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# EFFECT OF DEPOLARIZING AGENTS ON THE $Ca^{2+}$-INDEPENDENT AND $Ca^{2+}$-DEPENDENT RELEASE OF [$^3$H]GABA FROM SHEEP BRAIN SYNAPTOSOMES

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**Abstract**—The purpose of the present study was to compare the effects of several depolarizing agents on both the membrane potential and on the release of [$^3$H] γ-aminobutyric acid (GABA) from sheep brain cortex synaptosomes. We examined the effects of KCl, 4-aminopyridine (4-AP), veratridine, ouabain and tetraphenylphosphonium cation ($TPP^+$) on $Ca^{2+}$-independent (carrier-mediated) and $Ca^{2+}$-dependent (exocytotic) release. We found that, in the absence of $Ca^{2+}$, KCl at 40 mM releases 7.57 ± 0.65%, veratridine at 50 μM releases 45.85 ± 2.48%, ouabain at 1 mM releases 8.62 ± 0.93% and $TPP^+$ at 1 mM releases 4.09 ± 0.37% of the total accumulated neurotransmitter, provided that the external medium contains $Na^+$. These are about the maximal values of release obtained with each depolarizing agent in a $Na^+$ medium and in the absence of $Ca^{2+}$. Replacing external $Na^+$ with choline blocks the release observed in the presence of the depolarizing agents in the absence of $Ca^{2+}$, and this divalent ion can increase [$^3$H]GABA release only for $K^+$ or 4-AP. Synaptosomal depolarization requires $Na^+$ except for $K^+$ depolarization. Furthermore, although $Ca^{2+}$ stimulates the release of [$^3$H]GABA due to $K^+$ depolarization (13.56 ± 0.44%) or due to 4-AP (4.26 ± 0.51%), it inhibits the release due to the other depolarizing agents. The amount of [$^3$H]GABA released by 4-AP in $Na^+$ medium (4.26 ± 0.51%) is similar to that induced by KCl in the presence of $Ca^{2+}$ in the absence of $Na^+$ (3.39 ± 0.29%) which represents only exocytotic release. This suggests that the $Ca^{2+}$-dependent exocytotic release of [$^3$H]GABA can be specifically induced by 4-AP in a $Na^+$ medium, or by KCl in the absence of $Na^+$, as reported by us earlier. The observation that $Ca^{2+}$ inhibits the $Ca^{2+}$-independent release is of interest because it suggests that $Ca^{2+}$ may modulate the release of cytoplasmic GABA probably by inhibiting the carrier-mediated release of GABA. It is of interest as to whether $Ca^{2+}$ regulation depends on intracellular $Ca^{2+}$.

*In vitro* studies have demonstrated that γ-aminobutyric acid (GABA‡) can be released from central synaptic terminals by two distinct mechanisms: one mechanism is $Ca^{2+}$ independent and occurs by thermodynamic reversal of the GABA carrier whereas the other is $Ca^{2+}$ dependent and involves exocytosis from the synaptic vesicles [1–4].

The release of GABA has normally been induced by high KCl concentrations [5–14], veratridine [15–19], ouabain [20–22] and 4-AP [23, 24]. All these agents are known to induce membrane depolarization: KCl by decreasing the $K^+$ gradient, veratridine by opening voltage-sensitive $Na^+$ channels inducing an influx of $Na^+$ ions into synaptosomes [25] and ouabain by inhibiting the $Na^+$, $K^+$-ATPase, which also causes intracellular accumulation of $Na^+$ [26, 27]. 4-aminopyridine (4-AP), a $K^+$ channel antagonist, prolongs the action potential and the period of $Ca^{2+}$ entry through the voltage-dependent $Ca^{2+}$ channels which remain open for an extended period [28]. Under the experimental conditions used in most of these studies, the mechanism of GABA release was referred to as $Ca^{2+}$ dependent, $Ca^{2+}$ independent or as including both components.

The purpose of the present study was to systematically compare the effects of various depolarizing conditions on [$^3$H]GABA release from sheep brain cortex synaptosomes in which we had studied previously in detail the $Ca^{2+}$-dependent and the $Ca^{2+}$independent release of GABA [11, 21, 22]. In previous studies we used predominantly $K^+$ as a depolarizing agent; only occasionally were the other depolarizing agents tested. We examined here, under similar conditions the effects of KCl, 4-AP, veratridine, ouabain and $TPP^+$ on the $Ca^{2+}$-dependent and $Ca^{2+}$-independent release measured both in the presence and in the absence of $Na^+$. The results bring up important fundamental differences in the mechanisms of stimulus–secretion coupling controlled by the different depolarizing agents. Only $K^+$ and 4-AP are capable of coupling changes in membrane potential to exocytotic $Ca^{2+}$-dependent release, but all depolarizing agents influence carrier-mediated [$^3$H]GABA release which seems to be inhibited by

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‡ Abbreviations: GABA, γ-aminobutyric acid; 4-AP, 4-aminopyridine; $TPP^+$, tetraphenylphosphonium cation.

