Biochemical Pharmacology, Vol. 34, No. 14, pp. 2471–2475, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

INHIBITION OF PHOSPHATIDYLINOSITOL PHOSPHODIESTERASE ACTIVITY IN SKELETAL MUSCLE BY METAL IONS AND DRUGS WHICH BLOCK NEUROMUSCULAR TRANSMISSION

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(*Received* 21 *May* 1984; *accepted* 7 *February* 1985)

Abstract—The calcium-dependent phosphatidylinositol phosphodiesterase activity of skeletal muscle cytosol was determined. The enzyme was inhibited by Zn^{2+}, Cu^{2+} and Pb^{2+} ions but Mg^{2+} and Mn^{2+} were without effect. The antimalarial drugs chloroquine and quinine and the aminoglycoside antibiotics gentamicin and neomycin all of which, like Zn^{2+}, have been shown to block neuromuscular transmission, also inhibited the enzyme.

Zn^{2+} has been shown to reversibly block transmission at the frog neuromuscular junction [1]. The effects of Zn^{2+} paralleled those of quinine [2], which lowered the excitability of the motor endplate in mammalian, avian and amphibian muscle. The related antimalarial drug chloroquine, induced muscle weakness in nerve-muscle preparations from frog and cat [3]. Furthermore, neuromuscular blockade is a recognised complication of antimicrobial therapy [4].

In this laboratory, a Ca^{2+}-dependent phospholipase C, phosphatidylinositol (PtdIns) phosphodiesterase, was recently demonstrated in rat skeletal muscle cytosol [5] and in nerve cytosol [6]. This enzyme hydrolysed PtdIns to liberate inositol phosphate [5]. It was also shown that PtdIns phospholipase C was released following nerve stimulation in an isolated phrenic nerve–diaphragm preparation [7]. Furthermore purified bacterial phospholipase *C* preparations were shown to increase the acetylcholine sensitivity of skeletal muscles and a role for the muscle enzyme in the activation of acetylcholine receptors was proposed [8, 9]. As the acetylcholine receptors are largely restricted to the endplate region of skeletal muscle it was of interest to investigate the effect of agents which are known to block neuromuscular transmission on the activity of muscle PtdIns phosphodiesterase.

MATERIALS AND METHODS

Enzyme determination. The activity of PtdIns phosphodiesterase was determined in cytosol prepared from soleus muscle as described previously [5]. The PtdIns was prepared by biosynthesis on isolated sarcoplasmic reticulum and either the labelled membranes themselves were used as substrate or the PtdIns was first extracted and purified before addition to the assay as an aqueous suspension. When the membrane-bound substrate was used the incubation mixture contained sarcoplasmic reticulum protein (800 μm/ml), sodium deoxycholate (0.6 mM), tris maleate buffer, (50 mM, pH 7.4), cytosol protein (400 μg/ml) and $CaCl_2$ (either 1.2 mM or 7.2 mM as stated in the text). When the isolated PtdIns was used, it was incubated in a concentration of 1.4 mM with Tris maleate buffer, (50 mM, pH 5.5), cytosol protein (100 μg/ml) and $CaCl_2$ (either 1.2 mM or 7.2 mM as stated). In all experiments, incubations were carried out in duplicate. No-enzyme controls were included for each set of conditions, i.e. for each concentration of metal ion or drug. Metal ions were added in each case as the chloride salt.

Drugs. Chloroquine sulphate was obtained from May & Baker Ltd. (Dagenham, Essex) and quinine hydrochloride and neomycin sulphate were obtained from Sigma Co. Ltd. (London). Gentamicin (570 U/mg) was a gift from Dr. S. Comis in this Department.

RESULTS

Effect of divalent metal ions on PtdIns phosphodiesterase activity

It has been shown previously that the PtdIns phosphodiesterase of skeletal muscle cytosol is activated by Ca^{2+} in millimolar concentrations [5]. This dependence was specific for Ca^{2+} ions in that they could not be replaced by Mg^{2+}, Mn^{2+}, or Zn^{2+} ions.

