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TWO TYPES OF γ-AMINOBUTYRIC ACID RECEPTOR ON EMBRYONIC SENSORY NEURONES

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1 Embryonic sensory neurones of the chick grown in dissociated cell culture respond to application of low concentrations of γ-aminobutyric acid (GABA) with a change in resting membrane resistance (R_{in}) and/or a change in action potential duration (APD) (Dunlap & Fischbach, 1978; Choi & Fischbach, 1981). Intracellular microelectrode recording techniques were employed to determine if these two effects are mediated by the same, or different, GABA receptors.

2 Cells responded, for the most part, with a change in either R_{in} or APD, but 10% of the cells exhibited both effects. In the latter cells the two responses were clearly distinguishable as discussed below.

3 The proportion of neurones exhibiting a GABA-induced decrease in R_{in} declined during the first week *in vitro* while the proportion exhibiting a decrease in APD increased during that time.

4 The two effects were pharmacologically distinct. Muscimol, a GABA analogue, produced only the change in R_{in} ($ED_{50} = 5.5\ \mu M$) while baclofen, another analogue of GABA, produced only the change in APD ($ED_{50} = 1\ \mu M$). The analogues were approximately equipotent with GABA. Bicuculline, a GABA antagonist, blocked the muscimol-induced change in R_{in} (but not the baclofen-induced change in APD) in a dose-dependent fashion with an $ID_{50} = 0.7\ \mu M$.

5 The time courses of the two effects were different. The change in APD resulting from a brief application of GABA (or baclofen) was prolonged relative to the rapid return to control associated with the GABA- (or muscimol-) induced change in R_{in}.

6 Desensitization of the two responses exhibited separate time courses. In the continual presence of the agonists, GABA- and muscimol-induced decreases in R_{in} completely desensitized in ca. 10 s while GABA- and baclofen-induced decreases in APD persisted undiminished throughout a prolonged (1 min) application of the drugs and returned to control only after cessation of application.

7 It is concluded that embryonic chick sensory neurones in culture exhibit two types of GABA receptor that differ in their functional and pharmacological properties. Implications of these results are discussed.

Introduction

The effects of γ-aminobutyric acid (GABA) on the excitability of peripheral neurones are well documented. GABA produces a depolarization of the resting membrane potential in sensory and sympathetic neurone cell bodies and axons. This depolarization is mediated by an increase in Cl^- permeability which is blocked by picrotoxin and/or bicuculline (DeGroat, 1970; 1972; Bowery & Brown, 1974; Nishi, Minota & Karczmar, 1974; Adams & Brown, 1975; Brown & Marsh, 1978; Gallagher, Higashi & Nishi, 1978).

Recently, another effect of GABA on embryonic sensory neurones has been described: GABA decreased the soma Ca action potential duration by a mechanism which did not involve a change in resting membrane permeability (Dunlap & Fischbach, 1978) but resulted instead from a selective decrease in the voltage-sensitive Ca channel conductance (Dunlap & Fischbach, 1981). This effect was not blocked by bicuculline.

In response to GABA application, embryonic chick sensory neurones (in culture for less than eight days) exhibit changes in both input resistance and action potential duration. This study was undertaken to determine whether these two GABA-mediated effects result from a single type of GABA receptor with dual function or from two separate GABA receptor types. Parallels between the effects of GABA on sensory neurones and on sympathetic ganglion cells are discussed in relation to possible mechanisms for inhibition of transmitter release.

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Methods

Dissociated cell cultures were prepared from 10–12 day old chick embryos by methods described previously (Dichter & Fischbach, 1977; Dunlap & Fischbach, 1978). Non-neuronal background cell growth was halted by gamma-irradiation (5000 R) of the dissociated single-cell suspension prior to plating at a density of ca. 25,000 cells/35 mm tissue culture dish.

Standard intracellular microelectrode recording techniques (Dichter & Fischbach, 1977; Dunlap & Fischbach, 1978) were used on cells less than two weeks in culture. Current was passed through the recording electrode by means of an active bridge circuit. GABA and other drugs were applied in known concentration by pressure ejection from pipettes with tip diameters of ca. 3–5 μm (Choi, Farb & Fischbach, 1977; Choi & Fischbach, 1981). All experiments were performed at room temperature (25°C) in HEPES buffered saline solution (with 5 mM Ca^{2+}) supplemented with 5 mM glucose (pH 7.3).