$Ca^{2+}$. It is not clear whether a rise in intracellular $Ca^{2+}$ concentration is necessary for this inhibition of the GABA carrier.

## MATERIALS AND METHODS

*Isolation of synaptosomes.* Synaptosomes were isolated from sheep brain cortex according to the method of Hajos [29] slightly modified by Carvalho and Carvalho [30]. The final synaptosomal pellets were washed and resuspended in 0.32 M sucrose buffered with 10 mM HEPES–Tris pH 7.4, at a final protein concentration of about 20 mg/mL.

*Release experiments.* The release of [$^3$H]GABA was studied by the superfusion technique described previously [31]. This technique is a modification of that described initially by Raiteri *et al.* [32]. Synaptosomes (0.5 mg protein/mL) were pre-incubated at 30° for 5 min in a $Na^+$ medium, containing (in mM): NaCl 128, KCl 5, $MgCl_2$ 1.2, glucose 10, HEPES–Tris 10 at pH 7.4 and aminooxyacetic acid 0.01. GABA + [$^3$H]GABA (0.5 $\mu$M, 0.25 $\mu$Ci) was then added and, after a 15-min labelling period, aliquots at 0.5 mL were aspired and collected on Whatman GF/B filters. The retained synaptosomes were continuously superfused with a $Na^+$ medium for 15 min at a flow rate of 0.3–0.4 mL/min to remove excess radiolabel and the superfusate was discarded. Fractions were then collected directly into liquid scintillation vials at 1-min intervals. After 2 min of spontaneous release, the superfusion medium was changed with various test solutions as described in the legends of the figures. When test solutions contained 40 mM KCl the isoosmolarity was maintained by equivalent reduction of NaCl or choline ion concentration. The composition of choline was identical to the $Na^+$ medium except that the NaCl was replaced by choline chloride. Under these conditions (experiments with choline media) the synaptosomal samples, loaded with [$^3$H]GABA, were superfused with $Na^+$ medium during 12 min and then with choline medium before the addition of KCl or 4-AP. The radioactivity in each sample and that remaining in the filters was determined by liquid scintillation fluid. The amount of [$^3$H]GABA release per fraction is expressed as a percentage of the total radioactivity (radioactivity released from min 16 to 24 of superfusion plus that remained associated with the synaptosomes at the end of superfusion). Total evoked release (histograms) represents the release above baseline ($Na^+$ or choline medium) from min 18 to 23.

*Membrane potential measurements.* The membrane potential of synaptosomes was determined from the accumulation of tetraphenylphosphonium ($TPP^+$) as described previously [21] except that 5 $\mu$M $TPP^+$ was used in the assay medium. The values given for the membrane potential of synaptosomes were determined after correcting for $TPP^+$ taken up in the absence of $K^+$ gradient (in a medium containing 133 mM KCl) which gives approximately the contribution of mitochondrial membrane potential and that of $TPP^+$ binding for the total $TPP^+$ accumulation.

*Statistical analysis.* All results are expressed as means ± SEM values of the number or experiments indicated. Statistical evaluation of differences between means was determined using the two tailed student's *t*-test and a probability level of $P < 0.05$ was considered to be statistically significant.

*Protein assay.* The amounts of protein from synaptosomes were determined by the Biuret method as described by Layne [33] using bovine albumin as the standard.

*Materials.* [$^3$H]GABA (sp. act. = 65 Ci/mmol) was obtained from Amersham International, (Amersham, U.K.). Aminooxyacetic acid, veratridine, 4-AP and ouabain were supplied by the Sigma Chemical Co. (Poole, U.K.). The other reagents used were of analytical grade.

