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Divalent cation modulation of the ionic permeability of the synaptosomal plasma membrane

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Synaptosomes from guinea-pig cerebral cortex reveal two distinct Na^+ permeabilities when divalent cations are removed from the incubation. In the presence of Mg^{2+}, Ca^{2+} chelation by EGTA causes a partial activation of a voltage-dependent tetrodotoxin-sensitive pathway, manifested as a ouabain-sensitive respiratory increase, a partial depolarization of the plasma membrane, and a lowered gradient of γ-amino[^{14}C]butyrate. In addition there is a hyperpolarization of the mitochondrial membrane potential. When Mg^{2+} is omitted from the incubation, Ca^{2+} chelation induces a substantially larger permeability which is only partially sensitive to tetrodotoxin. The tetrodotoxin-insensitive component is not associated with a non-specific permeabilization of the plasma membrane and may be reversed by either Mg^{2+} or Ca^{2+}.

Introduction

Synaptosomes appear to retain the plasma membrane transport properties characteristic of the intact terminal, and maintain plasma membrane potentials ($\Delta\psi_p$), extrude Ca^{2+} and accumulate neurotransmitters by a Na^+-dependent mechanism [1–4]. In media containing millimolar concentrations of Mg^{2+} and Ca^{2+}, the permeability of the plasma membrane for K^+ is at least 20-times greater than for Na^+ [5,6]. The low Na^+ permeability implies that $\Delta\psi_p$ is close to the K^+ diffusion potential, that little energy is dissipated in maintaining $\Delta\psi_p$ and that the maximal Na^+ electrochemical potential is available for the accumulation of metabolites by Na^+ cotransport.

Schmalzing [6] has reported that the Na^+ permeability of the synaptosomal plasma membrane may be increased by lowering Ca^{2+} or Mg^{2+} in the incubation. The permeability increase was insensitive to tetrodotoxin (TTX), indicating a pathway other than the voltage-dependent Na^+ channel. Since it is established that Ca^{2+} removal also increases the excitability of the voltage-dependent Na^+ channel [7], we set out to investigate the relationship between these two permeabilities in isolated synaptosomes.

We conclude that two distinct pathways for electrophoretic Na^+ entry may be seen when synaptosomes are suspended in media deficient in divalent cations. In the presence of Mg^{2+}, Ca^{2+} chelation specifically increases the conductance of the voltage-dependent Na^+ channel, while in the absence of both divalent cations the tetrodotoxin-insensitive pathway described by Schmalzing [6] is induced.

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Abbreviations: TTX, tetrodotoxin; FCCP, carbonylcyanide *p*-trifluoromethoxy phenylhydrazone; Tes, 2-([2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino)ethanesulphonate; TPP, tetraphenylphosphonium; GABA, γ-aminobutyrate; $\Delta\psi_m$ and $\Delta\psi_p$, membrane potentials across the plasma and mitochondrial membranes of intact synaptosomes.

Methods

Synaptosomes were prepared from the cerebral cortices of Dunkin-Hartley guinea pigs as described previously [8], and were stored as pellets at 0°C. Protein was measured with the biuret method.

Synaptosomes were incubated in a medium containing 122 mM NaCl, 3.1 mM KCl, 0.4 mM KH_2PO_4, 5 mM $NaHCO_3$, 20 mM Na-TES, 10 mM D-glucose and 16 μM bovine serum albumin, pH 7.4, 30°C. Where indicated 1.2 mm $MgSO_4$ was initially present. In some experiments $CaCl_2$ or Na-EGTA was added after 5 min preincubation to give a final concentration of 1.3 mM. Where EGTA was added to media already containing 1.3 mM $CaCl_2$, its pH was adjusted with NaOH to counteract the acidification due to the displacement of protons from the chelator.

Respiration and membrane potentials. Synaptosomal respiration was determined in a Hansa-Tech oxygen electrode chamber (Hansa-Tech, Kings Lynn, U.K.).