The effect of Zn^{2+} on the enzyme activity was determined using the isolated substrate only, since deoxycholate, a cofactor in the assay when the membrane-bound substrate was used, was precipitated by Zn^{2+} at the concentrations used. $ZnCl_2$ was a potent inhibitor of the enzyme activity (Fig. 1a) when it was determined in the presence of 7.2 mM Ca^{2+} at pH 5.5, with approx. 50% inhibition by 0.4 mM Zn^{2+} with all concentrations of the substrate used (Fig. 1b).

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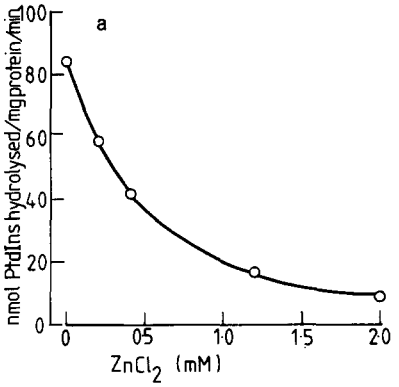

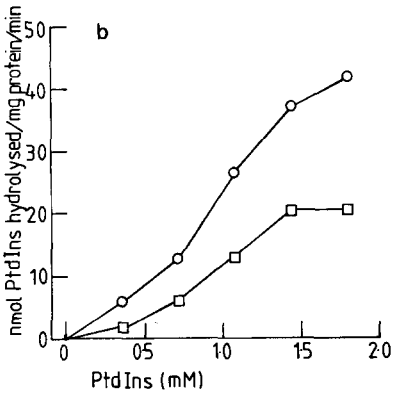

Fig. 1. Effect of Zn^{2+} on PtdIns phosphodiesterase activity. (a) Inhibition by Zn^{2+} in the presence of 7.2 mM $CaCl_2$. (b) PtdIns phosphodiesterase activity at different concentrations of isolated PtdIns in the absence (○) and presence (□) of Zn^{2+} (0.4 mM). The activity was determined at pH 5.5 in the presence of 7.2 mM $CaCl_2$. The results are typical of (a) five experiments, (b) two experiments.

Table 1. Inhibition (%) of PtdIns phosphodiesterase activity by divalent metal ions

	Inhibition (%)				
$CaCl_2$ conc.	Mn^{2+}	Mg^{2+}	Pb^{2+}	Cu^{2+}	Zn^{2+}
1.2 mM	0	0	100	99	75
7.2 mM	0	0	43	54	59

Enzyme activity was determined at pH 5.5 using isolated PtdIns as substrate. Divalent metal ions were present at a concentration of 0.4 mM, except for Ca^{2+} which was present at the concentrations indicated. Activity is expressed as inhibition (%) of the control, which contained no divalent metal ions except Ca^{2+}.

The inhibition by Zn^{2+} was more marked at lower concentrations of Ca^{2+} (Table 1). The effects of different divalent metal ions on the enzyme activity at two concentrations of Ca^{2+}, 7.2 mM and 1.2 mM, are compared in Table 1. Mg^{2+} and Mn^{2+}, at a concentration of 0.4 mM, were without effect at either concentration of Ca^{2+}. However, Pb^{2+} and Cu^{2+} ions were, like Zn^{2+}, potent inhibitors. At a Ca^{2+} concentration of 1.2 mM, inhibition by these two ions, present in a concentration of 0.4 mM, was virtually complete.

Effect of aminoglycoside antibiotics

Gentamicin and neomycin were potent inhibitors of PtdIns phosphodiesterase activity in the presence of 7.2 mM $CaCl_2$ (Fig. 2). In the presence of 1.2 mM $CaCl_2$ low concentrations of the antibiotics stimulated the enzyme activity. The inhibitory effect observed at the higher concentrations of the drugs was more marked at the lower Ca^{2+} concentration.