## RESULTS

### *Effect of depolarizing agents on plasma membrane potential*

In Fig. 1 we report the effect of depolarizing agents, which induce the release of [$^3$H]GABA, on the plasma membrane potential of synaptosomes in $Na^+$ (−52.8 ± 0.74 mV) or choline medium (−56.58 ± 1.91 mV). Membrane depolarization was measured from the efflux of $TPP^+$ previously accumulated to steady state by polarized synaptosomes (about 5 min). KCl at 40 mM, 1 mM aminopyridine, 50 $\mu$M veratridine and 1 mM ouabain, when added in the presence of external $Na^+$, depolarized the synaptosomes to a value of −27.41 ± 2.03 mV, −50.73 ± 1.87 mV, −37.92 ± 2.02 mV and −39.97 ± 1.37 mV, respectively (Fig. 1A). Note that the kinetics of the depolarization as measured by the $TPP^+$ efflux are different for the several substances tested, indicating that the mechanisms responsible for membrane depolarization are different. In $Na^+$-free medium (Fig. 1B) only $K^+$ was able to induce membrane depolarization (to a value of −28.03 ± 2.09 mV).

### *Release of [$^3$H]GABA in $Na^+$ medium: $Ca^{2+}$-dependent and $Ca^{2+}$-independent release*

Figure 2 shows the effect of $K^+$ depolarization on [$^3$H]GABA release from synaptosomes. In the presence of 1 mM $Ca^{2+}$, KCl at 40 mM evoked the release of 13.56 ± 0.43% (N = 7) of the total [$^3$H]-GABA initially accumulated by synaptosomes. The removal of extracellular $Ca^{2+}$ decreased the total amount of [$^3$H]GABA released by about 60% (7.57 ± 0.66% of total/5 min, N = 7). Control studies showed that the amount of [$^3$H]GABA released during the superfusion with $Na^+$ medium, just before depolarization (min 16–18), was not significantly affected by the presence of $Ca^{2+}$ (result not shown).

4-AP also induced the release of [$^3$H]GABA provided that $Ca^{2+}$ was present in the external medium (Fig. 3). However, the amount of [$^3$H]-GABA released by 4-AP (4.25 ± 0.5% of total/5 min, N = 5) was lower than the $Ca^{2+}$-dependent release induced by KCl depolarization (7.57 ± 0.66% of total/5 min, N = 7).

The results depicted in Figs 4 and 5 show that the depolarizing agents veratridine, ouabain and $TPP^+$ also induced release of [$^3$H]GABA in the absence of $Ca^{2+}$, but this $Ca^{2+}$-independent release was

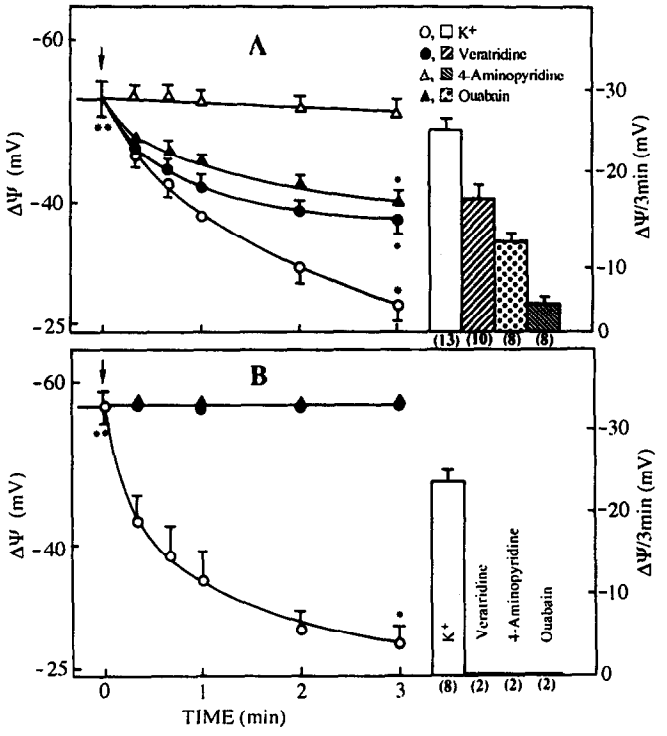


Fig. 1. Comparative effects of KCl, veratridine, ouabain and 4-AP on synaptosomal plasma membrane potential. Membrane potential (in mV) was determined by following the uptake of $TPP^+$ by synaptosomes in $Na^+$ (A) or in choline chloride medium (B) supplemented with 5 $\mu$M $TPP^+$, as described in Materials and Methods. Where indicated, 40 mM KCl, 50 $\mu$M veratridine, 1 mM ouabain or 1 mM 4-AP was added. Histograms represent the difference between the values of membrane potential determined immediately before the addition of the test substances and the membrane potential 3 min later. Data are means ± SEM (bars) for the number of experiments shown in parentheses.* Values significantly different ($P < 0.01$) from the value of membrane potential in $Na^+$ or choline medium.**