Tetraphenylphosphonium (TPP) accumulation was quantified with a TPP-selective electrode as described previously [9]. Synaptosomes (2 mg protein/ml) were incubated in 2 ml of incubation medium, with additions of divalent cations as detailed in the legends. 1 μM TPP was added at 5 min, and its accumulation calculated from a calibration curve. A synaptosomal volume of 3.23 μl/mg protein was used in calculating the mean intrasynaptosomal concentration of TPP (ignoring compartmentalization).

Plasma and mitochondrial membrane potentials ($\Delta\psi_p$ and $\Delta\psi_m$, respectively) were determined isotopically as described previously [1] with subsequent modifications [10]. TPP was used in place of triphenylmethylphosphonium, and tetraphenylboron was omitted.

^{14}C-GABA uptake. Synaptosomes (1.5 mg protein/ml) were suspended in medium with the addition of 1 mM aminoxyacetate. At 5 min ^{14}C-GABA (0.1 μCi/ml; 0.5 μM) was added. At defined times 150-μl samples were centrifuged for 1 min in an Eppendorf 5412 centrifuge, and 50 μl of supernatant radioassayed. The gradient across the synaptosomal plasma membrane was calculated using a volume of 3.23 μl per mg protein [1], and assuming that all synaptosomes accumulated the amino acid.

Lactate dehydrogenase. Synaptosomes (2 mg protein/ml) were incubated in the presence of the appropriate divalent cations. Lactate dehydrogenase (EC 1.1.1.27) was assayed [11] in the total incubation and in supernatants following 1 min centrifugation in an Eppendorf centrifuge.

Reagents. ^{3}H-TPP, ^{14}C-GABA, ^{86}Rb and ^{14}C-sucrose were from the Amersham International, Amersham, U.K. Unlabelled TPP was from Fluorochem, Glossop, U.K. TTX, ouabain, Ruthenium red and other reagents were obtained from Sigma (London) Chemical Co., Poole, U.K.

Results

The respiration of synaptosomes incubated in the presence of a physiological Mg^{2+} concentration is increased by some 25% when Ca^{2+} is chelated by the addition of EGTA [12,13], see also Table I. The EGTA-induced increment is sensitive to ouabain [13] suggesting that it is caused by enhanced Na^+-cycling across the plasma membrane. Table I shows that both in the presence and absence of Ca^{2+} there is a tetrodotoxin-sensitive component of respiration, the extent of which is doubled by Ca^{2+} chelation. Therefore, under polarized conditions, the voltage-dependent Na^+ channel of synaptosomes can provide a pathway

TABLE I

SYNAPTOSOMAL RESPIRATION, INFLUENCE OF DIVALENT CATIONS

Synaptosomes (2 mg protein/ml) were incubated in oxygen-electrode chambers in the presence of 0.1 mM EGTA with the further addition of 1.4 mM $CaCl_2$ and/or 1.2 mM $MgSO_4$ as indicated. At 20 min 2 μg/ml of TTX and at 27 min 1 μM FCCP were added to each incubation. Values are means ± S.E. for four or five experiments.

		Respiration (nmol·min^{-1}·(mg protein)$^{-1}$)	
		$+Ca^{2+}$	$-Ca^{2+}$
$+Mg^{2+}$			
	basal	2.5 ± 0.08	3.2 ± 0.04
	+TTX	2.2 ± 0.15	2.4 ± 0.10
	+TTX+FCCP	11.3 ± 0.9	10.1 ± 0.6
$-Mg^{2+}$			
	basal	2.8 ± 0.24	4.7 ± 0.21
	+TTX	2.2 ± 0.11	3.2 ± 0.23
	+TTX+FCCP	10.1 ± 1.0	11.1 ± 1.4

