shown that frog DRG may have a more positive resting membrane potential than frog DRT (c.f. Nishi
et al., 1974 and Padjen & Hashiguchi, 1983).

No matter what the cause of the differential effect on resting membrane potential, it is reasonable to conclude that depolarizing responses on DRT would be more likely to be depressed than those on DRG due to the greater barium-induced membrane depolarization. The use of voltage clamp technique would therefore be advantageous to quantitate the contribution of membrane depolarization itself on neutral amino acid responses. This has proven too difficult to do on DRT because of physical limitations. Two groups, recording intracellularly from frog DRG (Nishi et al., 1974) and dorsal root axons (Padmin & Hashiguchi, 1982) have shown the equilibrium potential of GABA-induced depolarization to be about -30 mV. If such values are the same throughout the neuron, then a 10 mV depolarization of DRT (with resting membrane potential of -75 mV) on its own would not theoretically account for much of the depression of the response to GABA in the presence of 5 mM barium (see figure 3.2 of Akaike et al., 1985). Furthermore, using K as depolarizing agent in bullfrog spinal cord, Shefner & Levy (1980) found the depolarization of DRT by 3 mM GABA to be reduced by less than 20% with [K]o equal to or less than 10 mM (which causes a depolarization of magnitude similar to that produced by 5 mM barium under our conditions). It should be stressed however that membrane depolarization induced by K and barium cannot be compared interchangeably because of differing mechanisms, especially since the depolarizing responses may be mediated (at least partially) by K influx.

II. Effect of barium on membrane resistance

Blockade of the resting K conductance leads to an increase in total membrane resistance (Krnjevic et al., 1971) which would in turn cause a greater voltage deflection for the same current passing through a membrane (eg. as a result of receptor-activated ionophore opening). This effect could
explain, at least partially, the increased amplitudes of neutral amino acid responses on DRG after potassium channel blockade by barium. The reason why the DRT responses were not similarly increased is not clear. At least two possibilities should be considered: (i) it is possible that a concomitant blockade by barium of an indirect potassium component of amino acid responses on DRT masks this barium effect. However, the method and the approach used does not allow a more precise quantitative evaluation of this contribution (ii) barium directly blocks the ionic mechanism underlying amino acid responses on DRT. This would then suggest a difference between the electrogenic mechanism involved in amino acid responses on DRT vs. DRG.

III. Contribution of electrical synapses

It has been suggested so far that the responses to neutral amino acids during barium incubation may represent predominantly direct actions of these agonists on DRT. There is good evidence on frog spinal cord however suggesting the existence of electrical synapses (gap junctions) between DRT and motoneurons (Grinnell, 1970; Shapovalov et al., 1978; Alvarez-Leefmans et al., 1979). Barium should be ineffective at blocking such synapses. What is seen in barium with the neutral amino acids may therefore still be indirect since these agonists can produce electrical effects on motoneurons. But because such effects are predominantly hyperpolarizations (Nicoll et al., 1976), it is doubtful that they contribute substantially to the depolarizing responses on DRT. The emergence of hyperpolarizing components in these responses during barium incubation may, on the other hand, be due to such effects, especially since the neutral amino acid-induced hyperpolarizing responses on motoneurons are enhanced by barium (Padjen & Smith, 1981).
Physiological significance

Almost since its discovery, the K-sensitive microelectrode has been extensively used in the vertebrate spinal cord to study the contribution of changes in $[K]_o$ in PAD (Sykova, 1983). Most of these studies down-played any major role of this ion in producing PAD evoked by single afferent volleys or short application of GABA while emphasizing the contribution of GABAergic axoaxonic synapses under such conditions.

A number of groups (Krnjevic & Morris, 1975; Barker et al., 1975b; Nicoll, 1979) have however suggested that PAD is made up of both components. Each phase could then contribute towards the observed PAD to varying degrees depending upon neuronal conditions (e.g. activity). Nicoll (1979) reports that 10% of the amplitude of DRP set up by single afferent volleys may be due to changes in $[K]_o$ while this percentage could rise to 80-90% (most of which is dependent on synaptic transmission) during high-frequency sustained neuronal activity. Such high frequency sustained neuronal activity has been observed in frog dorsal horn in response to stimulation of high threshold nociceptive afferents (Czech et al., 1981) so that such conditions may not be all that non-physiological. The large, sustained PAD seen under these conditions would logically be expected to be due to mechanisms other than GABAergic axoaxonic receptors because (i) such receptors (responses) are known to quickly desensitize (Hackman et al., 1982) (ii) responses would be depressed due to a decreased electrochemical gradient for ions (iii) Krnjevic & Morris (1981), working in hippocampus, have suggested that an increase in $[K]_o$ produced by tetanic stimulation may itself cause an apparent desensitization to GABA.