(+)-Bicuculline (Sigma) and (±)-baclofen (Lioresal: CIBA-Geigy) were dissolved at 10 mM in 100 mM HCl and diluted to final concentration in HEPES buffered saline at pH 7.3.

Results

The effects of GABA on the input resistance (R_{in}) and action potential duration (APD) recorded from two different sensory neurone cell bodies are shown in Figure 1a and b. Two superimposed oscilloscope traces are shown before and after (arrow) application of 10^{-4} M GABA. In the first cell (Figure 1a), GABA produced a 40% decrease in R_{in} (accompanied by an 8 mV depolarization), without decreasing the APD. Frequently, the changes in R_{in} and membrane potential (V_m) were accompanied by decreases in the peak amplitude of the action potential (predominantly a function of inward Na^+ current). This might reflect either Na^+ channel inactivation resulting from the depolarization or a simple shunting of the action potential by the drop in resistance. The decrease in R_{in}, however, was always (except with extremely large changes in R_{in}) associated with either no change or an increase in APD. (The increase in APD sometimes seen but not shown here might be due to K^+ channel inactivation, resulting from the depolarization, which has been observed in these cells under voltage clamp: K. Dunlap, unpublished observations). A shunting of the action potential, therefore, becomes an unlikely possibility since such an effect would decrease action potential duration as well as

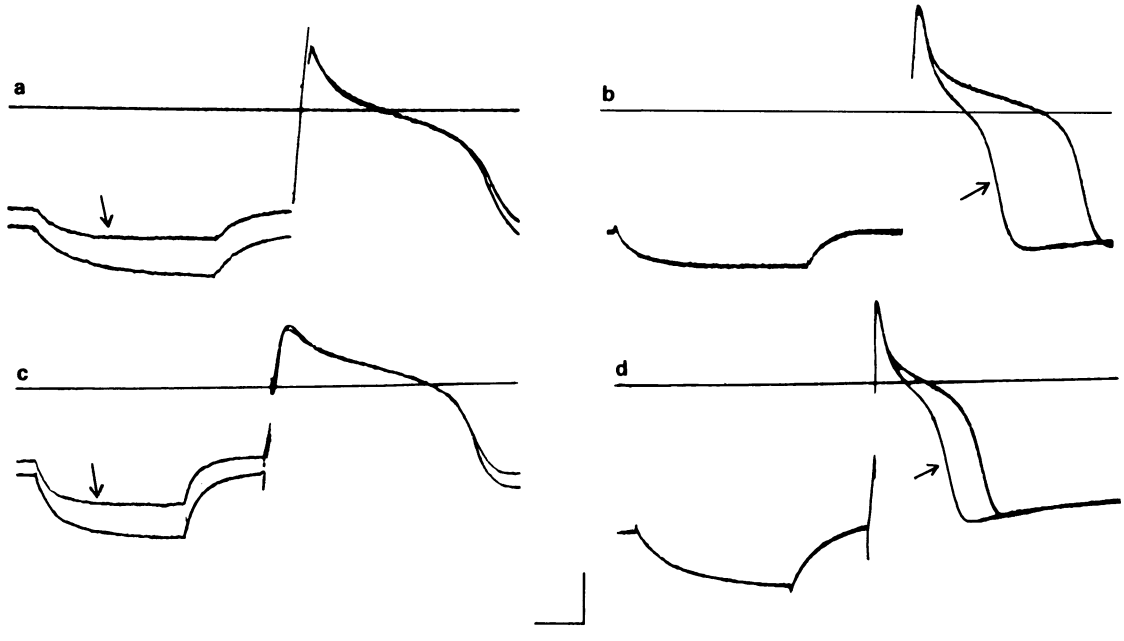

Figure 1 Effect of γ-aminobutyric acid (GABA), muscimol and baclofen on R_{in} and APD of sensory neurone cell bodies. In each panel are shown two superimposed oscilloscope traces of membrane hyperpolarizations followed by action potentials evoked by current pulses passed through the recording electrode before and after (arrows) drug application. (a and b) 10^{-4} M GABA; (c) 10^{-4} M muscimol; (d) 10^{-4} M baclofen. Horizontal lines mark 0 mV in each panel. Calibration bar is 20 mV, 2 ms.

peak amplitude. The rate of rise of the action potential did not appear to change significantly although precise measurements were prevented by uncertainty of the bridge balance during passage of the relatively large currents used to evoke action potentials (2–5 nA).