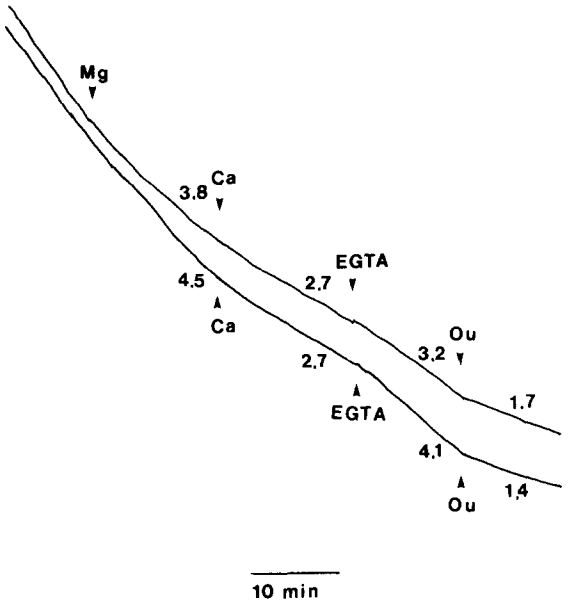

Fig. 1. Effects of Ca^{2+} and Mg^{2+} on ouabain-sensitive respiration. Synaptosomes (2 mg protein/ml) were incubated in divalent cation-deficient medium with the addition of 0.1 mM EGTA. The following additions were made as indicated: 1.2 mM $MgSO_4$ (Mg); 1.4 mM $CaCl_2$ (Ca); 2.6 mM EGTA (EGTA) and 0.1 mM ouabain (Ou). Values indicate oxygen uptake in $nmol \cdot min^{-1} \cdot (mg\ protein)^{-1}$.

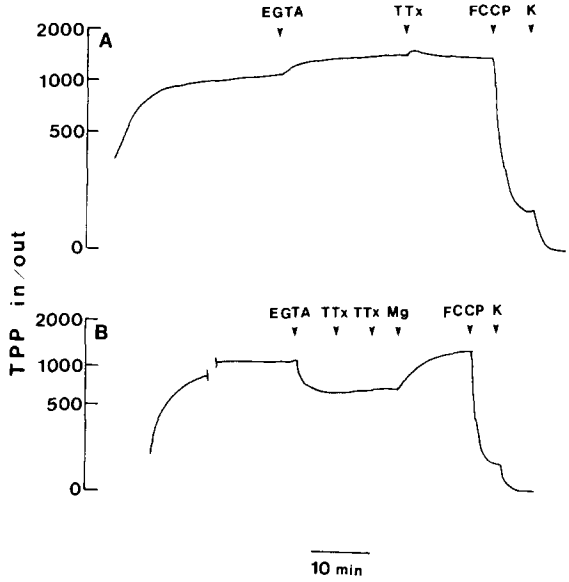

Fig. 2. Synaptosomal TPP accumulation. Opposite effects of Ca^{2+} chelation in the presence and absence of Mg^{2+}. Synaptosomes (2 mg protein/ml) were incubated in the presence (A) or absence (B) of 1.2 mM $MgSO_4$. 1.3 mM $CaCl_2$ was added to both incubations at 5 min. TPP accumulation was determined with the electrode as described in Methods. The following additions were made as indicated: 2.6 mM EGTA (EGTA), 1 μg/ml tetrodotoxin (TTX), 1.2 mM $MgSO_4$ (Mg), 4 μM FCCP (FCCP) and 30 mM KCl (K).

for the slow re-entry of Na^+ when Ca^{2+} is absent.

The respiratory increase induced by EGTA is enhanced almost 3-fold in the absence of external Mg^{2+} (Table I). As in the presence of Mg^{2+} the increase is ouabain-sensitive (Fig. 1). However, while the response in the presence of Ca^{2+} shows a high sensitivity to TTX, in the absence of both divalent cations there is a significantly elevated TTX-insensitive rate (Table I). Schmalzing [6] has reported that Ca^{2+} chelators increase the Na^+ permeability of synaptosomes incubated in the absence of Mg^{2+}; thus at 16 μM free Ca^{2+} the Na^+ permeability was largely TTX-sensitive, while a further decrease to 20 nM Ca^{2+} disclosed TTX-insensitive cation channels [6].

While the respiratory consequences of Ca^{2+} chelation are similar in the presence and absence of Mg^{2+}, opposite effects are produced on TPP distribution (Fig. 2), an indicator of changes in either $\Delta\psi_m$ or $\Delta\psi_p$ [9]. In the presence of 1.2 mM Mg^{2+} the accumulation of TPP increases by about 25% when EGTA is added, while in the absence of Mg^{2+}, EGTA causes an efflux of TPP which lowers the gradient by 35%. Prior to Ca^{2+} chelation, the TPP accumulation is the same in the presence or absence of Mg^{2+}.