Consequently, it is difficult to determine what physiological conditions are most relevant to studies such as ours where amino acids are experimentally applied over the whole intraspinal length of primary afferent fibres for unphysiological time frames (two to six minutes in our
experiments). This type of application may be inducing changes in \([K]_0\) that would not occur via very localized release of neurotransmitters onto subsynaptic membrane. Alternatively, it is possible that our experimental conditions may resemble most a physiological condition in between the two extremes of neuronal activity mentioned above.
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Figure 3.1 Effect of barium on responses of DRT to amino acids and K. Abbreviations: GABA - gamma-aminobutyric acid; GLU - glutamate; KJA - kojic amine; HT - hypotaurine; 6K - 6 mM potassium; BALA - beta-alanine; TAU - taurine; arrow indicates start of perfusion with barium (Ba). On this and other figures: drug application is marked by a horizontal bar; depolarization is upwards; calibrations - mV (vertical), minutes (horizontal). Note that the 6K-evoked response is reversed while all amino acid-evoked responses are depressed in Ba.
Figure 3.2 Concentration-dependent effect of barium on membrane potential and K-evoked responses on DRT. RMP - resting membrane potential. Results from a single experiment. Note pronounced depolarizing effect of barium on resting membrane potential. Note also complete blockade and reversal of K-evoked response in presence of 5 mM Ba.
EFFECT OF BARIUM ON RMP & K-RESPONSE
ON DRT (Rana pipiens)

my response

Concentration of Barium (mM)

RMP  5 mM K

0  2  4  6  8  10
Figure 3.3 Effect of barium on concentration-response curve of K-evoked responses on DRT. Note reversal of all potassium-evoked responses at 5 mM Ba. (Mean +/- S.E., n = 2-22; where bars are not shown, S.E. is within symbol).
EFFECT OF BARIUM ON POTASSIUM (K)

CONC-RESPONSE ON DRT (R. pipiens)

Concentration of K (mM)

- K
- K + 0.5mM Ba
- K + 5.0mM Ba
Figure 3.4 Effect of barium on concentration-response curves of GABA and taurine-evoked responses on DRT.

A. Graph shows taurine-evoked responses to be more depressed than those evoked by application of GABA.

B. Responses to taurine regularly exhibit a hyperpolarizing component (unlike GABA) in presence of 5 mM Ba. Only depolarizing component is plotted in A. (mean +/- S.E., n = 2-65; difference between the 2 test curves is significant at 5% level, using student t-test, only with 1.0 mM agonist concentration).
EFFECT OF BARIUM ON GABA AND TAURINE

CONC-RESPONSE ON DRT (R. pipiens)

A

\[ \text{my depolarizing response} \]

\[ \text{Concentration (mM)} \]

\[ \begin{align*}
\square \text{ GABA} & \\
\text{TAURINE} & \\
\text{GABA + 5mM Ba} & \\
\text{TAURINE + 5mM Ba} & 
\end{align*} \]

B

Control

1 5 15

L

\[ 5\text{mM Ba} \]

1 15
Figure 3.5 Concentration-dependent depression by barium of DRT responses to GABA, taurine and K. Note that K-evoked responses are more sensitive to Ba than taurine-evoked (depolarizing) responses. GABA-evoked responses are the least affected by barium. (mean ± S.E., n = 2–10; all three curves are significantly different, using student t-test, at 5% level in presence of 5 mM barium).
EFFECT OF BARIUM ON RESPONSES ON DRT (R. pipien)

Concentration of Barium (mM)

- □ 1 mM GABA
- ○ 1 mM TAURINE
- ▼ 4 mM K

MN response (% of control)

0 2 4

0 10 20 30 40 50 60 70 80 90 100

-10 -5 0 5 10
Figure 3.6 Effect of barium on responses of DRG to amino acids and K. Note that the Ba-evoked depolarization of the resting membrane potential is much smaller than on DRT (cf. figure 1). The 4K-evoked response is severely depressed but amino acid-evoked responses are all enhanced.
Figure 3.7 Concentration-dependent effect of barium on responses of DRG to GABA, taurine and K. Results from a single experiment. Note that the K-evoked response is almost completely blocked while responses to GABA and taurine are greatly enhanced.
EFFECT OF BARIUM ON RESPONSES
ON DRG (R. pipiens)

[Graph showing the effect of barium concentration on depolarizing response (% of control) for different substances: 0.05 mM GABA, 10 mM Taurine, and 6 mM K.]

- 0.05 mM GABA
- 10 mM Taurine
- 6 mM K

Concentration of Barium (mM)
Figure 3.8 Concentration-response curves of GABA and taurine-evoked responses on DRG. Note that GABA is more potent on DRG than on DRT with the reverse being true for taurine, but maximal response of both amino acids is less than on DRT (cf. Figure 3-4). (mean +/- S.E., n = 2-26).
CONCENTRATION—RESPONSE GABA AND TAURINE
ON DRG (R. pipiens)

Mv depolarizing response

Concentration (mM)