Neomycin (10^{-4} g/ml) in the presence of 7.2 mM $CaCl_2$ inhibited PtdIns phosphodiesterase at all concentrations of PtdIns tested (Fig. 3). Inhibition by

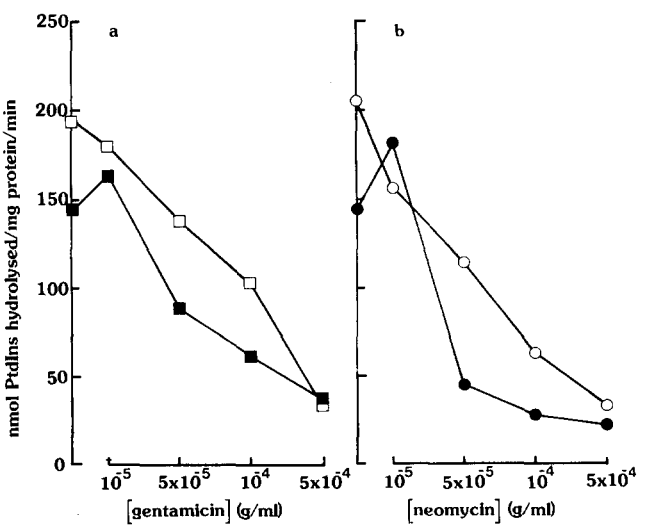

Fig. 2. Effect of aminoglycoside antibiotics on PtdIns phosphodiesterase activity. (a) Gentamicin, (b) neomycin in the presence of 7.2 mM (open symbols) and 1.2 mM (closed symbols) $CaCl_2$. The determinations were carried out at pH 5.5 using isolated PtdIns as substrate. The results are typical of three experiments.

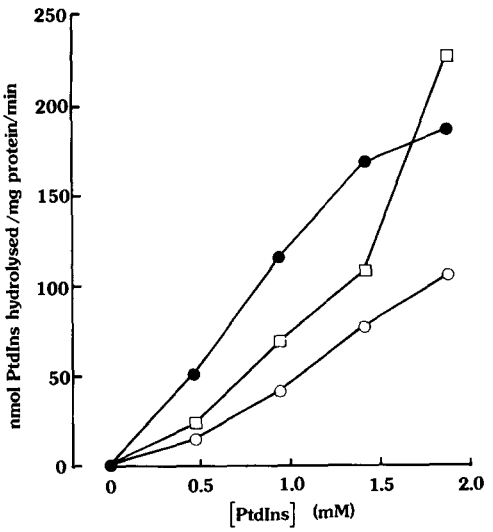

Fig. 3. Effect of aminoglycoside antibiotics on PtdIns phosphodiesterase activity at different concentrations of isolated PtdIns: (●) no drug present, (□) gentamicin and (○) neomycin. The antibiotics were present at a concentration of 10^{-4} g/ml. Activity was determined at pH 5.5 in the presence of 7.2 mM $CaCl_2$.

gentamicin under the same conditions was observed for all concentrations of the substrate tested, except at 1.88 mM PtdIns when the drug stimulated the enzyme activity.

Effect of chloroquine sulphate and quinine hydrochloride

The effect of chloroquine sulphate on PtdIns phosphodiesterase activity was tested using either the isolated substrate at pH 5.5 (Fig. 4a) or the membrane-bound substrate at pH 7.4 (Fig. 4b). The pH values chosen were previously found to be nearly optimum under the conditions used. The Ca^{2+} concentration was 7.2 mM. The drug was inhibitory under both sets of conditions. When the isolated substrate was used the inhibition was virtually complete at 9 mM chloroquine sulphate. However, a slight stimulation of the enzyme activity was seen at low concentrations (2–4 mM) of the drug (Fig. 4a).

