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Effects of depressant drugs and sulfhydryl reagents on the transport of calcium by isolated nerve endings

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One effect of neuronal depolarization is to open the synaptic membrane channels that allow calcium to flow down the electrochemical gradient and enter the nerve terminal [1]. The resultant increase in the concentration of free calcium in the nerve terminal leads to the release of neurotransmitter substances [2]. To terminate the release of neurotransmitter, the excess calcium in the nerve ending must be either sequestered or removed. The mechanisms involved in the removal of calcium are not well understood, but a high affinity, non-mitochondrial, ATP-dependent calcium uptake has been described recently in lysed synaptosomes [3]. This intrasynaptosomal calcium transport system may play a significant role in buffering the intracellular calcium concentration of nerve endings [3]. Since synaptic function is likely to depend upon both the depolarization-dependent influx of calcium and the ATP-dependent sequestration of calcium, we have compared the effects of *in vitro* addition of various drugs on both of these processes in isolated synaptosomes to evaluate the role of calcium transport in the neuropharmacological effects of these drugs. Of particular interest were the observations of increased spontaneous release of neurotransmitters from nerve terminals exposed to ethanol [4–6], barbiturates [7] or sulfhydryl reagents [8–10]. These findings have been taken as evidence to indicate that these agents directly affect the membrane processes responsible for the release of neurotransmitters. However, it is possible that these compounds might also increase the release of neurotransmitters indirectly by inhibiting the intraneuronal sequestration of calcium. In addition, we were interested in the similarities between the ATP-dependent uptake of calcium by brain membranes and by muscle sarcoplasmic reticulum. Accordingly we selected several sulfhydryl reagents and other drugs which are known to inhibit the transport of calcium by sarcoplasmic reticulum and studied their effects on ATP-dependent calcium transport by lysed synaptosomes.

Male Sprague–Dawley rats (200–250 g) (Charles River Breeding Laboratories, Wilmington, MA) were decapitated, and synaptosomes were isolated from whole brain homogenates as described previously [11]. ATP-dependent and potassium-stimulated calcium uptakes were assayed as described by Blaustein *et al.* [3,12]. For both assays, the synaptosomal band was removed from the Ficoll gradient, slowly diluted 5-fold with ice-cold calcium-free Na^+-$5K^+$ (132 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 10 mM glucose, and 20 mM Tris; pH 7.4) and pelleted at 15,000 *g* for 6 min. For the ATP-dependent uptake, the pellet was then resuspended in hypotonic lysis solution [1.3 mM $MgCl_2$, 2.4 mM NaH_2PO_4 and 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (Hepes), pH 7.4] and incubated at 37° for 3 min to disrupt the synaptosomes. This suspension was placed on ice and diluted with an equal volume of a solution containing 362 mM KCl, 1.95 mM $MgCl_2$, 3.6 mM NaH_2PO_4, 30 mM Hepes, 0.5 mM NaN_3, 0.5 mM dinitrophenol and 5 μg/ml oligomycin, pH 7.4. Aliquots (0.8 ml; 0.4–0.8 mg protein) were added to tubes containing 0.2 ml of various drug solutions or distilled water (control) and incubated at room temperature for 15 min, followed by incubation at 37° for 1 min. At this point, 1 ml of the ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA)-buffered ^{45}Ca solution (100 μM $CaCl_2$, 0.5 μCi/ml $^{45}CaCl_2$, 107 μM EGTA, 145 mM KCl, 1.3 mM $MgCl_2$ and 0 or 2 mM MgATP) was added and incubation was continued for 5 min at 37°. The final concentration of free calcium was calculated to be 2 μM [13]. The uptake was terminated by rapid filtration through GF/C discs which were washed three times with 5 ml of a solution containing 145 mM KCl, 1.2 mM $CaCl_2$ and 1.4 mM $MgCl_2$ The ATP-dependent uptake (ΔATP) was considered to be the difference between the uptake in the absence of ATP (−ATP) and in the presence of ATP (+ATP) (see Table 1). For potassium-stimulated calcium uptake by intact synaptosomes, the synaptosomal pellet was resuspended in Na^+-$5K^+$ (132 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 10 mM glucose, 20 mM Tris and 1.2 mM $CaCl_2$; pH 7.4) to give 1–2 mg/ml protein. Portions (0.5 ml) of this suspension were incubated for 15 min at 37° except for experiments involving ethanol or pentobarbital, which were carried out at 30°. Aliquots of the drug solutions were then added and the incubation was continued for 12 min. Next, 0.5 ml of either a solution of Na^+-$5K^+$ containing ^{45}Ca or a similar solution in which 132 mM KCl was substituted for the NaCl (depolarizing solution) was added. Incubation continued for 1 min and uptake was stopped by the addition of 0.5 ml of an ice-cold EGTA-halting solution (132 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 30 mM Tris and 30 mM EGTA) plus 5.0 ml of calcium-free Na^+-$5K^+$. Membranes were filtered immediately on GF/C discs and washed twice with 10 ml of calcium-free Na^+-$5K^+$. The ^{45}Ca on the discs was determined by liquid scintillation spectrometry. The depolarization-dependent uptake (ΔK^+) was considered to be the difference between the uptake in a low concentration of potassium ($-K^+$) and in a high concentration of potassium ($+K^+$). Protein concentrations were determined by a modification of the phenol method [14].