0 0.01 0.1 1 10 100

GABA TAURINE
CHAPTER 4

SOME CHARACTERISTICS OF BACLOFEN-EVOKED RESPONSES

ON PRIMARY AFFERENTS OF FROG

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Abstract

GABA is recognized as a neurotransmitter mediating presynaptic inhibition on the first sensory synapse in vertebrate spinal cord. It was previously reported that responses of intraspinal terminals of dorsal roots (DRT) to GABA and its analogues (e.g., kojic amine) in isolated frog spinal cord are dual in nature, consisting of both a bicuculline-sensitive depolarizing component (GABA-D) and a bicuculline-resistant hyperpolarizing component (GABA-H). Baclofen has been shown to be a selective agonist at a subclass of GABA receptor (GABA-B) in many regions of the vertebrate nervous system. Since it evokes a pure hyperpolarizing response on DRT, we have examined some characteristics of these responses under the working hypothesis that GABA-H responses are mediated by activation of GABA-B receptors. We have found that baclofen-evoked responses on DRT are stereospecific, dependent on [K]o, blocked by barium and unaffected by 4-AP, inorganic calcium channel blockers (manganese, cobalt, cadmium) or proposed GABA-B antagonists (delta-aminovaleric acid, delta-aminoisovalulinic acid). These results are consistent with the idea that baclofen-evoked responses on DRT are mediated by an increase in conductance to K ions.
INTRODUCTION

GABA is recognized as being one of the most widely used inhibitory neurotransmitters in the vertebrate CNS (Krnjevic, 1974), including presynaptic inhibition on the first sensory synapse in spinal cord (Eccles, 1964; Levy, 1977). In the last few years, convincing data has surfaced in support of GABA receptor multiplicity in the vertebrate CNS (Bowery et al., 1984). Two of these binding sites have been designated by Bowery and colleagues as GABA-A and GABA-B. Presently, the chief distinction between the two receptors is that the former are sensitive to the antagonistic action of bicuculline while the latter are bicuculline-resistant and selectively activated by baclofen, a chlorophenyl derivative of GABA. Baclofen is also capable of depressing neurotransmission across the first sensory synapse by a mechanism involving a presynaptic site (Davidoff & Sears, 1974). Consequently, it is possible that GABA can produce presynaptic inhibition through more than one mechanism.

On DRT of isolated frog spinal cord, responses to GABA as well as other amino acids (e.g. kojic amine, taurine) are dual in nature, consisting of both a depolarizing component (GABA-D) and a hyperpolarizing component (GABA-H), which becomes more evident at low agonist concentrations or in the presence of bicuculline (Bourne & Padjen, 1980; Padjen & Hashiguchi, 1982; Padjen & Hashiguchi, unpublished observations). Responses to baclofen, on the other hand, consist of a pure hyperpolarization under all conditions.

Under the working hypothesis that all the hyperpolarizing effects are mediated by the same receptor (GABA-B?), we have chosen to further characterize responses of DRT to baclofen because of its apparent selectivity for these effects. This was done in two ways. The first was to see if such responses had ionic requirements consistent with those of GABA-B responses found elsewhere in the vertebrate nervous system. The second approach was by testing the capability of
proposed GABA-B antagonists to block these responses.

Preliminary results have been communicated (Mitsoglou & Padjen, 1985).
METHODS

Experiments were done on isolated hemisected spinal cord from leopard frog, Rana pipiens (both northern and southern varieties), kept in aquaria at 4 degrees celsius for periods of 1 week to several months (cf. Barker et al., 1975a; Padjen & Smith, 1983). Frogs were cooled to an anesthetic state by placing in crushed ice and decapitated before dorsal or ventral laminectomy was performed. The spinal cord with attached roots was carefully removed to a dissecting dish. Sagittally hemisected spinal cord with attached 9th or 10th dorsal roots was then placed in the central compartment (bath volume 0.15 ml) of a sucrose gap chamber. Dorsal roots were led out by way of slits through the sucrose compartment into two separate pools of normal Ringer solution. All slits, as well as a closely fitted cover, were coated with Vaseline (USP) to provide a leak-proof separation of compartments.

The central and sucrose compartments were continuously perfused with unoxygenated Ringer (flow rate 1.5-2.0 ml/min) and 244 mM sucrose (0.5-1.0 ml/min) respectively. Temperature (10-12 degrees celsius) was controlled by means of a Peltier device. Normal Ringer solution (pH 7.2) contained the following (in mM): sodium chloride, 115; potassium chloride, 2; calcium chloride, 2; HEPES (pH 7.3), 5; D-glucose, 10. Tetrodotoxin (0.2 uM) was present in all solutions superfusing the spinal cord to minimize indirect drug responses. Drugs were applied by immediate interchange of solutions superfusing the central compartment via a set of stopcocks. Drug solutions were made by diluting appropriate amounts of concentrated stock solution (in water) in normal Ringer solution. All drugs were purchased from Sigma, St. Louis, MO., U.S.A. except for kojic amine (gift from Merck Frosst Laboratories, Kirkland, QC., Canada) and baclofen (gift from Ciba-Geigy).

Electrical activity was recorded by monitoring the potential difference between two silver-silver chloride pellet
electrodes making contact with solution in the central (ground) and root compartments respectively via salt bridges (4% agar Ringer). Permanent records were obtained from a rectilinear pen recorder (Brush model 2400). Signals were filtered (1 second time constant on amplifier and 5 Hz low-pass filter on polygraph) without distortion of drug responses.