In the second cell (Figure 1b), no change in R_{in} or V_m could be detected, but GABA produced a 60% decrease in APD. Both (a) and (b) were recorded within 2 s of a 1 s application of GABA. Cells generally exhibited either a change in R_{in} or a change in APD, but some (ca. 10%) showed a concomitant decrease in R_{in} and APD. Of 341 cells tested (within 7 days of plating) with 10^{-4} M GABA, 84 responded solely with a change in R_{in}, 171 solely with a change in APD, 39 with both, and 47 were unaffected.

The proportion of neurones exhibiting changes in

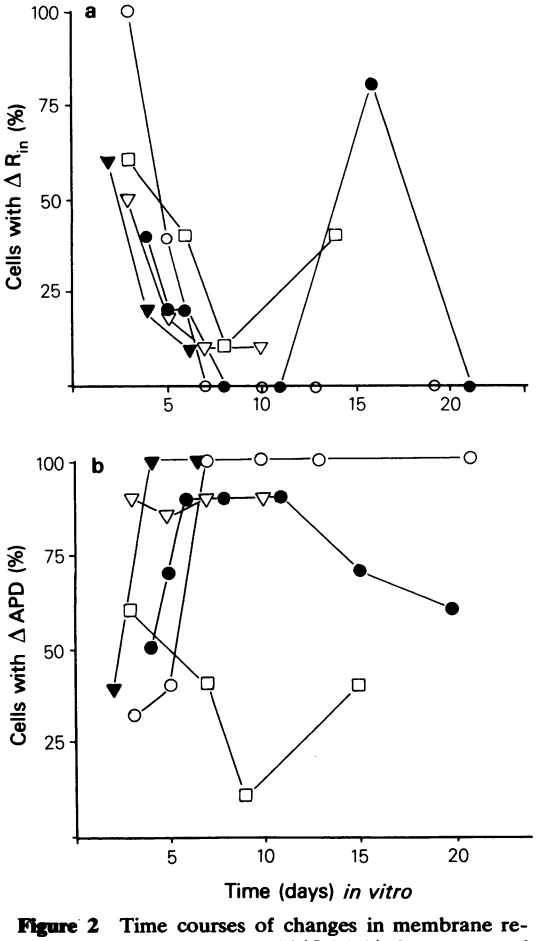

Figure 2 Time courses of changes in membrane responses to γ-aminobutyric acid (GABA). Percentages of cells exhibiting GABA-induced changes in R_{in} (a) or APD (b) are plotted against days *in vitro* for five separate platings, each marked with a different symbol. Each point is the result of ten cells tested in a given plating.

APD compared to those exhibiting changes in R_{in} altered with development, or time, in culture. This phenomenon was studied in five separate platings which were tested with 10^{-4} M GABA every 1–2 days for up to 3 weeks. In all platings the proportion of neurones showing a GABA-induced change in R_{in} declined from ca. 50% to near 0 during the first week in culture. In two of the platings a secondary rise in this fraction was seen during the second week, reaching its peak around day 15 (Figure 2a). In contrast, three of the five platings showed an increase in the proportion of neurones exhibiting a GABA-induced change in APD during the first four days *in vitro*. In the remaining two platings, one showed a decrease and one no change in the percentage of cells responding to GABA with a decrease in APD (Figure 2b). In cases where GABA produced changes in both R_{in} and APD, the two responses were clearly separable and could be distinguished on the basis of several criteria.