When the membrane-bound substrate was used the drug was maximally effective at a concentration of 2 mM (Fig. 4b). At this concentration, however, a low (less than 20%) activity remained. Since the sarcoplasmic reticulum membranes used as substrate exhibited no endogenous enzyme activity [5], the residual activity in the presence of concentrations of the drug which produced the maximum inhibition represented a proportion of the soluble activity which

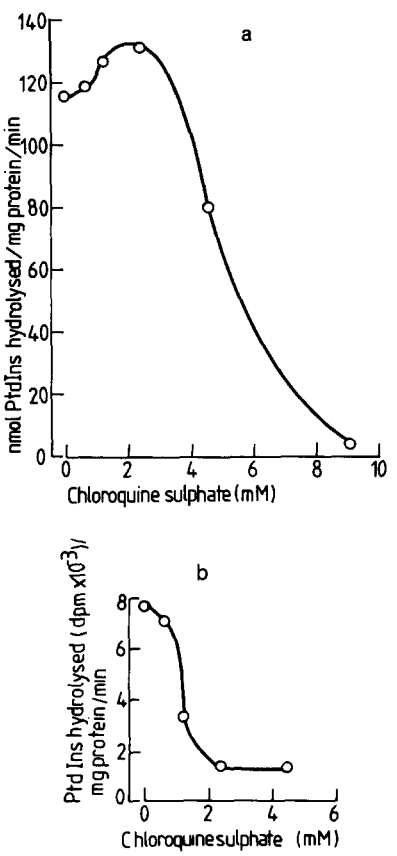

Fig. 4. The effect of chloroquine sulphate on PtdIns phosphodiesterase activity. (a) The enzyme was determined using the isolated substrate at pH 5.5. (b) The determinations were carried out using the membrane-bound substrate at pH 7.4. All incubations contained 7.2 mM $CaCl_2$. The results are typical of three experiments.

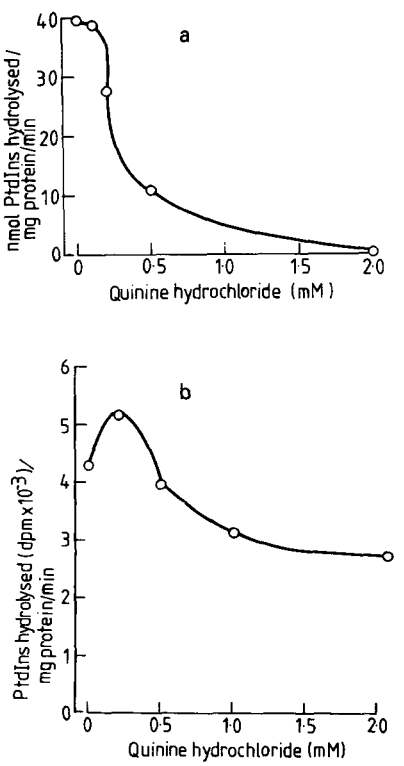

Fig. 5. The effect of quinine hydrochloride on the activity of PtdIns phosphodiesterase activity. (a) The determinations were carried out using the isolated substrate at pH 5.5. (b) The determinations were carried out using the membrane-bound substrate at pH 7.4. All incubations contained $CaCl_2$ at 7.2 mM. The results are typical (three experiments).

was protected from the action of the drug. Association of some of the soluble enzyme with the membranes might be responsible for this protection.

The effect of quinine hydrochloride on the soluble PtdIns activity was, like that of chloroquine sulphate, inhibitory (Fig. 5a and b). However in the case of quinine hydrochloride complete inhibition of the enzyme determined with the isolated substrate was seen at a concentration of 2 mM (Fig. 5a). Again, as with chloroquine a proportion of the enzyme was protected from the drug when the membrane-bound substrate was employed at pH 7.4 (Fig. 5b). In this case the maximum inhibition seen was only 35%. A slight stimulation of the activity was seen at low concentrations of quinine hydrochloride when the membrane-bound substrate was used.

DISCUSSION

Evidence is presented that some agents which are known to block neuromuscular transmission also inhibit the PtdIns phosphodiesterase activity of soleus muscle cytosol. The observations are at least consistent with the hypothesis that this enzyme has a role in the control of acetylcholine sensitivity at the neuromuscular junction.

