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Effect of electrical stimulation and high potassium concentrations on the efflux of [^{14}C] glycine from slices of spinal cord

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Summary

1. The effects of electrical stimulation and solutions containing a high concentration of potassium on the efflux of [^{14}C] glycine from slices of rat spinal cord have been studied.

2. Slices of cord were incubated with [^{14}C] glycine which rapidly accumulated in the tissue. The slices were then superfused in a small chamber and the radioactivity released from the tissue was measured. After superfusion for 60 min, 98% of the radioactivity remaining in the tissue was present as unchanged glycine.

3. The spontaneous efflux of [^{14}C] glycine consisted of an initial rapid phase followed by a much slower release of [^{14}C] glycine. After superfusion for 60 min, more than 65% of the radioactivity taken up during the incubation period was retained by the tissue.

4. When the slices were depolarized by electrical stimulation or by solutions containing a high concentration of potassium (40 mM), a striking increase in the efflux of [^{14}C] glycine was produced. This effect was not reduced by the absence of calcium ions in the superfusion medium.

5. Electrical stimulation produced similar increases in the efflux of [^{3}H] GABA and [^{14}C] glutamate from slices of cord but had no significant effects on the efflux of [^{3}H] alanine or [^{14}C] urea.

6. The results are consistent with the suggestion that glycine may be an inhibitory synaptic transmitter substance in the mammalian spinal cord.

Introduction

There is a considerable amount of neurophysiological and neurochemical evidence to support the suggestion that glycine may be an inhibitory synaptic transmitter substance in the mammalian spinal cord (Aprison & Werman, 1965 ; Davidoff, Shank, Graham, Aprison & Werman, 1967 ; Werman, Davidoff & Aprison, 1967, 1968 ; Curtis, Hösli & Johnston, 1967 ; Curtis, Hösli, Johnston & Johnston, 1968). In establishing a neurotransmitter role for a substance, it is usually considered important to show that it is released from nerve terminals as a result of presynaptic stimulation. However, it has proved difficult to demonstrate changes in the release of glycine from the cord after nerve stimulation. Some of this difficulty may be due to the efficient uptake mechanism for glycine which is present in the cord (Neal & Pickles, 1969 ; Neal, 1971) and which may provide a mechanism for terminating

the action of glycine after its release from nerve terminals (Neal & Pickles, 1969; Curtis, Duggan & Johnston, 1970; Neal, 1971). A similar uptake mechanism may be responsible for terminating the inhibitory actions of gamma-aminobutyric acid (GABA) on cortical neurones (Iversen & Neal, 1968), and it is thought that an uptake mechanism is responsible for terminating the actions of noradrenaline after its release from nerve terminals (Iversen, 1967). The demonstration of transmitter release at adrenergic synapses has been greatly facilitated by the use of drugs which block the uptake of noradrenaline into nerve terminals (Brown, 1965). Unfortunately, no drug is known which will selectively block the uptake of glycine into nervous tissue without exerting unwanted pharmacological actions.

Because of the difficulties of studying the release of glycine from the mammalian spinal cord *in vivo*, the factors affecting release of [^{14}C] glycine from slices of rat spinal cord *in vitro* have been investigated. Preliminary results of some of these studies have been reported previously (Hopkin & Neal, 1970).

Methods

Male Wistar rats (100–150 g) were killed by a blow on the head and the spinal cords removed rapidly and chilled. Sections of cord weighing approximately 100 mg were sliced transversely at 1 mm intervals with a mechanical tissue chopper (McIlwain & Buddle, 1953). The resultant slices were incubated at 37° C in 10 ml of Krebs bicarbonate medium which was gassed with a mixture of 5% carbon dioxide and 95% oxygen. After a preliminary incubation of 15 min, [^{14}C] glycine was added to give a final concentration of 6×10^{-7}M. The incubation was continued for 30 min and the slices were then recovered by rapid filtration on to a small nylon gauze disc. This disc containing the slices of cord was transferred to a superfusion chamber similar to that described by Srinivasan, Neal & Mitchell (1969). The slices were retained in the chamber by the addition of a second gauze disc, placed lightly on top of the tissue slices. The cord was superfused with medium at a rate of 0·5 ml/minute. The superfusate was removed by suction using a roller pump (Watson Marlow, H.R. Flow-Inducer) and was collected into test tubes which were changed automatically every 2 min by a fraction collector.

Samples of 0·2 ml were transferred to scintillation vials and the radioactivity was measured by liquid scintillation counting after the addition of 4·0 ml ethoxyethanol and 10 ml of phosphor (1% Butyl PBD, Ciba, in toluene). The radioactivity remaining in the tissue at the end of the experiment was measured after extraction with 1·0 N hydrochloric acid.