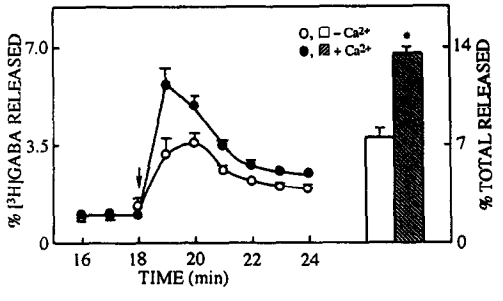


Fig. 2. Effect of KCl on synaptosomal [$^3$H]GABA release measured in the absence or in the presence of $Ca^{2+}$. Synaptosomes (0.5 mg/mL) were preincubated with 0.5 $\mu$M of [$^3$H]GABA, at 30°, for 15 min and then were superfused with $Ca^{2+}$-free $Na^+$ medium, as described in Materials and Methods. Where indicated by the arrow, the medium was replaced with medium containing 40 mM KCl + 93 mM NaCl, in either the absence of added $Ca^{2+}$ (○) or in the presence of 1 mM $Ca^{2+}$ (●). Histogram represents the total amount of [$^3$H]GABA released during 5 min (from min 18 to 23) minus the radioactivity released during the same period in $Na^+$ medium. Data are means ± SEM (bars) from seven independent experiments.* Value significantly different ($P < 0.01$) from respective control obtained in the absence of $Ca^{2+}$.

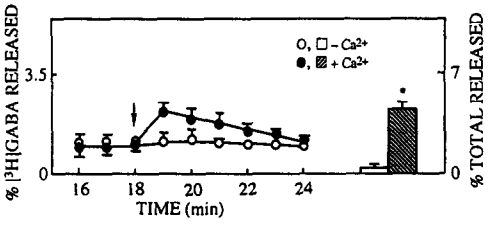


Fig. 3. Effect of 4-AP on synaptosomal [$^3$H]GABA release measured in the absence or in the presence of $Ca^{2+}$. Synaptosomes labeled with [$^3$H]GABA were superfused with $Ca^{2+}$-free $Na^+$ medium, as described in Materials and Methods. Where indicated by the arrow, 1 mM 4-AP was added to the $Na^+$ superfusion medium in either the absence of added $Ca^{2+}$ (○) or in the presence of 1 mM $Ca^{2+}$ (●). Histogram represents the total amount of [$^3$H]GABA released during 5 min (from min 18 to 23) minus the radioactivity released during the same period in $Na^+$ medium. Data are means ± SEM (bars) from seven independent experiments.* Value significantly different ($P < 0.01$) from respective control obtained in the absence of $Ca^{2+}$.

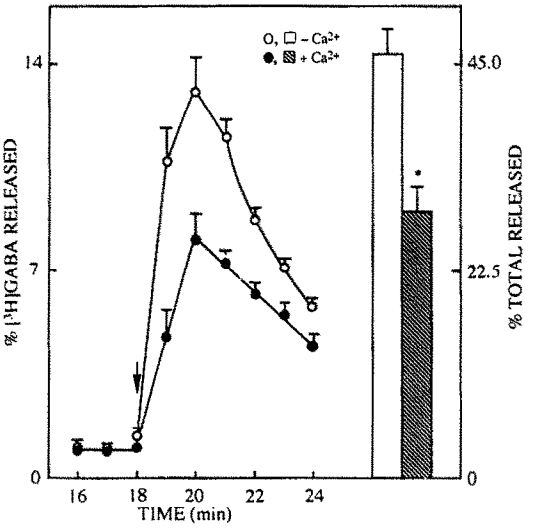


Fig. 4. Effect of veratridine on synaptosomal [$^3$H]GABA release measured in the absence or in the presence of $Ca^{2+}$. Synaptosomes labeled with [$^3$H]GABA were superfused with $Ca^{2+}$-free $Na^+$ medium, as described in Materials and Methods. Where indicated by arrow, 50 $\mu$M veratridine was added to the $Na^+$ superfusion medium in either the absence of added $Ca^{2+}$ (○) or in the presence of 1 mM $Ca^{2+}$ (●). Histogram represents the total amount of [$^3$H]-GABA released during 5 min (from min 18 to 23) minus the radioactivity released during the same period in $Na^+$ medium. Data are means ± SEM (bars) from five or six independent experiments.* Value significantly different ($P < 0.01$) from respective control obtained in the absence of $Ca^{2+}$.

