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Catecholamines inhibit Na–Ca ATPase

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Neurotransmitters are released from nerve endings in discrete packets or quanta by a calcium dependent process [1] which is similar to exocytosis [2, 3]. Many studies have shown that within the nerve ending, the neurotransmitters are bound to synaptic vesicles. These vesicles would appear to be storage sites for the neurotransmitters, although there is some doubt as to their role in transmitter release [4]. Vesicles have also been shown to have properties similar to the sarcoplasmic reticulum of muscles, including the ability to sequester calcium ions [5]. One possible role for the vesicles would be that of buffering the internal calcium ion concentration of the nerve ending. The resting calcium concentration of nerve cells is 10^5-fold less than that of the external medium. During the depolarisation of the nerve ending, prior to the release of the transmitter substance, the inward calcium ion movement increases the internal calcium ion concentration from 10^{-7} M to around 5×10^{-6} M [6]. Therefore, it seems likely that if transmitter release is to be effectively controlled, the internal calcium ion concentration will require to be adequately buffered. De Langen and Mulder [6] have suggested that the presynaptic autoregulation of noradrenaline release results from a decrease of the availability of calcium ions for the stimulus–secretion coupling process. In theory, the mechanisms available for maintaining a low calcium ion concentration inside nerve endings are active extrusion (a calcium pump) and an accumulation in subcellular organelles or binding to some cytoplasmic proteins.

Active transport of calcium is sodium dependent and involves the hydrolysis of ATP either directly or indirectly. If noradrenaline regulates its own release by modulating the internal calcium ion concentration of nerve endings, we might therefore expect it either to interfere with the mechanism responsible for the extrusion of the calcium from nerve endings or perhaps modify the binding of calcium to subsynaptic organelles.

Synaptosomes and synaptic membranes were prepared by the method previously described [7]. The ATPase activity of synaptic membranes was studied by measuring the rate of release of inorganic phosphate [8].

The conditions for the assay of Ca-ATPases were 1 mM ATP and 2 mM $CaCl_2$ in 50 mM imidazole buffer, pH 7.4. A sodium stimulated ouabain insensitive ATPase, Na–Ca-ATPase, was found to be present in synaptic membranes and intra-synaptosomal vesicle preparations. The optimum conditions for Na–Ca-ATPase of synaptic membranes were, 37°C, 150 mM NaCl, 2 mM $CaCl_2$ and 1 mM ATP in 50 mM imidazole buffer at pH 7.4.

The kinetic constants of Ca-ATPases were K_{Ca} $1.0 \pm 0.3 \times 10^{-7}$ M, K_m $7.69 \pm 0.17 \times 10^{-5}$ M Ca-ATP and the V_{max} 7.17 ± 0.65 μmoles P_i/mg/hr. The Na–Ca-ATPase kinetic constants were, half max. stimulations 15 mM Na^+ and K_{Ca} $2.02 \pm 0.37 \times 10^5$ M, K_m $5.11 \pm 1.7 \times 10^{-5}$ M Ca-ATP and V_{max} 6.4 ± 1.01 μmoles P_i/mg/hr. The specific activities of these enzymes were 2.01 ± 0.36 and 1.43 ± 0.19 μmoles P_i/mg/hr respectively. 10^{-5} M noradrenaline inhibited synaptic membrane Na–Ca-ATPase by 50%. Adrenaline (10^{-6} M) and dopamine (10^{-5} M) also inhibited this enzyme. The concentration of adrenaline required to inhibit Na–Ca-ATPase by 50% was 1.1×10^{-6} M. In this respect adrenaline was a more potent inhibitor of Na–Ca-ATPase than was noradrenaline or dopamine (Table 1). The extent of the amine inhibition was dependent on the free calcium ion concentration. The K_{Ca} which was $2.02 \pm 0.37 \times 10^{-5}$ M (control) was increased to $6.57 \pm 0.5 \times 10^{-5}$ M in the presence of 10^{-5} M NA. Dopamine (10^{-5} M) similarly increased the K_{Ca} constant to $4.7 \pm 0.3 \times 10^{-5}$ M.

Table 1. The IC_{50} values for catecholamine inhibition of Na–Ca-ATPase

	IC_{50}
Noradrenaline	$1.1 \pm 0.2 \times 10^{-5}$ M
Adrenaline	$0.9 \pm 0.17 \times 10^{-6}$ M
Dopamine	$0.9 \pm 0.23 \times 10^{-5}$ M

IC_{50} values quoted are means ± S.D. of three experiments.

Adrenaline (10^{-6} M): this effect of adrenaline was antagonised by 10^{-6} M phentolamine ($P < 0.01$) and 10^{-5} M propanalol ($P < 0.05$), which might suggest that an adrenergic receptor mediated in the inhibition of Na–Ca-ATPase (Table 2). The effects of these adrenergic receptor antagonists on the inhibition of Na–Ca-ATPase by noradrenaline and dopamine were studied. In this respect the inhibition of Na–Ca-ATPase by dopamine was not significantly influenced by phentolamine or propanalol. The inhibitory effect of noradrenaline was antagonised by 10^{-6} M ($p < 0.01$) phentolamine and 10^{-5} M propanalol ($P < 0.05$).