The medium in the superfusion vessel was maintained at 37° C and gassed with a mixture of 5% carbon dioxide and 95% oxygen. The tissue slices could be stimulated electrically via two silver electrodes. A Grass SD9 stimulator was used to provide rectangular pulses (5 ms duration, 100 Hz, 20 mA) and the stimulus current was monitored continuously. Similar superfusion experiments were performed after incubating tissue slices with [^{14}C]-L-glutamic acid (5×10^{-7}M), [^{3}H] GABA (5×10^{-7}M), [^{14}C]-L-alanine (5×10^{-6}M), or [^{14}C] urea (10^{-4}M). In order to take into account the differences in uptake of the various amino-acids by the cord, the results are expressed as the efflux rate constant (*f*) which is the fractional rate of loss per unit time.

$$f = \frac{\triangle A}{\Delta t . A_t}$$

Where ΔA represents the counts lost in the time interval Δt, and A_t is the amount of radioactive compound in the tissue at the midpoint of the interval Δt. In order to determine A_t, the counts released from the cord during the whole experiment were added to the amount of radioactivity extracted from the tissue at the end of the experiment. This gives the value A_o. A_t is the difference between A_o and the total counts lost up to the middle of Δt.

Metabolism experiments

In two experiments, spinal cord was incubated for 40 min with [^{14}C] glycine at 37° C and superfused as described previously with fresh medium for a further 40 minutes. The tissue was then recovered by filtration, washed with 5 ml of ice cold medium and extracted by homogenization with 1·0 ml of 70% ethanol. The ethanol extracts were centrifuged and samples of the supernatant were applied to paper chromatograms after the application of non-radioactive glycine (25 μg) as a carrier. The chromatograms were run overnight in an ascending manner using *n*-butanol: acetic acid: water (8:5:3) as the solvent system. The chromatograms were dried and the radioactivity was measured with a radiochromatograph (Nuclear Chicago, Actigraph III). The glycine spots were then made visible by spraying the chromatogram with ninhydrin (0·2% in acetone) followed by heating. The pellets obtained after centrifugation of the ethanol extract were dissolved in Soluene 100 (Packard) and the radioactivity was measured by liquid scintillation counting after the addition of 10 ml of phosphor.

Materials

The incubation and superfusion medium was Krebs-bicarbonate Ringers of the following composition (g/l.); $NaCl=6{\cdot}92$, $KCl=0{\cdot}354$, $CaCl_2=0{\cdot}28$, $MgSO_4=0{\cdot}144$, $KH_2PO_4=0{\cdot}162$, $NaHCO_3=2{\cdot}1$, D-glucose$=2{\cdot}0$.

Glycine-1-[^{14}C], specific activity = 41·4 mCi/mmol; [^{14}C] urea, specific activity = 15·4 mCi/mmol; L-glutamic acid-[^{14}C] (U), specific activity = 260 mCi/mmol; L-alanine-[^{3}H] (G), specific activity = 100 mCi/mmol, were obtained from the Radiochemical Centre, Amersham, England, GABA-[2,3-^{3}H], specific activity = 2 Ci/mmol, was obtained from New England Nuclear Chemical GmbH, 6072 Dreieichenhain, West Germany.

Results

[^{14}C] Glycine—spontaneous efflux

The release of [^{14}C] glycine from slices of spinal cord was multiphasic and consisted of at least two major components. An initial rapid release of [^{14}C] glycine, which probably represented the extracellular washout of glycine was followed after about 10 min by a steady, and much slower efflux ($f \simeq 0{\cdot}008$ min^{-1}). After superfusion for 60 min, 65–70% of the radioactivity taken up during the incubation period was retained by the tissue.

Metabolism experiments

In two experiments in which spinal cord was incubated for 40 min with [^{14}C] glycine and then superfused with fresh medium for a further 40 min, tissue extracts

were subjected to paper chromatography. Ninety-eight per cent of the radioactivity in the tissue was unchanged [^{14}C] glycine. Less than 2% of the total radioactivity remained in the tissue after extraction with 70% ethanol, indicating that under the conditions of these experiments, there was negligible incorporation of glycine into proteins or nucleic acids. Paper chromatography indicated that the radioactivity taken up by the tissue during incubation with [^{14}C] glycine, and retained during the subsequent superfusion, chromatographed as a single spot with an R_F value identical to that of authentic glycine. The efflux of radioactivity was therefore taken as a measure of glycine release.