Firstly, a pharmacological distinction between receptor types was made. Muscimol (a GABA analogue, Tridom Chemicals) was tested for its potency on these two types of GABA responses (Figure 1c). When applied in a saturating concentration of 10^{-4} M, muscimol decreased R_{in} without affecting the APD. In 63 cells tested between 3 and 10 days *in vitro*, 36 responded with an average 42% decrease in R_{in} and none with a change in APD. The effect of muscimol was dose-dependent with an ED_{50} of 5.5×10^{-6} M; maximal decreases in R_{in} were produced at ca. 10^{-4} M (Figure 3). This is comparable to the concentration of GABA required to produce a maximal decrease in R_{in} in these cells (Choi & Fischbach, 1981). GABA and muscimol appear to be equipotent: when applied at 10^{-4} M they each produced an average decrease in R_{in} of ca. 40% (Figure 3). In cultures older than 1 week in which no GABA-induced decreases in R_{in} were observed, muscimol produced no changes in R_{in} or APD.

(±)-Baclofen, another GABA analogue, when applied at 10^{-4} M did not, in 46 cells tested, produce any changes in R_{in}, but 76% of the cells (5 to 13 days *in vitro*) responded with an average 35% decrease in APD (Figure 1d).

The effect of baclofen on APD was also dose-dependent with an ED_{50} of ca. 1 μM; maximal decreases in APD were produced at 10^{-4} M (Figure 4). The ED_{50}s and maximal concentrations of GABA and baclofen were comparable; GABA, at 10^{-4} M evoked an average 44% decrease in APD compared to the average 35% decrease evoked by 10^{-4} M baclofen (Figure 4). In cultures lacking GABA- and muscimol-induced decreases in R_{in}, baclofen remained effective (as did GABA) in producing decreases in APD.

Baclofen appears to exert its effect via the GABA

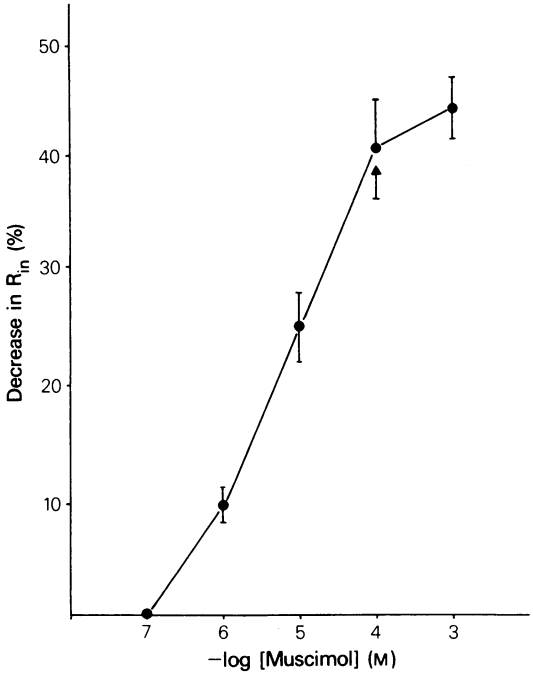

Figure 3 Muscimol dose-response curve. The % decrease in R_{in} is plotted against the logarithm of the muscimol concentration. Each point is the mean of measurements made on a minimum of 5 cells; vertical lines show s.e.mean. The average % decrease in R_{in} produced by a maximal γ-aminobutyric acid dose (10^{-4} M) is shown for comparison (▲).

receptor. GABA (10^{-4} M) was tested for its effects on R_{in} and APD in two populations of cells, before and after addition of 10^{-4} M baclofen to the bathing solution. Prior to the addition of baclofen, 2 out of 5 cells showed a decrease in R_{in} and 3 out of 5 a decrease in APD. Following addition of baclofen to the bath, 4 out of 10 responded with a decrease in R_{in} and none with a change in APD. In contrast, it has been reported that saturating doses of noradrenaline (which decrease APD via a separate receptor) do not interfere with the ability of GABA to produce a decrease in APD in these cells (Dunlap & Fischbach, 1981).