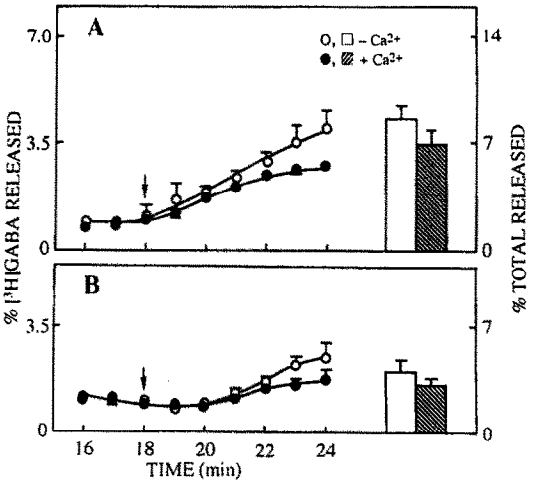


Fig. 5. Effect of ouabain or $TPP^+$ on synaptosomal [$^3$H]-GABA release measured in the absence or in the presence of $Ca^{2+}$. Synaptosomes labeled with [$^3$H]GABA were superfused with $Ca^{2+}$-free $Na^+$ medium, as described in Materials and Methods. Where indicated by the arrow, 1 mM ouabain (A) or 1 mM $TPP^+$ (B) was added to the $Na^+$ superfusion medium in either the absence of added $Ca^{2+}$ (○) or in the presence of 1 mM $Ca^{2+}$ (●). Histogram represents the total amount of [$^3$H]GABA released during 5 min (from min 18 to 23) minus the radioactivity released during the same period in $Na^+$ medium. Data are means ± SEM (bars) from four independent experiments for ouabain and seven for $TPP^+$. The amount of [$^3$H]-GABA released in the presence of $Ca^{2+}$ did not show a statistically significant difference when compared with that obtained in the absence of $Ca^{2+}$ ($P > 0.05$).

partially inhibited by $Ca^{2+}$, contrary to the effect of $Ca^{2+}$ on the [$^3$H]GABA release induced by $K^+$ depolarization or by 4-AP referred to above. Figure 4 shows that the presence of $Ca^{2+}$ decreased the amount of [$^3$H]GABA released by veratridine from a value of 45.86 ± 2.48% (N = 6) to a value of 28.89 ± 2.74% (N = 5) of the total accumulated [$^3$H]GABA, which corresponds to an inhibition of about 40%. This inhibition is less evident, and not significantly different from the control measured in the absence of $Ca^{2+}$, for the case of [$^3$H]GABA release by ouabain or by $TPP^+$. Thus, Fig. 5 shows that $Ca^{2+}$ inhibited the release induced by ouabain from 8.26 ± 0.93% to 6.94 ± 0.92% of total and inhibited the release induced by $TPP^+$ from 4.09 ± 0.8% to 3.21 ± 0.37% of total.

The rate of release observed in the presence of $K^+$ or 4-AP was different from that observed in the presence of veratridine, ouabain and $TPP^+$. Figures 2 and 3 show that $Ca^{2+}$ evoked a rapid release of [$^3$H]GABA during the first minute, which immediately began to decay, even with $K^+$ or 4-AP still present. In contrast, the rate of release induced by ouabain and $TPP^+$ (Fig. 5), measured either in the absence or in the presence of $Ca^{2+}$, was considerably lower, and the release increased during the experimental period (min 18–24) without returning to basal level. It is interesting that the release induced by veratridine was also transient as in the case of $K^+$ or 4-AP, but this transient response to veratridine probably indicates that a large fraction of the [$^3$H]GABA (45%) was released when the synaptosomes were first challenged with veratridine, thus depleting the stores of [$^3$H]GABA. With respect to the response to $Ca^{2+}$, the release of [$^3$H]GABA induced by veratridine was similar to the release induced by ouabain or $TPP^+$, i.e. $Ca^{2+}$ inhibited the release (Fig. 4).

### *Release of* [$^3$H]*GABA in* $Na^+$*-free medium*

Previous results from our laboratory [11, 22] show that in a $Na^+$-free medium (choline medium) $K^+$ depolarization-dependent release of [$^3$H]GABA is absolutely dependent on external $Ca^{2+}$, which suggests that under these conditions all release occurs by exocytosis. The results of Fig. 6 confirm this and show that 4-AP (1 mM) has an effect on [$^3$H]GABA release similar to that of $K^+$, i.e. the release was absolutely dependent on $Ca^{2+}$. However, the same dependency on $Ca^{2+}$ was also observed in a $Na^+$ medium in the case of 4-AP, but not in the case of $K^+$ depolarization (Figs 2 and 3).