Electrical stimulation

The slices of cord were electrically stimulated for 30 s (duration, 5 ms ; frequency, 100 Hz ; current, 20 mA) after they had been superfused for 20 min, that is during the steady, slower phase of [^{14}C] glycine efflux. This produced a striking increase in [^{14}C] glycine release, the efflux being increased to a maximum of 2·1 times the prestimulation value (Fig. 1). This maximum effect occurred during the collection period when the tissue was being stimulated. The results are the mean of eight experiments and the standard error of the mean for each point was less than 15%. This effect of electrical stimulation was not dependent on the presence of calcium ions in the superfusion medium because a similar increase in [^{14}C] glycine efflux was obtained using a calcium-free medium, the maximum electrically evoked release being 2·3 times the prestimulation value.

Effect of high potassium concentrations

When slices of cord were superfused with medium containing a high potassium concentration (40 mM), a similar increase in [^{14}C] glycine efflux was produced (Fig. 2). The maximum efflux, which was 2·4 times the resting efflux, occurred during the second or third period after superfusing the tissue with high potassium medium.

The effect of high potassium on the efflux of [^{14}C] glycine was not significantly reduced by the absence of calcium ions in the medium.

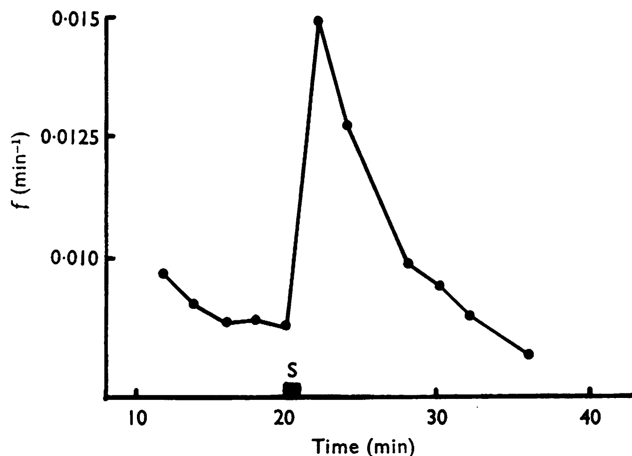

FIG. 1. Effect of electrical stimulation (S) on the efflux rate constant (*f*) of [^{14}C] glycine from spinal cord slices. Each point is the mean of eight results. The standard errors of the means were less than 15%.

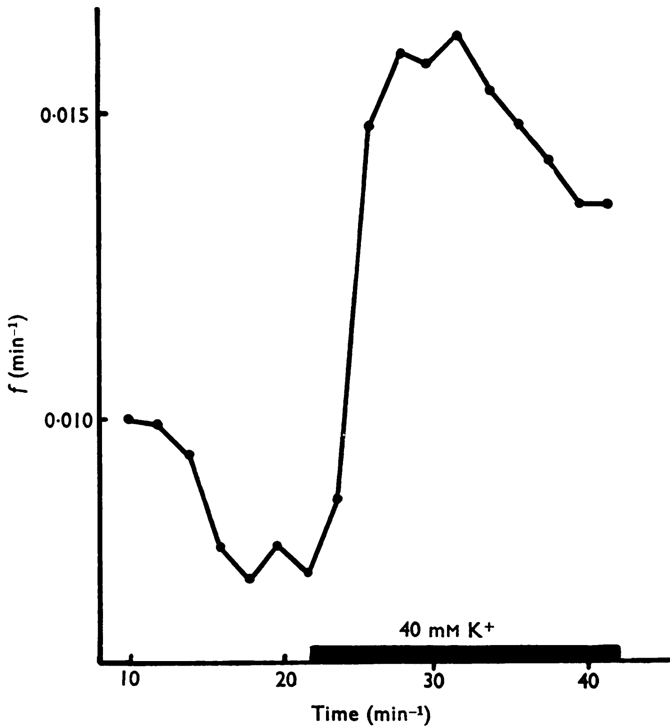

FIG. 2. Effect of high potassium (40 mM) on the efflux rate constant (*f*) of [^{14}C] glycine from slices of spinal cord. Each point is the mean of seven results. The standard errors of the means were less than 15%.