Observed potential changes represent an attenuated version of the average changes in membrane potential of intraspinal axons of a population of primary afferents. Under our recording conditions an upward deflection of the pen represents depolarization. Results are expressed as mean +/- standard error of the mean wherever possible.
RESULTS

Differential sensitivity of DRT to GABA and baclofen.

Sucrose gap recording from DRT (figures 4.2-4.9) reveals that baclofen consistently produces a concentration-dependent hyperpolarizing response, unlike its chemical analogue (ie. GABA). Such responses are characteristically slow to equilibrate (time to peak about 4 minutes) and very slow to recover, especially at higher concentrations (up to 1.5 hours at 0.1 mM). At concentrations greater than 0.1 mM, they often never fully recover, even after several hours of wash out. Furthermore, DRT from different segments of the spinal cord are differentially sensitive to baclofen and GABA (not illustrated). Baclofen-evoked responses have greater amplitudes on DRT of segment IX (DRT9) than on DRT of segment X (DRT10) for the same agonist concentrations, with the reverse being true for GABA.

Stereospecificity of baclofen-evoked responses

Unlike GABA, baclofen exists in either the dextrarotatory (D-) or levorotatory (L-) structural configurations. Figure 4.1, displaying concentration-responses curves for the two baclofen stereoisomers on DRT9, illustrates, in quantitative fashion, that baclofen responses are stereospecific. By comparing ED50's, it can be seen that the L isomer is about 100 times more potent than the D (see figure 4.3 for typical recordings). It is also clear from this figure that the maximal absolute response amplitude of DRT to baclofen is relatively small (mean of 1.25 mV hyperpolarization at 1mM) being about 10 times less than that of GABA (mean of 10 mV depolarization at 40mM on DRT9; see chapter 3). Baclofen is however about 100 times more potent than GABA in producing electrical effects on DRT, with an EC50 of 0.01 mM.
Sensitivity to changes in [K]o

As mentioned above, responses of DRT to neutral amino acids exhibit a hyperpolarizing component (GABA-H) under certain conditions, such as at low agonist concentrations and in the presence of bicuculline (e.g. see kojic amine response in figures 4.2 and 4.9) (Bourne & Padjen, 1980). However, even when duality is not evident under these conditions, lowering [K]o can bring out such a component, as exemplified by the GABA and taurine-evoked responses in figure 4.2. Also illustrated is the observation that hyperpolarizing responses already visible in the presence of bicuculline (i.e. kojic amine and baclofen), are increased when [K]o is decreased (see also figure 4.5). This point is further demonstrated in a typical experiment shown on figure 4.3 whereby [K]o is changed in both directions. Lowering [K]o causes an increase in the amplitude of the baclofen-evoked response and reveals a hyperpolarizing component of the GABA-evoked response. Baclofen response potentiation depends on the extent of [K]o reduction: in 0.5 mM K, these responses increase to 120% (n = 2) of control (figure 4.3) while in 0.1 mM K, the amplitudes approximately double (215% +/− 25%; n = 6; figures 4.2 & 4.5). Parallel increases in the slope of the responses is also evident, indicating an effect on ionic gradient. In contrast, raising the level of [K]o depresses the baclofen-evoked response in a totally reversible manner (to 15% in 4 mM K; n = 2; figure 4.3).

Effect of Barium

Barium is known to block a variety of K currents (Krnjevic et al., 1971; Standen & Stanfield, 1978, Arhem, 1980). Two concentration-dependent effects which are indicative of this channel-blocking ability are its prominent,
depolarizing effect on the resting membrane potential of DRT as well as its blockade and/or reversal of K-evoked depolarizing responses (Figures 4.4 & 4.5). A typical experiment testing the effects of this divalent cation (0.5 mM) on responses of DRT to a set of agonists and K is displayed in Figure 4.4. Responses to all amino acids (i.e. GABA, taurine, kojic amine, baclofen, glutamate) are depressed to varying degrees with baclofen exhibiting the greatest sensitivity (amplitude 27% of control; mean 37% +/- 7%, n = 4). The taurine-evoked response also shows considerable depression with the emergence of a hyperpolarizing component (see chapter 3). Figure 4.5 further demonstrates that making conditions more unfavorable for K conductance by increasing barium concentration in low [K]o (reflected in a much greater baseline depolarization; see chapter 3) produces total blockade of baclofen-evoked responses while that to GABA is only depressed.

Effect of 4-aminopyridine (4-AP)

4-AP is another well-known blocker of voltage-dependent potassium conductance (Ulbricht & Wagner, 1976; Dubois, 1983). On frog DRT (Figure 4.6), this agent proved to be totally ineffective at depressing baclofen-evoked responses at concentrations of 2-5 mM (n = 4). It did however cause a small depolarization of the resting membrane potential (1.43 +/- 0.50 mV at 5 mM, n = 4) and increased the amplitudes of GABA (160% of control +/- 10%, n = 4) and K (not shown; Padjen & Smith, in press) evoked responses. On the other hand, taurine-evoked responses were depressed (42% of control +/- 7%, n = 4; not shown).