The muscimol- and baclofen-induced effects differed not only in their agonist specificity but also in their sensitivity to the antagonist bicuculline. In two groups of 10 cells each, 6 out of 10 responded with a muscimol-induced change in R_{in} and 9 out of 10 with a baclofen-induced change in APD. Following incubation of the cells in a maximal concentration of 10^{-4} M bicuculline, muscimol was not effective in any of the 10 cells tested while baclofen remained effective in all 10 cells tested. The inhibition of the muscimol response was reversible and dose-dependent with an ID_{50} of 0.7 μM (Figure 5). The sensitivity to bicuculline of these two responses would, of course, be predicted given recent studies concerned with GABA effects on sensory neurones *in vitro* which showed that the GABA-induced change in APD was bicuculline-insensitive while its effect on R_{in} was blocked reversibly by bicuculline (Dunlap & Fischbach, 1978; Choi, 1978; Choi & Fischbach, 1981).

In addition to the pharmacological differences observed, these two responses exhibited dramatic differences in both their time course and 'desensitization'. The change in R_{in} produced by a 1 s application of 10^{-4} M GABA was sustained only about 10 s while the change in APD produced by a 1 s application of GABA was a longer lasting one, its return to control APD requiring up to 2 min. This difference may be due, at least in part, to differences in the desensitization properties of these two responses to GABA.

In response to a continuous application of GABA, the change in R_{in} desensitized while the change in APD did not. The term desensitization is used loosely, since alterations in receptor binding are not being investigated. Here, the term refers to the decline in magnitude of the membrane response during continual exposure to the drug. Desensitization of the GABA-induced changes in R_{in} and APD is shown in

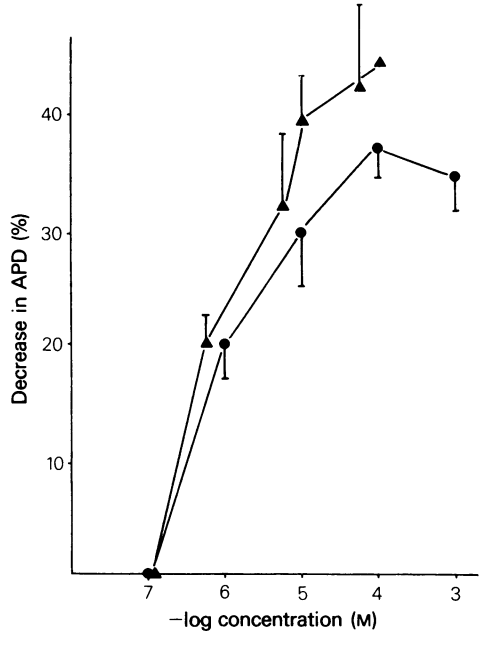

Figure 4 Baclofen dose-response curve. The % decrease in APD is plotted against the logarithm of the baclofen (●) and γ-aminobutyric acid (▲) concentrations. Each point is the mean of measurements made on a mimimum of five neurones; vertical lines show s.e.mean.

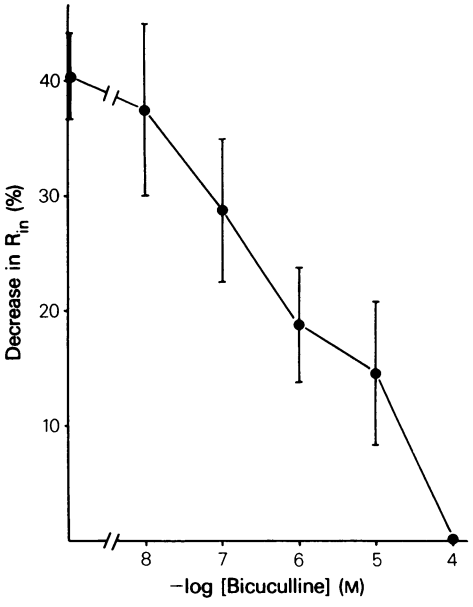

Figure 5 Bicuculline dose-response curve. The % decrease in R_{in} produced by 10^{-4} M muscimol was assayed in the presence and absence of varying concentrations of bicuculline and plotted as the average % decrease in R_{in} (5 neurones per point) against the logarithm of the bicuculline concentration; vertical lines show s.e.mean. Successively increasing concentrations of bicuculline were added both to the recording bath solution and to the puffer pipette containing 10^{-4} M muscimol. Cells were assayed for effects of muscimol on R_{in} immediately following addition of bucuculline solutions to the bath.