In order to differentiate between $\Delta\psi_m$ and $\Delta\psi_p$, the uptake of ^{86}Rb and ^3H-TPP were compared [1,10]. Both in the presence and absence of Mg^{2+}, a slight but significant increase in $\Delta\psi_m$ was observed after addition of EGTA (Fig. 3). The high $\Delta\psi_m$ together with the sensitivity of respiration to ouabain (Fig. 1) and the high FCCP-releasable respiratory control (Table I) all indicate that no uncoupling of the intra-synaptosomal mitochondria occurs when the incubation is deprived of divalent cations. Opposed to the increase in $\Delta\psi_m$ is a depolarization of the plasma membrane, which is enhanced 7-fold in the absence of Mg^{2+}, accounting for the opposite net effects of EGTA seen with the TPP-electrode (Fig. 2).

If divalent cation depletion were to increase the availability of non-specific cation binding sites in the terminals, an increase in TPP accumulation

could occur due to enhanced binding, rather than a change in membrane potential. To assess the extent of binding, the accumulation of TPP and ^{86}Rb were compared in the presence of valinomycin and a range of K^+ concentrations [1]. The relation between TPP and ^{86}Rb accumulation was not affected by Ca^{2+} depletion (not shown).

No significant effect of TTX upon synaptosomal potentials could be seen in the presence of both Mg^{2+} and Ca^{2+} (Fig. 3B), consistent with the low TTX-sensitive respiration in the presence of both cations (Table I). In the presence of Mg^{2+}, the EGTA-induced hyperpolarization detected with the TPP-electrode was slightly enhanced when TTX was added (Fig. 2A). Isotopic analysis (Fig. 3A) revealed that the toxin reversed the effect of EGTA upon $\Delta\psi_p$, but did not abolish the mitochondrial hyperpolarization.