The possibility that this increase in the K_{Ca} value might reflect an increase Ca binding to the membrane was studied. Synaptic membranes were incubated at a variety of temperatures with radiocative ^{45}Ca. The incubation media always contained 10^{-5} M EGTA and the calcium ion concentration ($10^{-6} \rightarrow 5 \times 10^{-5}$ M ^{45}Ca) was varied using the data of Nanninga [9]. The membranes were isolated from the incubation medium by millipore filtration (0.45 μ diameter). Filters were washed twice with 3 ml of 5×10^{-5} M EGTA solution. The ^{45}Ca content of the membranes was estimated by liquid scintillation spectroscopy using Filter Count (Packard Instruments Ltd.). The specific binding of ^{45}Ca to the membrane was estimated as the difference between binding in the presence and absence of 10^{-3} M $CaCl_2$. In this study of specific calcium binding, there is an inherent assumption that the specific receptor does not show specificity for the labelled or 'cold' calcium. Likewise it is also assumed that the chelating properties of the EGTA are not influenced by the atomic number of the calcium species. The observed K_{Ca} for ^{45}Ca binding to the synaptic membranes was $1.74 \pm 0.3 \times 10^{-5}$ M. This value is relatively high and it could be argued that a significant proportion of the specifically bound calcium was removed in the EGTA wash procedure. If this were so, it would not provide us with an accurate measure of specific binding. On the other hand the catecholamine induced change in the K_{Ca} for the Na–Ca-ATPase were of the order of 3- to

Table 2. The influence of phentolamine and propanalol on the effects of adrenaline on Na–Ca-ATPase

	K_{Ca} constant ($\times 10^{-5}$ M)	
	Control	10^{-6} M Adrenaline
No drug	2.02 ± 0.37	7.8 ± 0.5
Phentolamine 10^{-6}	2.17 ± 0.4	3.17 ± 0.16
Propanalol 10^{-5}	1.91 ± 0.27	5.11 ± 0.37

K_{Ca} values are means ± S.D. of 4 experiments.

4-fold. If the catecholamines were to achieve their inhibitory effects by altering the specific binding we should reasonably expect to see this reflected in the K_{Ca} value for ^{45}Ca binding in our studies. However in our experiments the K_{Ca} for ^{45}Ca binding to synaptic membranes in the presence of 10^{-6} M adrenaline was $2.01 \pm 0.4 \times 10^{-5}$ M and $1.89 \pm 0.27 \times 10^{-5}$ M in the presence of noradrenaline. It is reasonable to assume that specific calcium binding can take place on either surface but from a functional point of view it is likely that the affinity for binding would be greater on the inside surface of the membrane.

If noradrenaline were to increase calcium binding to synaptic membrane it could on one hand inhibit calcium efflux from synaptosomes. On the other hand, enhanced binding of calcium to the inside surface and decreased binding to the external surface could induce an increased calcium efflux from the synaptosomes.

However, in a series of experiments to investigate the extrusion of calcium from synaptosomes, the rate of efflux was insensitive to noradrenaline at concentrations up to 5×10^{-5} M (Logan, unpublished data).

Therefore it is our view that the inhibition of Na–Ca-ATPase is not the consequence of an enhancement of calcium binding to membranes.

To summarise, the catecholamines inhibit Na–Ca-ATPase by a mechanism which may be receptor-mediated and which is most probably a result of direct interaction with the enzyme rather than by some modulation of the free calcium ion concentration.

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On the interaction of diazepam with human, rat and mouse plasma proteins and erythrocytes

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The binding of a drug to plasma proteins usually affects its activity, distribution, rate of metabolism, and glomerular filtration [1–4]. Drugs bind to plasma proteins to different degrees depending upon their physico-chemical characteristics. Binding of drugs occurs mainly in the albumin fraction of plasma proteins [5]. Some lipophilic drugs, however, are also dissolved in the lipid phase of serum lipoproteins to an extent almost equal to that bound to serum albumin. To predict changes in protein binding of a specific drug in plasma of an individual patient, the extent of binding to the different protein fractions of plasma should be known because of the variations in the plasma protein pattern. In the comparison with albumin, other proteins are often subject to greater changes. Consequently, if proteins other than albumin contribute to the binding of drugs, more frequent variations in the binding are to be expected [6].

Although the possible influence of the erythrocytes and other cellular blood components in pharmacokinetics has been neglected by most authors, high binding of some lipophilic drugs to erythrocytes has recently been demonstrated [7–9].

The binding of diazepam to serum albumin and whole plasma of different species has been evaluated by others [10–12]. This communication is concerned with the binding of diazepam to human, rat and mouse plasma protein fractions and erythrocytes.

Human blood was obtained from healthy volunteers, fasted overnight, 18–30 years of age; rat blood from male, albino Wistar rats fasted 18 hr, weighing 200–250 g; mouse blood from male mice, strain H Konárovice, fasted 18 hr, weighing 18–30 g. Plasma was obtained by centrifugation of heparinized (approx. 5000 IU·l^{-1}) blood at 1000 *g* for 30 min.

[*N*-$^{14}CH_3$]Diazepam (sp. act. 4.92 GBq/g) was supplied by ÚVVVR (Prague, Czechoslovakia). The radiochemical purity of [*N*-$^{14}CH_3$]diazepam verified by TLC on silica gel (in the system heptane–choloroform–ethanol 5:5:2) was found to be greater than 98%. Diazepam was dissolved in acetone and mixed with unlabelled drug to achieve suitable concentrations. The required amount of diazepam was dissolved, after evaporation of acetone, in the blood, plasma or erythrocytic suspension in the period of 30 min. In the case of incubation of blood or erythrocytic suspension the samples were subsequently centrifuged and the drug was determined in the centrifugate and supernatant.

The binding of diazepam to whole plasma was studied by a method of equilibrium dialysis [13]. The samples were dialysed against 0.15 M NaCl solution buffered with 0.01 M phosphate (pH 7.4) for 20 hr at 37°.

The interaction between diazepam and various plasma proteins by molecular exclusion chromatography was studied by applying 1 ml fresh plasma, containing diazepam of a suitable concentration, to 11 × 350 mm column of Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) previously equilibrated with 0.15 M NaCl solution buffered with 0.01 M phosphate (pH 7.4) containing diazepam, 0.1