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Reversal by local anaesthetics of ouabain-induced [^{14}C]ACh and [^{14}C]choline release from synaptosomes

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We have recently shown that ouabain not only causes a significant increase in the release of labelled [^{14}C]ACh from cerebral cortex synaptosomes but also in that of its precursor [^{14}C]choline [1]. It was suggested that this action of ouabain was due to depolarization of the synaptosomal membrane resulting in Ca^{2+}-dependent release of transmitter and, in addition, increased diffusion of the bases down the electrochemical gradient. It was observed that when ouabain was added to synaptosomes in depolarizing (high K^+) media, a further increase in the release of both [^{14}C]ACh and [^{14}C]choline was produced. Ouabain also caused a small, but significant, further increase in the release of [^{14}C]ACh in synaptosomes depolarized by veratrine. These results indicated that ouabain may have an additional effect on the synaptosomal membrane, perhaps a general destabilization or non-specific increase in membrane permeability.

In order to test this possible additional property of ouabain, the effect of two local anaesthetics, procaine and cinchocaine, on the ouabain-induced release of synaptosomal [^{14}C]ACh and [^{14}C]choline was studied. Local anaesthetics are membrane stabilizers so that if ouabain had a destabilizing effect on the synaptosomal membrane, in addition to its inhibition of Na^+–K^+ ATPase then it would be predicted that its action could be partially counteracted by these compounds (to a degree related to their local anaesthetic action). In this paper, we report that two local anaesthetics, procaine and cinchocaine, appear to have this effect.

Materials and methods

Synaptosomes were prepared from scraped guinea-pig cortices by the method of Gray and Whittaker [2]. They were resuspended in 3 mM K^+ medium so that their final concentration was 3–5 mg protein/ml. 3 mM K^+ medium contained 180 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 8 mM NaPi (pH 7.2) and 10 mM glucose. In the case of 25 mM K^+ medium, the concentration of NaCl was decreased so that the ionic concentration remained unchanged.

Synaptosomes were preincubated with 0.1 μCi/ml (2 μM) [^{14}C]choline for 30 min at 37° during which [^{14}C]ACh was synthesized. They were washed twice with 3 mM K^+ medium containing no Ca^{2+} but 20 μM eserine to remove excess [^{14}C]choline. The synaptosomal pellet was then divided into aliquots of 0.3–0.5 g of brain tissue in Eppendorf microreaction tubes. To each aliquot, appropriate medium (either 3 or 25 mM K^+ medium) was added and where necessary the compounds veratrine (100 μM), ouabain (200 μM), procaine or cinchocaine (both 100 μM). The release was activated by incubating the tubes at 37° for 5 min after which it was terminated by rapid centrifugation in a Janetski TH 11 centrifuge; supernatants and pellets were separated and placed in ice. The supernatants were extracted for ACh and choline by the method of Marchbanks and Israël [3] and Wonnacott and Marchbanks [4]. [^{14}C]ACh and choline were separated by thin layer chromatography and the radioactivity was measured in a Nuclear Enterprises 8312 scintillation spectrometer.

Table 1. The effect of procaine and cinchocaine on Na^+–K^+ ATPase activity.

Condition	μmole Pi/mg Protein per hour	% Activity of the Enzyme still present
Control: Na^+–K^+ ATPase	10.75	100
+ 100 μM procaine	8.07	75.1
+ 100 μM cinchocaine	9.88	91.9
+ 200 μM ouabain	0.904	8.4
+ procaine + ouabain	1.82	16.9
+ cinchocaine + ouabain	1.54	14.3

Na^+–K^+ ATPase activity of an hypo-osmotically shocked synaptosomal preparation was measured by preincubating 2 ml aliquots of incubation medium containing Tris–HCl, pH 7.4 (50 mM), $MgCl_2$ (4 mM), NaCl (120 mM), KCl (30 mM) and ATP (5 mM) for 1 min at 37° with or without the test compounds. The reaction was started by the addition of hypo-osmotically shocked synaptosomal preparation containing 30 μg/ml protein. After 10 min incubation at 37° it was stopped by the addition of 1 ml 20% TCA, and the phosphate content determined by the method of Fiske and Subbarow [6]. The enzyme activity is expressed as μmole Pi/mg protein per hr and the above values represent mean of two determinations.

The method of Lowry *et al.* [5] was used to determine the protein concentrations of samples, using crystalline bovine plasma albumin as a standard.

Results and discussion

Firstly, the effect of either procaine or cinchocaine on Na^+–K^+ ATPase activity of synaptosomal membrane was measured to rule out any direct interaction between the two. The result of these two agents on the ATPase activity of hypo-osmotically shocked synaptosomes is shown in Table 1. Procaine reduced the enzyme activity slightly, cinchocaine had a negligible effect, whereas 200 μM ouabain almost completely inhibited the enzyme activity. Both these agents caused only a small change (a decrease of about 6–8%) in the inhibitory effect of ouabain. Figure 1 shows the effect of these two compounds on the release of [^{14}C]ACh and [^{14}C]choline in the normal and in the depolarizing medium, in the presence or absence of 200 μM ouabain.

In the absence of ouabain they caused a slight but insignificant decrease in [^{14}C]ACh release in the normal medium (Fig. 1a). However, their effect was more marked in high K^+ medium, the decrease in [^{14}C]ACh release caused by cinchocaine being statistically significant (Fig. 1c). As regards their action on [^{14}C]choline efflux (Fig. 1, a and c) procaine caused a slight reduction but cinchocaine had no effect.