Effect of inorganic calcium channel blockers

There were three main reasons for testing the effects of
agents known to block calcium conductance (Miledi, 1966; Adams, 1982): (i) if baclofen-evoked responses are directly mediated by an increase in K conductance, then the channels involved may be calcium-dependent. (ii) Responses of DRT to baclofen may be due to transient inhibition of the chronic release of a depolarizing substance onto DRT, even in the presence of tetrodotoxin. (iii) Baclofen effects have been explained as being due to a direct inactivation of voltage-dependent calcium current (Dunlap & Fischbach, 1978; 1981).

The three inorganic calcium channel blockers applied were manganese (2 mM; n = 2), cobalt (2-10 mM; n = 6), and cadmium (0.5 mM; n = 2). None of these agents had any significant effects on baclofen-evoked responses, as shown in figure 4.7. They did however have effects on resting membrane potential and on GABA-evoked responses, as previously reported (Padjen & Smith, 1981). Both manganese and cobalt depolarized the membrane potential and augmented GABA-evoked responses. Cadmium caused a small hyperpolarization of DRT.

Effects of proposed GABA-B antagonists

Two agents proposed to antagonize GABA-B effects were applied to test for any antagonism of baclofen-evoked responses on DRT (figures 4.8 and 4.9). These were delta-aminovalerl acid (Muhyaddin et al., 1982) and delta-aminolaevulinic acid (Brennan & Cantrill, 1979). Delta-aminovalerl acid produced a dual effect on the resting membrane potential at a concentration of 1 mM but was without significant effect at reducing the amplitude of baclofen-evoked responses (n = 4; figure 4.8). It nevertheless did depress the responses of DRT to 0.5 mM GABA (54% of control +/- 4%, n = 4). Delta-aminolaevulinic acid (1 mM) was also without significant effect on responses to baclofen (n = 2) but differed from the former agent since it did not cause any detectable changes in resting membrane potential of DRT nor in the depolarizing responses to GABA or taurine (figure 4.9).
DISCUSSION

On the basis of a number of physiological and binding studies, a separate site of GABA action has been described (GABA-B, Bowery et al. 1984) on presynaptic terminals in both peripheral and central nervous system of vertebrates. Although CNS terminals are rarely accessible for direct studies, hyperpolarizing responses to GABA, taurine and analogs on DRT of spinal cord have been described (GABA-H, Bourne & Padjen, 1980) with many attributes of a GABA-B site. In agreement with some binding studies (Price et al., 1984), the present study describes some characteristics of these responses, using baclofen as the most selective agonist. The results demonstrate that baclofen-evoked responses are: (i) tetrodotoxin and manganese resistant, and therefore evoked directly on DRT; (ii) stereospecific; (iii) dependent on [K]o; (iv) blocked by barium; (v) and insensitive to bicuculline, 4-AP, inorganic calcium channel blockers or proposed GABA-B antagonists.

Comparison with baclofen-evoked responses on other cells

Baclofen has been shown to directly evoke hyperpolarizing responses in other cells in the vertebrate CNS, particularly hippocampal pyramidal cells. The characteristics of such responses appear to be very similar to those of baclofen-evoked responses in our preparation. For example, the ratio of EC50 for the levarotatory versus the dextrorotatory isomers of baclofen on DRT (ie. approximately 100:1) is close to that obtained by Newberry & Nicoll (1985). Similarly, the observed dependence of our baclofen-evoked responses on [K]o and their sensitivity to barium ions is in agreement with the intracellular studies on hippocampal pyramidal cells (Newberry & Nicoll, 1985; Inoue et al., 1985a; Gahwiler & Brown, 1985). The observed effects of such manipulations are unlikely to be totally due to associated changes in membrane polarization. 
level since these would then be opposite to what is expected of hyperpolarizing responses. It has been reported (Inoue et al., 1985a; Newberry & Nicoll, 1985) that baclofen-evoked responses are voltage-sensitive in the depolarizing direction but this factor could not totally account for the depressant action of barium. Our experimental data are thus consistent with the hypothesis that responses to baclofen in vertebrate CNS reflect direct activation of a potassium conductance. More elaborate experiments on primary afferents utilizing intracellular technology would provide stronger evidence but this is not technically possible in DRT since such responses may be present predominantly on thin-diameter fibres (Pdjan & Hashiguchi, 1982; Price et al., 1984; Desarmonen et al., 1984) inaccessible with the conventional microelectrode.

The one clearly inconsistent result is the finding that 4-aminopyridine (Inoue et al., 1985a) is ineffective at depressing baclofen-evoked responses on DRT at a concentration 1000 times higher than that applied in hippocampus (Inoue et al., 1985a). Beyond obvious suggestions of possible tissue or species differences, the reason for this is unknown.