In the absence of external $Na^+$, but in the presence of $Ca^{2+}$, 40 mM KCl caused the release of 3.39 ± 0.29% (N = 7) and 4-AP caused the release of 2.30 ± 0.44% of the total accumulated [$^3$H]-GABA (N = 6). The observation that the total

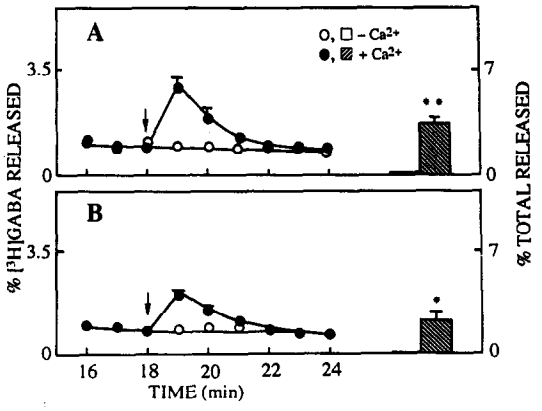


Fig. 6. $Ca^{2+}$-dependent [$^3$H]GABA release from synaptosomes induced by KCl and 4-AP in the absence of $Na^+$ (choline medium). Loaded synaptosomes were treated as described in the legend to Fig. 1, except that at 12 min of superfusion washing period, the $Na^+$ medium was replaced with choline chloride medium containing 1 mM $Ca^{2+}$. Where indicated by the arrow, the choline medium was replaced by a medium containing 40 mM KCl + 90 mM choline chloride (A) or by a similar medium containing 1 mM 4-AP (B) in either the absence of added $Ca^{2+}$ (○) or in the presence of 1 mM $Ca^{2+}$ (●). Histogram represents the total amount of [$^3$H]GABA released during 5 min (from min 18 to 23) minus the radioactivity released during the same period in choline medium. Data are means ± SEM (bars) from six or seven independent experiments.* $P < 0.02$, when compared with the value of [$^3$H]GABA released by KCl.**

release of [$^3$H]GABA induced by 4-AP in choline medium (2.30 ± 0.44%, Fig. 6B) was lower than in the presence of $Na^+$ (4.25 ± 0.5%, Fig. 3) suggests that, besides the $Ca^{2+}$ dependence, the effect of 4-AP may also be partially $Na^+$ dependent. Veratridine or ouabain, in $Na^+$-free medium, did not have any significant effect on the release of [$^3$H]GABA, measured in the absence or in the presence of $Ca^{2+}$ (not shown). Thus, in the case of these two depolarizing agents, $Na^+$ is an absolute requirement for [$^3$H]GABA release. As observed above, the $Na^+$ requirement exists in the case of $K^+$ for $Ca^{2+}$-independent release, but not for $Ca^{2+}$-dependent release (Figs 2 and 6), and 4-AP is the only agent tested which causes $Ca^{2+}$-dependent release only of [$^3$H]GABA (Figs 3 and 6B).

## DISCUSSION

The purpose of this study was to compare the effects of various depolarizing agents, normally used to induce the release of neurotransmitters, on the release of [$^3$H]GABA from sheep brain cortex synaptosomes. These studies extended those reported previously by our laboratory [11, 21–22], and are elucidative of the two distinct mechanisms triggered by different agents by which [$^3$H]GABA can be released: one is $Ca^{2+}$ dependent and involves exocytosis; the other is $Ca^{2+}$ independent and occurs after the reversal of the $Na^+$-dependent carrier of GABA.

It has been reported that the $Ca^{2+}$-independent release of [$^3$H]GABA can be induced by agents that depolarize the plasma membrane [2]. Our results confirm this and indicate that the depolarizing agents KCl, veratridine, ouabain and $TPP^+$, whatever their mode of action on the membrane potential, induced the release of [$^3$H]GABA initially accumulated by synaptosomes. An elevated KCl concentration that decreases the $K^+$ gradient and the inward $Na^+$ gradient ($Na^+$ was replaced by $K^+$ outside) and veratridine, which induces an influx of $Na^+$ through the opening of voltage-sensitive $Na^+$ channels, lead to a stable depolarization of synaptosomes and induced the release of [$^3$H]GABA.