The effect of procaine and cinchocaine on ouabain-induced [^{14}C]ACh and choline release is also shown in Fig. 1 (b and d). Both procaine and cinchocaine reduced [^{14}C]ACh as well as [^{14}C]choline release in normal and in the depolarizing medium. The decrease by cinchocaine in [^{14}C]choline efflux (in both the normal and in the depolarizing media) to the level seen in the absence of ouabain was statistically significant. Cinchocaine did not reverse the [^{14}C]ACh release evoked in the non-depolarizing medium but its inhibition of [^{14}C]ACh release was greater in high K^+ medium.

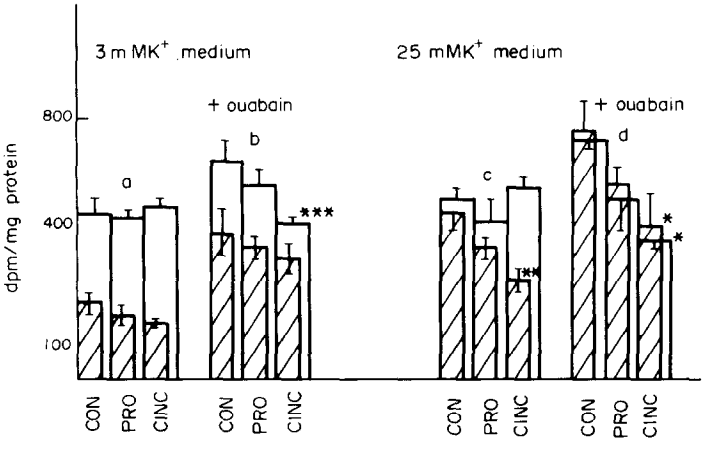

Fig. 1. The release of [^{14}C]ACh (inside columns ▨) and choline (□) in 3 and 25 mM K^+ in the presence of procaine or cinchocaine (100 μM) was measured as described in Materials and Methods. Ouabain (200 μM) was also included as indicated. The results are expressed as mean ± S.E.M. of 3–4 experiments. Asterisks show ***$P < 0.02$ (3 mM K^+ + ouabain as control), **$P < 0.05$ (25 mM K^- as control) and *$P < 0.001$ (25 mM K^+ + ouabain as control); Student's *t*-test two-tailed.

These results indicate that cinchocaine is more effective than procaine in inhibiting [^{14}C]ACh and choline release from synaptosomes, and this correlates with their relative local anaesthetic potency. There is a substantial evidence [7–9] which suggests that local anaesthetics bind specifically to Na^+ channels of the nerve membrane, preventing the normal Na^+ flux and as a result causing a nerve conduction block. The effect of procaine and cinchocaine in reducing [^{14}C]ACh release in high K^+ medium (in the absence of ouabain) suggests that they cause a reduction in synaptosomal depolarization by the same mechanism. This is also supported by the fact that the concentrations of the two anaesthetics used in these experiments, although slightly lower, are still within the range that has been used previously. For example, concentrations of about 1 mM procaine and 0.5 mM cinchocaine have been employed by Narahashi *et al.* to inhibit Na^+ conductance in nerve membrane [10, 11].

In these experiments, cinchocaine reversed the effect of ouabain on [^{14}C]ACh release even in depolarizing concentrations of high K^+. Its effect was even more marked on ouabain-induced [^{14}C]choline efflux, suggesting an additional effect of ouabain. In our previous work we showed that the effect of ouabain on [^{14}C]ACh and choline release is independent of Ca^{2+} [1]. In addition to its specific effect on Na^+–K^+ ATPase activity and the related ACh release, it seems likely that ouabain at a concentration of 200 μM has a detergent action which would increase the leakiness of the synaptosomal membrane. Such leakiness would be reversed or counteracted by the membrane stabilization that is a feature of local anaesthetic action [12].

Summary. Procaine and cinchocaine do not affect Na^+–K^+ ATPase but reverse the [^{14}C]ACh and [^{14}C]choline release evoked by ouabain with a relative effectiveness correlating with their local anaesthetic potency. They reduce the [^{14}C]ACh release evoked by high K^+. In addition, cinchocaine reversed the [^{14}C]ACh and [^{14}C]choline efflux induced by ouabain in synaptosomes already depolarized by high K^+. This suggests that ouabain (200 μM) has an additional detergent-type action that can be counteracted by local anaesthetic membrane stabilization.

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Uptake and metabolism of doxorubicin in isolated perfused rat lung*

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Primary bone and soft-tissue sarcomas frequently and almost exclusively metastasize to the lungs [1, 2]. Without treatment, over 75% of patients developing pulmonary metastatic osteosarcoma die within 12 months [3]. Surgical procedures have proven successful with a 26% five-year survival for soft-tissue sarcoma and 28% for osteogenic sarcoma [4–6]. At present, doxorubicin (DOX) is considered the most useful anticancer agent for the treatment of these forms of cancer with a 33–40% remission rate in patients with soft tissue sarcomas [7, 8]. However, a significant proportion of patients developing pulmonary metastatic disease are unresectable and unresponsive to chemotherapy.

Recently, studies were undertaken to develop an isolated perfused lung procedure for the treatment of patients with metastatic soft-tissue sarcomas to the lungs where other surgical and chemotherapeutic approaches have been exhausted [9]. The procedure entails short-term isolated pulmonary perfusion with blood concentrations of doxorubicin normally not tolerated *in vivo*. It is hoped that such a procedure may offer a means to deliver tumoricidal drug concentrations but avoid systemic toxicity.

Little is known regarding the disposition of anthracyclines in lung although this organ is often the site of drug

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