Physiological significance

The possible physiological role of baclofen-evoked responses, particularly on presynaptic terminals of primary afferents, deserves some attention. By virtue of its electrophysiological characteristics, baclofen could be assumed to inhibit release of transmitter from DRT. Indeed, this has been shown in frog spinal cord (Davidoff & Sears, 1974; Fukuda et al., 1977). But, it is uncertain whether or not the site of action of baclofen is on subsynaptic axoaxonic receptors, mainly because of the polarity of its evoked responses. Hyperpolarization of primary afferents (PAH) has been associated with presynaptic inhibition in invertebrate preparations (Fuchs, 1977; Kawai & Nawa, 1977;
Baxter & Bittner, 1981) but has not been identified in vertebrate spinal cord, where depolarization of primary afferents (PAD) is normally detected after stimulation of afferent inputs. Even though this suggests that PAH is not a synaptic entity therein, it is possible that both PAD and PAH occur simultaneously on DRT but that the more dominant depolarizing responses are masking the presence of PAH. Although depolarizing responses to both GABA and PAD could be blocked by bicuculline or picrotoxin (Nicoll & Alger, 1979) the associated convulsions would further mask any possible recognition of PAH. This problem is further complicated by the nature of the recording technique which measures a composite (averaged) response from a heterogenous population of fibres, as is the case with our studies.

The dual nature of GABA-evoked responses on DRT (Bourne & Padjén, 1980) suggests that GABA could be the physiological mediator of both PAD and (possibly) PAH via interaction at GABA-D (GABA-A) and GABA-H (GABA-B) receptors respectively. Although these receptors may be different, the mechanisms for producing presynaptic inhibition could be similar. In the invertebrate nervous system, presynaptic inhibition is thought to occur through a current shunting mechanism mediated by an increased conductance for one or more ion species whose combined equilibrium potential is lower than the threshold potential for transmitter release or impulse propagation (Nicoll & Alger, 1979). If this were to occur in vertebrate spinal cord, the same effect could be achieved if the equilibrium potential were more or less negative than the resting membrane potential such that the accompanying voltage response is either hyperpolarization or depolarization (Padjén & Hashiguchi, 1983), respectively. In addition to response polarity, amplitude of voltage deflection is also not an important pre-requisite for current shunting. For example, if it occurs as a result of an increase in K conductance (as suggested by the baclofen-evoked responses), then a large change in membrane potential would not be evident if the K equilibrium potential were only slightly more negative than
the resting membrane potential. Thus, the observation that baclofen-evoked responses are small does not necessarily imply physiological insignificance. This is especially true since they may occur predominantly on small-diameter DRT.

PAH could therefore cause presynaptic inhibition by preventing the activation of voltage-dependent calcium conductance through a current shunt that keeps the membrane potential from substantial depolarization. Direct participation of a calcium-dependent potassium conductance in these responses is unlikely because of the ineffectiveness of inorganic calcium channel blockers in depressing baclofen-evoked responses, although tested concentrations of these agents may not have been sufficient to prevent a minor contribution of calcium conductance. Effects of baclofen on calcium components already activated by depolarizing stimuli have not been tested here as they have been on cultured, embryonic, chick (Dunlap & Fischbach, 1978, 1981) or adult rat (Desarmenien et al., 1984) DRG.

The issue of whether PAH is physiologically important awaits two major developments for its resolution: (i) its physiological detection using technology capable of recording from even the thinnest DRT; (ii) discovery of potent competitive antagonists specific for the receptors responsible for the synaptic event. Demonstrating a concurrent depression of presynaptic inhibition or PAH and responses evoked by agonists of hyperpolarizing responses (eg. baclofen) would be useful in this respect. Such antagonists would also provide independent evidence as to whether or not hyperpolarizing responses elicited by neutral amino acids (eg. GABA, taurine, kojic amine) are mediated by the same receptor as that responsible for responses to baclofen. Unfortunately, the present results, as well as similar studies on isolated rat spinal cord (Evans, 1986) using putative GABA-B antagonists suggest that this discovery is still in the future.
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Figure 4.1 Concentration-response curves of baclofen stereoisomers on DRT9. Note that L-baclofen is more potent than D-baclofen in hyperpolarizing DRT. (mean +/- S.E. where possible, n = 1-6; difference between curves is significant at 5% level, using student t-test, only with 0.1 mM agonist concentration).
CONCENTRATION–RESPONSE D– & L–BACLOFEN

on DRT9 (R. pipiens)

my hyperpolarizing response

Concentration (mM)