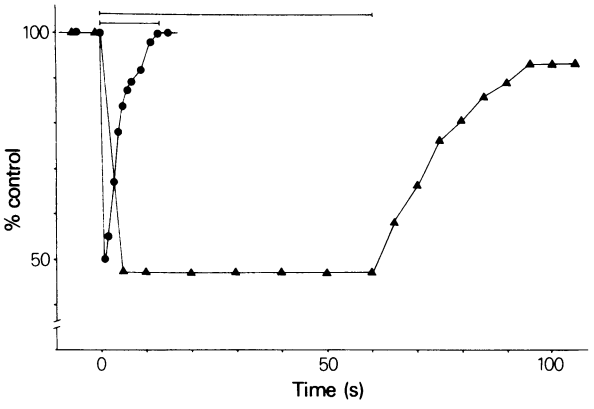

Figure 6 Desensitization of γ-aminobutyric acid (GABA) responses. The changes in R_{in} (●) and ADP (▲) were monitored before, during and after application of 10^{-4} M GABA. Each point is the average of 3 (▲) or 5 (●) cells. The duration of GABA application is indicated by the horizontal bars.

Figure 6. The circles represent the change in R_{in} (average of 5 cells) and the triangles the change in APD (average of three cells) induced by continual application of 10^{-4} M GABA throughout the measurement period (bars above curves). The decrease in R_{in} reached its peak 1–2 s after the start of GABA application, underwent strong desensitization and returned to control within 13 s, at which time GABA application was terminated. Similar results were also described by Choi (1978). In contrast, the decrease in APD reached its maximum 5 s after the onset of the GABA stimulus, remained at that value throughout the 1 min application, and returned to control only after the termination of the GABA pulse.

The desensitization curves of the responses to muscimol and baclofen also exhibit separate time courses similar to those observed in response to GABA (Figure 7). The muscimol-induced decrease in R_{in} completely desensitized in less than 10 s following the onset of muscimol application while the baclofen-induced decrease in APD persisted in the presence of the drug (1 min) and returned to control only following the removal of baclofen. A slight desensitization (ca. 15%) of the baclofen response did occur which may represent a difference in the actions of GABA and baclofen. Although the time course for the recovery from desensitization was not studied in detail, the neurones were found capable of responding to multiple, successive applications of GABA and its analogues.

Thus, muscimol and baclofen appear to act as specific agonists for the two separate GABA-mediated membrane responses which differ both in affected channels and in desensitization kinetics.

Discussion

The differences in functional and pharmacological properties of the two responses to GABA observed in sensory neurones suggest the involvement of two types of GABA receptor which differ in their sensitivity to agonists and antagonists and in their susceptibility to desensitization. These two receptor types appear to be functionally linked to different ionic channels. Activation of the muscimol-sensitive receptor increases the membrane permeability to Cl^- while the baclofen-sensitive receptor decreases the permeability of the voltage-senstive Ca^{2+} channel.

It is interesting that both bicuculline-sensitive and bicuculline-insensitive GABA responses have also been described in sympathetic neurones, and two classes of GABA receptor have been proposed for that system (Bowery & Hudson, 1979) with some of the same properties as those described in this paper. Only the response associated with the bicuculline-sensitive receptor has been studied electrophysiologically in these cells, and it is clear that GABA (and

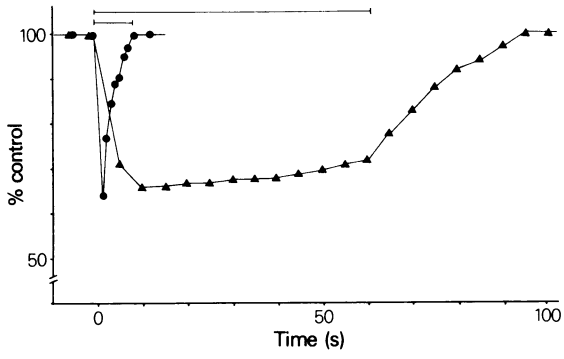

Figure 7 Desensitization of muscimol and baclofen responses. The muscimol-induced change in R_{in} (●) and the baclofen-induced change in APD (▲) were monitored before, during and after drug application. Each point is the average of 3 (●) or 5 (▲) cells. The periods of 10^{-4} M muscimol and 10^{-4} M baclofen application are indicated by the horizontal bars.