The results in Table 1 indicate that depressants such as ethanol (800 mM), pentobarbital (0.5 mM) and acetaldehyde (100 mM) significantly inhibited the ATP-dependent uptake, while chlorpromazine (10^{-5} M), diphenylhydantoin (10^{-4} M) and phencyclidine (10^{-5} M) were without effect. Ethanol and the barbiturates had been shown previously to decrease the ATP-dependent calcium uptake in cardiac sarcoplasmic recticulum vesicles [15–17], while

chlorpromazine has been shown both to increase and to decrease [18,19] the uptake by sarcoplasmic reticulum. In contrast to results obtained with the ATP-dependent uptake, the potassium-stimulated uptake was inhibited by pentobarbital, chlorpromazine, diphenylhydantoin, ethanol, phencyclidine and acetaldehyde. These results confirm the inhibitory effects of pentobarbital, chlorpromazine and diphenylhydantoin, which have been reported by others [20–25]. Although Blaustein and Ector [20] reported that 100 mM ethanol did not affect the potassium-stimulated uptake of calcium by synaptosomes, our data indicate that a consistent, dose-dependent inhibition was produced by this drug. With all the depressant drugs, the inhibition of the potassium-stimulated uptake was much greater than that seen with the ATP-dependent uptake. Regarding the local anesthetics, procaine and propranolol reduced the potassium-stimulated influx, while only propranolol inhibited the ATP-dependent calcium uptake. Similarly, the ATP-dependent uptake of calcium by sarcoplasmic reticulum has been reported to be inhibited by anesthetic concentrations of propranolol [26]. The concentrations of ethanol, pentobarbital, propranolol, diphenylhydantoin and chlorpromazine that were effective in inhibiting the ATP-dependent uptake were in the anesthetic range [27].

We also studied the effect of sulfhydryl reagents on the non-mitochondrial ATP-dependent uptake and the potassium-stimulated calcium uptake. In contrast to the depressants and anesthetics, the sulfhydryl reagents inhibited intrasynaptosomal ATP-dependent calcium uptake more strongly than they inhibited potassium-stimulated uptake. Sulfhydryl reagents have also been shown to inhibit the calcium uptake of sarcoplasmic reticulum [28]. The minimal effect of the more specific reagent 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) on potassium-stimulated uptake suggests that sulfhydryl groups, in contrast to the ATP-dependent transport, are not important for the potassium-stimulated uptake. The greater reduction observed with the mercury-containing compounds is probably due to their lower specificity. Not only do they react with thiol groups, but they also may compete with calcium [29] and react with amino, phosphate or carboxyl groups [30]. In regard to the drug effects, since ethanol has been shown to reduce the availability of membrane sulfhydryl groups [31], and since acetaldehyde is known to react with thiol groups [32], it is possible that their inhibitory effects on ATP-dependent calcium transport involve their effects on sulfhydryl groups.

Inhibition of ATP-dependent uptake may account for the increase in spontaneous release of neurotransmitters that has been observed with sulfhydryl reagents and with high concentrations of depressants and anesthetics [4–10]. Inhibition of the intrasynaptosomal ATP-dependent calcium uptake would tend to increase the intracellular calcium concentration and thus result in a greater release of neurotransmitter from the terminal. The drug concentrations which are required to increase the spontaneous release of neurotransmitters are similar to those which were found to inhibit the ATP-dependent uptake. However, interpretation of the effects of ethanol and pentobarbital is complicated by the inhibitory effects of these drugs on the resting (non-depolarized) influx of calcium noted in this study and others [20,25]. This effect would tend to lower the amount of calcium in the nerve ending, perhaps offsetting the inhibitory effect of the drugs on the ATP-dependent sequestration of calcium. In contrast, the sulfhydryl reagents selectively inhibited the ATP-dependent uptake without affecting the resting influx. As discussed above, this effect would be expected to increase the release

Table 1. Effects of *in vitro* addition of drugs and sulfhydryl reagents on ATP-dependent and potassium-stimulated calcium uptake by brain synaptosomes*