Strong inhibition of PtdIns phosphodiesterase activity by Zn^{2+} ions at a concentration (0.5 mM) which blocked neuromuscular transmission in the frog [1] was demonstrated. Cu^{2+} and Pb^{2+} also inhibited the activity whilst Mg^{2+} and Mn^{2+} were without effect. It was previously suggested that Ca^{2+} exerts its effect by acting on the enzyme directly rather than via an action on the substrate [5] and the present results demonstrating inhibition of this enzyme by divalent metal ions at different Ca^{2+} concentrations indicate that these ions may compete for the Ca^{2+}-binding site on the enzyme. The mechanism of action of the drugs could be similar. It has been shown, for example, that neomycin does not interact with PtdIns in the presence of 1.0 mM Ca^{2+} [10], thus competition at a Ca^{2+}-binding site on the enzyme is possible. This explanation has also been advanced for the inhibition of soluble Ca^{2+}-dependent PtdIns phosphodiesterase by neomycin and gentamicin in human amnion [11] and rat kidney [12]. The effects of the antibiotics were dependent on the concentrations of Ca^{2+}, PtdIns and aminoglycosides. Stimulation of enzyme activity was seen when the ratio of antibiotic to phosphatidylinositol was low (Figs. 2 and 3). Sagawa *et al.* [11] reported similar effects of gentamicin on the PtdIns phosphodiesterase from human amnion which may be due to a charge effect of the polyvalent antibiotic cations on the enzyme particles. In addition, inhibition by chloroquine of phosphatidylcholine hydrolysis by rat liver lysosomes has been demonstrated and a direct action of the drug on this enzyme was proposed [13].

The activity of the Ca^{2+}-dependent PtdIns phosphodiesterase of rat brain synaptosomes [14] has also been shown to be completely inhibited by Zn^{2+}, Cu^{2+} and Pb^{2+} at 0.5 mM concentration and unaffected by Mg^{2+} ions. The Ca^{2+}-independent PtdIns phosphodiesterases of rat liver and brain lysosomes [15, 16] and *Bacillus thuringiensis* [17] were also inhibited by Zn^{2+} ions but these enzymes, however, were also inhibited by Mg^{2+} and Mn^{2+}.

The low concentration of Ca^{2+} in the muscle cells (less than 10^{-6} M) probably precludes an intracellular role for PtdIns phosphodiesterase in normal muscles since the enzyme would not be active. However, the Ca^{2+} concentration in the extracellular fluid is approx. 2.0 mM, a concentration at which both the muscle and nerve soluble enzymes are optimally active [5, 6]. In isolated preparations at least, the enzyme can be released from muscle or nerve [9, 7]. An extracellular role for the enzyme in the control of chemosensitivity at the neuromuscular junction is therefore possible.

Disorders of neuromuscular transmission have been reported to be caused by a variety of drugs [18] including those used in this study. Both presynaptic and postsynaptic actions have been proposed for gentamicin [19], neomycin [20, 24], chloroquine [3] and quinine [18]. The presynaptic effect of the aminoglycoside antibiotics on quantal release of acetylcholine [20] may involve the polyphosphoinositides, the hydrolysis of which has been postulated to play a role in transmitter release [21] whilst it has been suggested that the postsynaptic effect could be due to interaction of the drugs with the acetylcholine receptor [22] or with the ionic channel of the receptor [23, 24]. Quinine has been shown to block responses to stimulation of both nicotinic [25] and muscarinic [26] acetylcholine receptors and the effect at the neuromuscular junction was found to be due to channel blockade [27]. It is interesting with respect to these considerations that quinine therapy exacerbates myasthenia gravis [25], a condition which is characterised by a decreased acetylcholine sensitivity at the neuromuscular junction.

It has been shown [28] that PtdIns may be intimately associated with the nicotinic receptor purified from *Torpedo californica* and it has been proposed previously that hydrolysis of PtdIns activates the acetylcholine receptor on muscle [8]. In this laboratory it was shown that the effect of PtdIns phosphodiesterase to increase the ACh receptor number on sarcolemma was inhibited by chloroquine sulphate (unpublished). It is possible that the action of the drugs at the neuromuscular junction partly reflects an inhibition of a PtdIns phosphodiesterase which hydrolyses PtdIns associated with the acetylcholine receptor.

Acknowledgements—The work was supported by the Wellcome Trust.

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