The observation that in the presence of veratridine the amount of [$^3$H]GABA released (about 50% of the total accumulated, Fig. 4) was higher than that observed in the presence of KCl (8% of the total) suggests that, in addition to depolarizing the membrane, the $Na^+$ which enters probably changes the internal $Na^+$ concentration which will have an additional effect on the release of [$^3$H]GABA. Thus, the superfusion of synaptosomes with veratridine, in the presence of $Na^+$, could raise the internal $Na^+$ concentration and increase the carrier-dependent [$^3$H]GABA release both by depolarizing the membrane and by loading the cytoplasm with $Na^+$. The entry of $Na^+$ may provide the substrate for the GABA carrier in the intracellular side of the membrane [8, 34, 35]. It should also be noted that there is not a direct relationship between the level of membrane depolarization and the amount of [$^3$H]GABA released for the various depolarizing agents.

The effect of ouabain on release might be also explained by its effect on membrane potential (Fig. 5, [21]). The drug causes a slow depolarization of the synaptic plasma membrane [21, 36, 37] which may be due to its direct action on the $Na^+$, $K^+$-ATPase. The inhibition of the enzyme leads to a reduction of the membrane potential which favors the efflux of GABA through the $Na^+$-coupled GABA carrier.

The lipophilic cation $TPP^+$ was used as a different tool to depolarize the synaptosomal membrane. Since the plasma membrane is permeable to $TPP^+$ [36] it is expected that a high concentration of $TPP^+$ (1 mM) will depolarize completely the plasma membrane. Besides its effect as a depolarizing agent, $TPP^+$ seems to affect the $Na^+$, $K^+$-ATPase (0.1 mM inhibits the activity of the enzyme by 50%; manuscript in preparation) which suggests that the $Ca^{2+}$-independent release of [$^3$H]GABA observed in the presence of $TPP^+$ could be mediated by its effect on the $Na^+$, $K^+$-ATPase activity.

The observation that when $Na^+$ was replaced by choline only KCl could induce the depolarization of synaptosomes is in accordance with the idea that the influx of $Na^+$ is an important requirement for depolarization and consequently for reversing the GABA carrier. The $Ca^{2+}$-independent release of GABA was inhibited when the GABA carrier was selectively blocked by the non-transportable GABA uptake inhibitor SKF 89976 [38], which confirms the role of GABA carrier in mediating the $Ca^{2+}$-independent release induced by several agents in

various neuronal preparations [4, 39, 40]. Nipecotic acid has also been used as a competitive inhibitor of the carrier, but it has some disadvantages since it is transported by the carrier and may cause GABA release by way of heteroexchange [13, 41–43].

It has been suggested that the mechanism of neurotransmitter release in the absence of extracellular $Ca^{2+}$ involves the mobilization of $Ca^{2+}$ from intracellular pools. The increase in the intracellular $Na^+$ concentration, as a result of the entry of $Na^+$ ions, could activate the $Na^+/Ca^{2+}$ exchanger present in mitochondria or other intracellular organelles [44–46]. Mobilization of $Ca^{2+}$ from intracellular stores was also proposed for the $Ca^{2+}$-independent release of other neurotransmitters [37, 47, 48].

The presence of $Ca^{2+}$ in the extracellular medium inhibits the $Ca^{2+}$-independent release of [$^3$H]GABA observed in the presence of veratridine, ouabain and $TPP^+$. The release induced by veratridine was inhibited by about 40% when the synaptosomes were superfused with medium containing 1 mM $Ca^{2+}$ which agrees with previous observations that the release of GABA induced by veratridine in $Ca^{2+}$-free medium was higher than the release measured in $Ca^{2+}$-containing medium [15, 17–19, 49]. The hypothesis suggested by Levi *et al.* [15] that this inverse $Ca^{2+}$ dependence may be due to $Ca^{2+}$ inhibiting the veratridine activation of $Na^+$ channels was confirmed by Sihra *et al.* [18] who found that the level of membrane depolarization induced by veratridine was lower in the presence of $Ca^{2+}$. However, under our experimental conditions, the presence of $Ca^{2+}$ did not affect the synaptosomal membrane potential [22]. Recently, Bernath and Zigmond [50] proposed that in the absence of extracellular $Ca^{2+}$, $Ca^{2+}$ channels serve to permit the influx of $Na^+$, which will promote the $Ca^{2+}$-independent release of GABA through the carrier. The presence of $Ca^{2+}$ should increase the competition between $Na^+$ and $Ca^{2+}$ for $Na^+$ entry through the $Ca^{2+}$ channels, and thus decrease the influx of $Na^+$ ions. Decreasing the intracellular $Na^+$ concentration would be expected to increase the electrochemical gradient of $Na^+$ with a consequent decrease in GABA release. Another possible explanation for the inhibitory effect of $Ca^{2+}$ on the $Ca^{2+}$-independent release is that $Ca^{2+}$ may be inhibiting the GABA carrier. At present, it is not known if this effect is specific for $Ca^{2+}$.