several analogues) increase resting membrane Cl^- permeability (DeGroat, 1970; Adams & Brown, 1975). A second important effect of GABA in sympathetic neurones is the inhibition of transmitter release from both pre- and postganglionic nerve terminals (Koketsu, Shoji & Yamamoto, 1974; Kato, Kuba & Koketsu, 1978; Bowery & Hudson, 1979; Brown & Higgins, 1979). This action of GABA is unaffected by the presence of bicuculline and is mimicked by baclofen (Bowery, Doble, Hill, Hudson, Shaw & Turnbull, 1979). The action of baclofen is prolonged relative to the more rapid desensitization reported for the GABA-induced change in Cl^- conductance. Desensitization is complete in about 10 s (Bowery & Brown, 1974) whereas transmitter release is suppressed by baclofen for several minutes (Kato *et al.*, 1978; Bowery & Hudson, 1979; Kato & Kuba, 1980). The difference in the time course of desensitization of the two effects described in this paper may, therefore, explain this discrepancy.

GABA may have a similar effect on transmitter release in sensory neurones. Substance P, a putative sensory neurotransmitter, is released from cultured embryonic chick sensory neurones in a Ca-dependent process activated by either high K^+ or electrical stimulation (Mudge, Leeman & Fischbach, 1979; Mudge, 1979). Low concentrations of GABA decrease substance P release from these cells (Mudge, 1979). Baclofen has not been tested for its effects on this release process.

The physiological relevance of GABA-mediated alterations in sympathetic neurone excitability has always been unclear since neither GABA nor its synthetic enzyme, glutamic acid decarboxylase, have been detected in ganglia (Nagata, Yokoi & Tsukada, 1966; McBride & Klingman, 1972). In contrast, the role of GABA in presynaptic inhibition of 1a sensory neurones is well documented (Burke & Rudomin, 1977; Krnjević, 1979). Due to the complexity of the spinal cord and the inaccessibility of the 1a afferent terminals, direct studies of the effects of GABA on terminal membrane excitability are prohibitively difficult. Measurements of dorsal root potentials (primary afferent depolarization) or axon excitability may not provide the important information concerning the function of GABA on the nerve terminal membrane, the presumed site of presynaptic inhibition. To draw supportive evidence from studies on sympathetic ganglia once more, GABA has been shown to depolarize both pre- and postganglionic axons but in the presence of bicuculline (in sufficient concentration to completely block the depolarization) inhibition of transmitter release by GABA (or baclofen) remains unaffected (Koketsu *et al.*, 1974; Bowery & Hudson, 1979; Kato & Kuba, 1980). Effects of baclofen and/or muscimol on transmitter release at identified synapses between sensory neurones and spinal cord cells in low density cultures may clarify the relative contribution of changes in input resistance and action potential duration to presynaptic inhibition.

The regulation of these two types of GABA receptor is interesting. GABA has not been reported to alter the action potential duration of adult dorsal root ganglion (or sympathetic) cell bodies although the change in Cl^- conductance is well documented (DeGroat, 1972; Nishi *et al.*, 1974; Adams & Brown, 1975). Little is known about the electrophysiological effects of GABA on nerve terminals. As shown here, the number of cells showing R_{in}-sensitive or APD-sensitive responses to GABA change with time in culture. These alterations at present appear complex but may indicate any of a number of possibilities, including elimination or modification of one receptor type, or redistribution of one type of receptor to regions of the cell membrane electrophysiologically remote from the cell body. Receptor binding techniques, coupled with autoradiography, may provide information on the mechanism of this change.

In summary, pharmacological and electrophysiological characterization of the action of GABA on cultured embryonic sensory neurones suggests that two types of GABA receptor exist in these cells. The differences in the receptor-mediated permeability changes may have important implications for the mechanism of presynaptic inhibition.

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