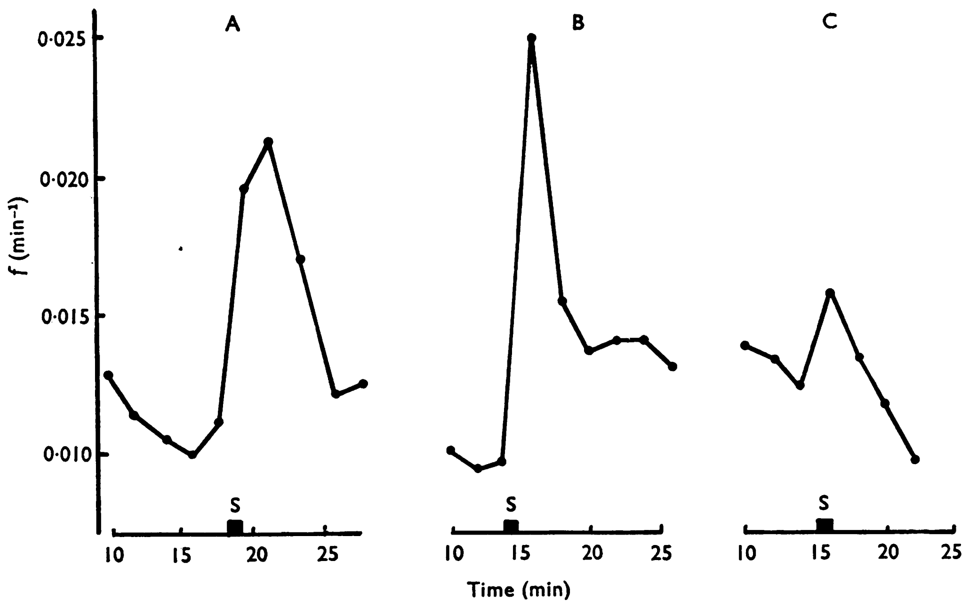

FIG. 3. Effect of electrical stimulation on the efflux rate constant (*f*) of [^{3}H] GABA (A); [^{14}C]-L-glutamate (B); and [^{3}H] alanine (C). Each curve represents the mean of two to four experiments.

[^{3}H] GABA ; [^{14}C] glutamate ; [^{3}H] alanine

Slices of cord were incubated in a medium containing one of these amino-acids and the slices were then superfused as described previously. The spontaneous efflux of these amino-acids from slices of spinal cord was similar to that described for glycine. The effect of electrical stimulation on the efflux of each of these amino-acids is shown in Fig. 3. The maximum efflux of [^{3}H] GABA and [^{14}C] glutamate following electrical stimulation was approximately 2 and 3 times the prestimulation value respectively. However, electrical stimulation of cord slices loaded with [^{3}H] alanine did not significantly increase the efflux of this amino-acid, the electrically evoked efflux being only 1·2 times the prestimulation efflux (Fig. 3). Each point is the mean of two to four experiments.

[^{14}C] urea

In three experiments, slices of cord were incubated in medium containing [^{14}C] urea and superfused as described previously. Electrical stimulation and high potassium concentrations failed to produce a change in the efflux of urea from cord slices (Fig. 4).

Discussion

The results demonstrate that [^{14}C] glycine is spontaneously released from slices of rat spinal cord. This efflux is multiphasic and a large proportion (60–70%) of the [^{14}C] glycine is retained by the tissue after superfusion for 60 minutes. A similar pattern of release from brain slices occurs with [^{3}H] GABA (Srinivasan, *et al.*, 1969) and with [^{3}H] noradrenaline (Baldessarini & Kopin, 1967). A pattern of multiphasic efflux associated with marked retention is consistent with the [^{14}C] glycine taken up by the tissue during incubation mixing with the endogenous glycine pool, although there is no direct evidence to support this suggestion.

In experiments where the measurement of radioactivity is taken to indicate the release of an amino-acid, it is important to show that the amino-acid is not significantly metabolized. In our experiments, more than 90% of the radioactivity released from the tissue, and more than 98% of the radioactivity remaining in the

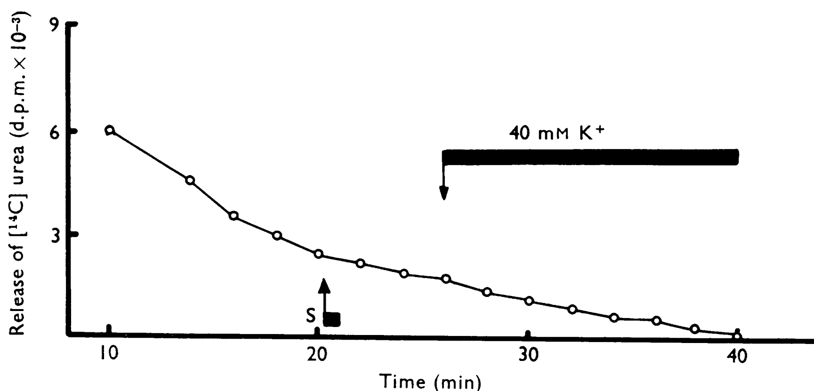

FIG. 4. Effect of electrical stimulation (S) and high potassium (40 mM) on the efflux of [^{14}C] urea from slices of spinal cord. Each point is the mean of three results.

tissue at the end of the experiment, was present as unchanged [^{14}C] glycine. The slow rate of metabolism of glycine taken up by slices of cord confirms the results of similar studies using brain slices, where only negligible metabolism of radioactive amino-acids was observed (Blasberg & Lajtha, 1965; Neal & Pickles, 1969; Srinivasan *et al.*, 1969).