It has been established that the exocytotic release of GABA is initiated by an elevation in the cytosolic free $Ca^{2+}$ following membrane depolarization and the opening of voltage-sensitive $Ca^{2+}$ channels [12, 14, 51]. We have reported that GABA exocytosis, in the absence of the carrier-mediated component, could be specifically induced by $K^+$ depolarization in choline medium [11, 22]. An interesting finding of this work is that this amount of [$^3$H]GABA released by exocytosis (Fig. 6) is very similar to that released by 4-AP, which releases GABA only in the presence of $Ca^{2+}$ (Figs 3 and 6). 4-AP is known to induce transmitter release from synaptosomes in a $Ca^{2+}$-dependent manner [23, 24, 52]. It has been considered to be a more physiological stimulus than KCl since 4-AP generates spontaneous action potentials which stimulate the synaptosomes in the absence of the sustained membrane depolarization observed in the presence of KCl (Fig. 1, [52, 53]). Nevertheless, we found that the $Ca^{2+}$-dependent release of [$^3$H]GABA induced by 4-AP in $Na^+$ medium was similar to that observed in the presence of 40 mM KCl in choline medium, in spite of the difference in level of membrane depolarization. This suggests that the release induced by 4-AP and $K^+$ could reflect a similar efficiency of the two agents in triggering the $Ca^{2+}$-dependent release of [$^3$H]GABA. It is known that both increase the cytosolic free $Ca^{2+}$ concentration [52, 54, 55].

The observation that in the absence of $Na^+$ the release of [$^3$H]GABA due to 4-AP was inhibited (Fig. 6B) indicates that $Na^+$ and probably the increases in intracellular $Na^+$ concentration are important for 4-AP effect. This result agrees with recent published data showing that TTX inhibits the 4-AP-induced neurotransmitter release [52, 56] and elevation of free intracellular $Ca^{2+}$ concentration [52, 55].

The results obtained in this study indicate that membrane depolarization or the presence of $Na^+$ in the external medium, or both, are essential for the $Ca^{2+}$-independent release of [$^3$H]GABA to occur. The change in membrane potential and the influx of $Na^+$ alter the equilibrium between the influx and the efflux of GABA. It is known that the GABA carrier, which transports GABA using an electrochemical gradient of $Na^+$, is reversible and may be used to release cytoplasmic GABA when the $Na^+$ gradient is reversed [57]. It is not clear to what extent this $Ca^{2+}$-independent, $Na^+$-dependent GABA release occurs under physiological conditions, although some release of cytoplasmic GABA of the nerve terminal probably occurs during the normal action potential. The presence of external $Na^+$ is not required for the $Ca^{2+}$-dependent release of [$^3$H]GABA due to $K^+$ depolarization. Under these conditions (choline medium), $K^+$ depolarization is likely to open $Ca^{2+}$ channels and the release of [$^3$H]-GABA occurs only by exocytosis without the carrier-mediated release component, since the carrier requires $Na^+$ [57–59]. The observation that, in the presence of $Na^+$, the carrier-mediated release is similar to the exocytotic release suggests that the large depolarization induced by 40 mM KCl could also activate the voltage-sensitive $Na^+$ channels which allowing the influx of $Na^+$ into synaptosomes. In fact, results previously published showed that when synaptosomes are depolarized by 10 mM KCl the exocytotic component of the release is much more important than the carrier-mediated component [22].

In conclusion we have shown that the $Ca^{2+}$-independent, $Na^+$-dependent release of [$^3$H]GABA from synaptosomes can be induced by agents that contribute to depolarization of the synaptic plasma membrane. Membrane depolarization seems to be an absolute requirement for the $Ca^{2+}$-dependent and the $Ca^{2+}$-independent mechanisms of GABA release. The $Ca^{2+}$-dependent exocytotic release of [$^3$H]-GABA can be specifically induced by 4-AP in a $Na^+$ medium, or by $K^+$ depolarization in the absence of

$Na^+$. Under these conditions, no $Ca^{2+}$ independent release occurs. The observation that calcium inhibits the $Ca^{2+}$-independent release induced by veratridine, ouabain or $TPP^+$ is of interest because it suggests that, besides its effect on exocytosis, $Ca^{2+}$ may modulate the release of cytoplasmic GABA.

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