L-baclofen  D-baclofen

0.001  0.1  10

4–21
Figure 4.2 Effect of low [K]o on neutral amino acid-evoked responses in the presence of bicuculline. Abbreviations: BIC - bicuculline, TAU - taurine, KJA - kojic amine; L-BAC - L-baclofen, .1K - 0.1 mM potassium. On this and other figures: drug application is marked by a horizontal bar; depolarization upwards; calibrations- mV (vertical), min (horizontal). Bicuculline depresses depolarizing responses (see text); in this case, the kojic amine-evoked depolarization (not shown) was changed to a hyperpolarizing response. Note that decreasing [K]o brings out the hyperpolarizing components of responses to GABA and taurine and increases the hyperpolarizing responses to kojic amine and L-baclofen.
Figure 4.3 Effect of changes in \([K]_0\) on responses of DRT to L-baclofen and GABA. Note that the amplitude of baclofen responses is inversely proportional to \([K]_0\). Note also that L-baclofen is more potent than D-baclofen in hyperpolarizing DRT (see also figure 1).
Figure 4.4 Effect of barium (0.5 mM) on responses of DRT to amino acids and K. Abbreviations: GLU - glutamate, Ba - barium (arrow indicates start of perfusion). Note that the hyperpolarizing response to D,L-baclofen and the depolarizing response to increased [K]o (3.5 mM) are depressed more than the other responses in 0.5 mM barium. Note also that the taurine-evoked response becomes multiphasic (see chapter 3).
CONTROL

GLU .5  3.5K  KJA .2  GABA .5  TAU 1

0.1·BAC .2

.5 mM Ba
Figure 4.5  Effect of barium (5 mM) in low [K]₀ on responses of DRT to amino acids and K. Note that the D,L-baclofen-evoked response is completely blocked and the K-evoked response is reversed while the response to GABA is only depressed.
Figure 4.6: Effect of 4-AP on responses of DRT to L-baclofen and GABA. Note the lack of effect of 4-AP on the amplitude of the response evoked by L-baclofen, while that evoked by GABA is increased.
DRT

CONTROL

L-BAC₃

5mM 4-AP

GABA₃  4-AP₃

KIA15

4-31
Figure 4.7 Effect of inorganic calcium channel blockers on responses of DRT to L-baclofen and GABA. Note that these agents (2 mM manganese, 2 mM cobalt, 0.5 mM cadmium) produce small changes in the resting membrane potential but have little effect on the baclofen-evoked responses.
Figure 4.8 Effect of delta-aminovaleric acid on responses of DRT to L-baclofen and GABA. Note that the baclofen-evoked response is not significantly depressed, unlike the response to GABA. Note also the biphasic effect on resting membrane potential.
DRT
CONTROL

GABA 5
L-BA 0.1

1mM 6-AVA

KMA 30
Figure 4.9 Effect of delta-aminolaevulinic acid on responses of DRT to neutral amino acids in the presence of bicuculline. There is no effect on resting membrane potential and little antagonistic action on any of the amino acid-evoked responses.
Abundant evidence points to a close association between the neutral amino acids, GABA and taurine, and presynaptic inhibition on primary afferent terminals (DRT) in vertebrate spinal cord (see chapter 1). Furthermore, evidence has been accumulating that these agonists act at multiple populations of receptors in the vertebrate CNS, including DRT. The specific questions addressed in this work were as follows:

(i) Do GABA and taurine interact at different receptors? It was demonstrated (chapter 2; Yarbrrough et al., 1981; Willow & Padjen, 1986) that taurine-evoked responses are blocked by AMBD while GABA-evoked responses are relatively unaffected. In addition, these two neutral amino acids have differential potency on DRT versus DRG and their evoked responses are differentially sensitive to depression by barium (ie. taurine > GABA) (chapter 3). 4-aminopyridine also differentially affects these responses since it enhances GABA-evoked responses but depresses those evoked by taurine (chapter 4; Zeise, 1985). Furthermore, the VR-DRP potential is blocked at a concentration of AMBD that is similar to that which blocks taurine-evoked responses on DRT (chapter 2). All this evidence is consistent with the existence of separate receptor-ionophore complexes for GABA and taurine on DRT and supports the hypothesis that taurine may be the final transmitter mediating the VR-DRP synaptic pathway in amphibian spinal cord, as previously suggested by other pharmacological studies (chapter 1; Barker et al., 1975a,b).

(ii) Does an indirect increase in [K]o contribute substantially to GABA and taurine-evoked responses on primary afferents? Data was presented (chapter 3) showing that depolarizing responses to neutral amino acids and [K]o are differentially sensitive to depression by barium. Responses evoked by GABA and taurine are enhanced on DRG and depressed on DRT in the presence of a concentration of barium that blocks or reverses responses evoked by increased [K]o on both sites. These results are therefore inconsistent with a substantial contribution of [K]o in neutral amino acid-evoked responses on DRG. On DRT, taurine-evoked responses are more
depressed by barium than are those evoked by GABA. In addition, these responses regularly show hyperpolarizing components in the presence of barium. It is thus concluded that, in the case of GABA-evoked responses, an increase in [K]o does not have a significant contribution (supporting the proposed role of GABA as a PAD neurotransmitter) but this may not be true for taurine-evoked responses. Alternatively, the possibility that the electrogenic mechanism of taurine-evoked responses is different (e.g. more barium-sensitive) from that of GABA-evoked responses cannot be excluded.