The efflux of [^{14}C] glycine was increased by electrical stimulation of the tissue and by superfusion with medium containing a high concentration of potassium ion, two procedures which cause depolarization of brain slices (Hillman & McIlwain, 1961; Hillman, Campbell & McIlwain, 1963). Electrical stimulation and solutions containing a high potassium concentration stimulate cell metabolism (McIlwain, 1955), but recent studies on the piriform cortex by McIlwain & Snyder (1970), in which the evoked release of putative transmitter substances and cell metabolism were studied simultaneously, suggest that the release of transmitter substances produced by electrical stimulation and high potassium concentrations is not merely a consequence of metabolic changes in the tissue produced by these procedures.

In our experiments, a significant evoked release of glycine was obtained from slices of spinal cord, but similar experiments failed to show any significant evoked increase in the efflux of this amino-acid from slices of rat cerebral cortex or guinea-pig piriform cortex (Mitchell, Neal & Srinivasan, 1969; McIlwain & Snyder, 1970). These apparent differences may be due to the different areas studied, or they may be merely due to differences in experimental techniques. In particular, the type of electrical stimulation seems to be important. Thus, although Katz, Chase & Kopin (1969) found that the effluxes of all the amino-acids they studied were increased by electrical stimulation, Mitchell *et al.* (1969) found that the effluxes of only [^{3}H] GABA and [^{14}C] glutamate were significantly increased.

The increased efflux of [^{14}C] glycine produced by potassium depolarization, and that evoked by electrical stimulation, were not significantly reduced when calcium-free medium was used to superfuse the tissue. Similarly, the electrically evoked release of [^{3}H] GABA and [^{3}H]-5-hydroxytryptamine from brain slices is unaffected by the absence of calcium ions from the medium (Srinivasan *et al.*, 1969; Katz *et al.*, 1969), but the release of [^{3}H] noradrenaline from brain slices is dependent on the presence of calcium ions in the medium (Baldessarini & Kopin, 1967). Although all known neurosecretory processes seem to be dependent on the presence of calcium ions, the lack of calcium dependence of the electrically evoked efflux of glycine from the cord does not necessarily preclude it from having a neurotransmitter function, since, although the release of [^{3}H] GABA from cortical slices also showed lack of calcium dependence, the neurally evoked release of GABA from the surface of the cerebral cortex *in vivo* was markedly reduced in the absence of calcium ions (Iversen, Mitchell, Neal & Srinivasan, 1970). Thus, the use of nervous tissue *in vitro* as a model for the study of central neurotransmitter release may have severe limitations. However, although there may be important differences in the release mechanisms of amino-acids from neural tissue *in vitro* and *in vivo*, the effect of electrical stimulation in evoking an increased efflux of [^{14}C] glycine from slices of cord is not due to an indiscriminate increase in membrane permeability since it has no significant effect on the efflux of [^{3}H] alanine or [^{14}C] urea.

The efflux of [^{3}H] GABA and [^{14}C] glutamate from slices of cord were both significantly increased by electrical stimulation, the maximum evoked efflux of these amino-acids being similar to that of glycine. These amino-acids have powerful

effects when applied by iontophoresis to nerve cells, and are believed to be transmitter substances in the central nervous system (Krnjević, 1970). Electrical stimulation of brain slices may also cause an increased release of amino-acids with no suspected neurohumoral function (Katz *et al.*, 1969) but it seems that the amino-acids with the most powerful actions on neurones are also those which are most effectively released from brain slices by electrical stimulation (Mitchell *et al.*, 1969).

Although studies performed on the release of putative transmitter substances from slices of nervous tissue *in vitro* must be interpreted with caution, the results of our experiments are not inconsistent with the suggestion that glycine may be an inhibitory transmitter substance in the mammalian spinal cord. Evidence that glycine is released from nervous tissue showing more structural integrity, *in vitro,* has recently been presented by Aprison (1970), who has shown that the release of [^{14}C] glycine from the superfused toad cord is increased by electrical stimulation of the dorsal roots.

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