Depressants	Conc (M)	−ATP	+ATP	ΔATP	$-K^+$	$+K^+$	ΔK^+
Ethanol	5×10^{-2}	99	95	97	106	90	79†
	10^{-1}	ND‡	ND	ND	91	86	79†
	2×10^{-1}	105	99	99	101	84§	70†
	4×10^{-1}	ND	ND	ND	89	71§	66†
	8×10^{-1}	92	83§	85§	76§	50†	29†
Pentobarbital	5×10^{-4}	102	78§	86§	86†	76†	55†
Acetaldehyde	10^{-1}	102	44†	24†	59†	56†	51†
Chlorpromazine	10^{-5}	88	104	112	86§	61†	45†
Diphenylhydantoin	10^{-4}	105	99	97	84	83§	82§
Phencyclidine	10^{-5}	103	92	96	92	77§	68§
Local anesthetics							
dl-Propranolol	10^{-3}	58†	66†	71†	58†	33†	19†
Procaine	10^{-3}	105	111	114	91	80§	73§
Sulfhydryl reagents							
DTNB	10^{-4}	100	43†	21†	ND	ND	ND
	10^{-5}	100	59†	43†	91	87	85
	10^{-6}	102	93	87§	ND	ND	ND
$HgCl_2$	10^{-5}	75†	25†	0†	77†	66§	60§
N-Ethylmaleimide	10^{-4}	91	61†	42†	ND	ND	ND
p-Chloromercuribenzoate	10^{-4}	57†	24†	5†	ND	ND	ND

* Values are presented as percent of control. Data are from a number of different experiments in which several drug treatments were compared with control preparations. Each experiment consisted of duplicate determinations of three to nine membrane preparations (see text for methodological details). The variation in drug effects between preparations was usually less than 10 per cent. The average control values (± S.E.M.) for all ATP-dependent uptake experiments were 0.31 ± 0.01 (−ATP), 1.21 ± 0.02 (+ATP) and 0.87 ± 0.02 (ΔATP) nmoles calcium/mg protein; N = 24. Control values (± S.E.M.) for the potassium-stimulated calcium uptake experiments were: 3.39 ± 0.20 ($-K^+$), 8.95 ± 0.64 ($+K^+$) and 5.56 ± 0.44 (ΔK^+) nmoles calcium/mg protein; N = 15.

† Significantly different from control, $P < 0.01$.

‡ Not determined.

§ Significantly different from control, $P < 0.05$.

of neurotransmitters, such as acetylcholine.

Alternatively, others have argued against the involvement of calcium in the release of acetylcholine by sulfhydryl reagents. This is based primarily on the observation that the effects of sulfhydryl reagents (e.g. *N*-ethymaleimide and *p*-hydroxymercuribenzoate) are not markedly affected by variations in the extracellular concentration of calcium [8–10]. However, removal of extracellular calcium does not necessarily reduce intracellular stores of calcium or preclude the participation of these stores in neurotransmitter release. In addition, a recent report demonstrated that the ethanol-induced release of norepinephrine from the vas deferens is dependent upon calcium availability [6]. A result apparently inconsistent with an intracellular site of action for sulfhydryl reagents is the observation that a sulfhydryl reagent which does not readily penetrate cell membranes is able to release acetylcholine from synaptosomes [10]. However, this compound appeared to be considerably less potent and to have a slower onset of action than sulfhydryl reagents which rapidly penetrate cell membranes [10], suggesting the possibility of an intracellular action despite limited permeability. These considerations, together with our present results, suggest that changes in intrasynaptosomal storage of calcium may be at least responsible for the effects of sulfhydryl reagents and depressants on the resting release of neurotransmitters.

Inhibition of the potassium-stimulated calcium uptake by depressants would tend to decrease the depolarization-stimulated calcium influx necessary for neurotransmitter release. This would result in a reduction in the release of neurotransmitter from the nerve terminal during stimulation. Indeed, ethanol and pentobarbital, at concentrations similar to those tested in this study, have been shown to inhibit the release of several neurotransmitters from brain tissue [33–35]. The greater sensitivity of the potassium-stimulated calcium uptake may indicate that this process is of greater importance in the mechanism of action of the depressants and anesthetics. It is of interest to note that, after chronic administration of ethanol, both calcium uptake systems display tolerance to the *in vitro* effects of the drug [36].

Our pharmacological data give experimental support to the suggestion of Blaustein *et al.* [3] that the ATP-dependent uptake of calcium by synaptosomes resembles the uptake of calcium by muscle sarcoplasmic reticulum. Both processes were inhibited by similar concentrations of the anesthetics, the depressants and the sulfhydryl reagents, although further studies are required to prove the identity of these two systems. Our data also demonstrate that the depolarization-stimulated uptake of calcium by intact synaptosomes is more sensitive to the inhibitory effects of depressants and anesthetics than is the ATP-dependent intrasynaptosomal calcium transport system. On the other hand, the ATP-dependent uptake is more sensitive to the effects of sulfhydryl reagents than is the depolarization-dependent uptake. These effects appear to be related to the known actions of these drugs on neurotransmitter release.

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