(iii) What is the nature of hyperpolarizing responses evoked by baclofen on DRT? It was demonstrated (chapter 4) that these are stereospecific, dependent on [K]o, blocked by barium and unaffected by 4-aminopyridine and inorganic calcium channel blockers (i.e. manganese, cobalt, cadmium). These data point to increased K conductance as the principal mechanism mediating baclofen-evoked responses on DRT. This mechanism would be compatible with the observed presynaptic inhibitory effect of baclofen on primary afferent transmission (Davidoff & Sears, 1974; Fukuda et al., 1977). Since baclofen could be a GABA-mimetic on DRT (Bourne & Padjen, 1980), as has been shown on other neuronal terminals (Bowery et al., 1984), it is possible that endogenous GABA can produce presynaptic inhibition through activation of more than one type of receptor.

Further interpretation of the results obtained in these studies requires consideration of several important points.

Experimental approach

All the data presented in this thesis were generated through sucrose gap recording from isolated frog spinal cord or dorsal root ganglia. There are many advantages to using such an in vitro set-up: (i) since it is at present very rarely possible to penetrate DRT with microelectrodes, the sucrose gap technique provides an excellent alternative for
studying electrical events originating from DRT with a resolution greatly surpassing conventional extracellular recording methodology; (ii) the technique also allows recording at a site on dorsal roots that is surpassed only by intrafibre recording (at dorsal root entry zone) in terms of electrotonic proximity to DRT; (iii) it is usually possible to obtain stable recordings for up to 48 hours with minimal deterioration of control responses and with all the benefits of in vitro conditions; (iv) the small volume of the central chamber compartment allows fast and complete equilibration to new steady-state concentrations following alterations of Ringer or application of drugs.

There are, however, several major disadvantages of the technique that limit the interpretation of recordings: (i) primary afferents are a highly heterogenous neuronal population. Sucrose gap records reflect a composite electrical response from thousands of neurons (eg. approximately 6000 axons in DRT10 of bullfrog, two thirds of which are unmyelinated (Wilhelm & Coggeshall, 1981) such that if one agent produces different effects on different (or same) cells, some of these effects may be electrically masked by other more predominant ones (ii) primary afferents make contact and are surrounded by a large network of interconnected neuronal populations. Pharmacological and physiological manipulations may therefore produce effects on neurons distant from the site of recording which in turn affect primary afferents through interneuronal communication. These indirect effects are minimized by inhibitors of synaptic transmission (eg. tetrodotoxin or manganese) but may not be completely abolished (eg. electrical junctions) (iii) being poikilothermic, frogs show seasonal variations in their physiological state (Muller, 1976).

But despite the limitations, the experimental methods used are the best available for examining the pharmacology of DRT (or of DRG), particularly when they are combined with intracellular recording from dorsal root axons (cf. Hashiguchi & Padjen, 1982).
Physiological significance of the results

Although it has been pointed out (chapters 1 and 3) that responses of DRG and DRT to the same neutral amino acids are likely to be mediated by the same receptor-ionophore complexes, the alternate possibility cannot be excluded. Evidence supporting site-specific receptor differences includes the differential effect of barium on amino acid-evoked responses, differential agonist potencies (eg. GABA) and the different appearance of responses (eg. presence of GABA shoulder in response of DRG to GABA, see figure 3.6) on the two primary afferent sites. Such a possibility would also be consistent with previous studies (Nicoll & Padjen, unpublished observations) demonstrating differences in sensitivity of neutral amino acid-evoked responses on the two sites to changes in extracellular sodium concentration. These possible receptor differences would mean that responses of DRG to neutral amino acids may not be a good model for PAD occurring at DRT.

Although their existence is fortuitous from a technical standpoint, the physiological significance of responses to neutral amino acids (especially GABA) on DRG can only be speculated at the present time since they appear to be mediated by extrasynaptic receptors. It has been suggested that GABA may also act in a hormonal capacity in the peripheral nervous system where it has been found to modulate release of transmitter from preganglionic terminals in a concentration range found in serum (Kato & Ruba, 1980). It may therefore also be possible that GABA interacts with receptors on DRG in this capacity. The relatively high potency of GABA in depolarizing DRG would be consistent with this hypothesis. The same argument may also be applied on DRT, considering our observations that low concentrations of GABA hyperpolarize this site (Bourne & Padjen, 1980).

The low potency of neutral amino acids in depolarizing
DRT (EC50's in the millimolar range) should be reconsidered. While such apparently high concentrations may be physiological (e.g. in synaptic clefts; Fonnus & Walberg, 1973), the mechanism of evoked responses could vary according to agonist concentration. Taking into account the concentration-dependent duality of the neutral amino acid-evoked responses on DRT (chapter 4; Bourne & Padjen, 1980) and the frequency-dependent increase in [K]o upon stimulation of afferent inputs (chapter 3; Nicoll, 1979), it is possible that the mechanism of presynaptic inhibition varies according to physiological conditions. Thus, depending upon the frequency of afferent or supraspinal volleys arriving into the dorsal horn and the consequent degree of endogenous release of neutral amino acids, the cause of presynaptic inhibition may vary from GABA-B to GABA-A to K.
CHAPTER 6

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