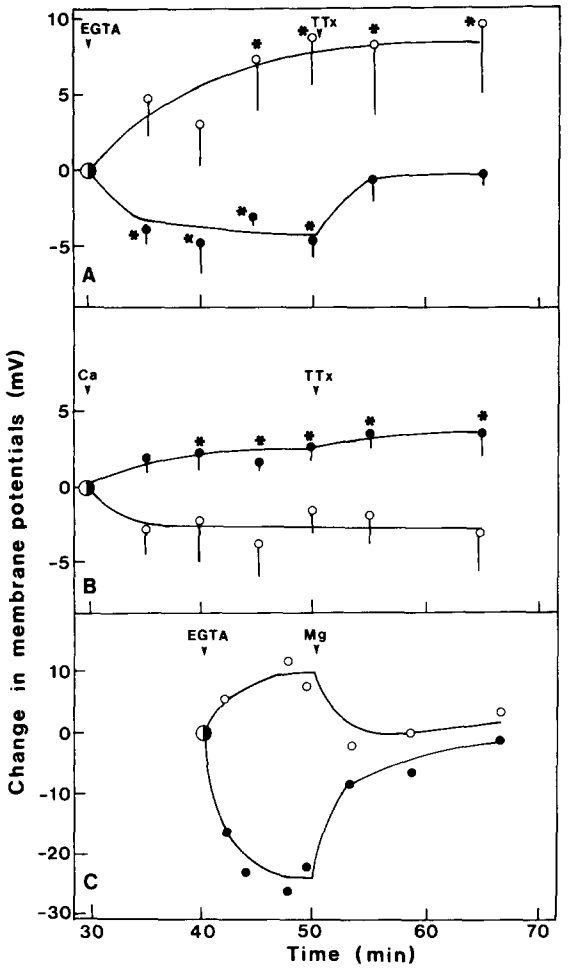

Fig. 3. Effects of Ca^{2+}, Mg^{2+} and TTX on $\Delta\psi_p$ and $\Delta\psi_m$. $\Delta\psi_p$ and $\Delta\psi_m$ were determined isotopically as described in Methods, and are presented as differences from the potentials in parallel control incubations. (A) 1.3 mM $CaCl_2$ and 1.2 mM $MgSO_4$ present. 2.6 mM EGTA and 1 μg/ml TTX were added as indicated. Initial potentials were $\Delta\psi_p$ 60.7 ± 3.3 mV ($n = 5$) and $\Delta\psi_m$ 157 ± 2 mV ($n = 5$). (B) 1.3 mM EGTA and 1.2 mM $MgSO_4$ present. 2.6 mM $CaCl_2$ and 1 μg/ml TTX added as indicated. Initial potentials, $\Delta\psi_p$ 58.4 ± 3.2 mV ($n = 5$), $\Delta\psi_m$ 165 ± 2 mV ($n = 5$). (C) 1.3 mM $CaCl_2$ initially present. 2.6 mM EGTA and 1.2 mM $MgSO_4$ added where indicated. Initial potentials, $\Delta\psi_p$ 59.2 mV ($n = 2$), $\Delta\psi_m$ 163 mV ($n = 2$). ●, $\Delta\psi_p$; ○, $\Delta\psi_m$. Symbols marked with a star are significantly different ($P < 0.05$) from paired controls.

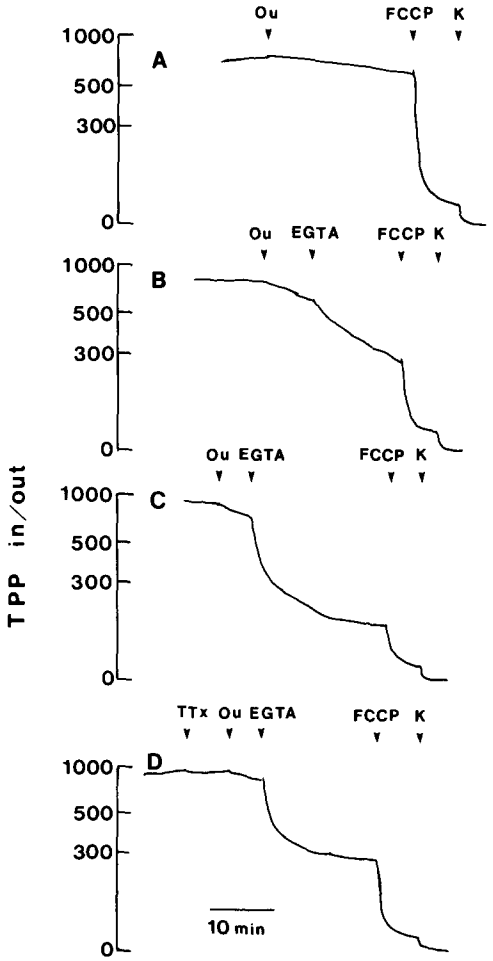

Fig. 4. Effects of divalent cations on the rate of depolarization of the plasma membrane following addition of ouabain (Ou). (A) 1.3 mM $CaCl_2$ present, Mg^{2+} absent; (B) $CaCl_2$ and 1.2 mM $MgSO_4$ present. (C) and (D) $CaCl_2$ present, Mg^{2+} absent. Additions as in Figs. 1 and 2.

In contrast, TTX could not restore TPP accumulation in the absence of divalent cations (Fig. 2b). This, together with the TTX-insensitive respiratory component revealed in the absence of divalent cations (Table I) supports the finding of Schmalzing [6] that a TTX-insensitive Na^+ permeability is induced under these conditions.

The addition of Mg^{2+} to divalent cation-free medium totally reverses the effects of EGTA both on the TPP-electrode (Fig. 2B) and on the individual potentials (Fig. 3C). Thus in contrast to the TTX-sensitive permeability seen in the presence of Mg^{2+}, which can be specifically inhibited by the addition of Ca^{2+}, the TTX-insensitive permeability is non-specifically inhibited by both Ca^{2+} and Mg^{2+}.

The depolarization of the plasma membrane following addition of ouabain is limited by the Na^+-permeability of the membrane. The characteristic slow depolarization seen in the presence of Ca^{2+} and Mg^{2+} (Fig. 4B) is indicative of the low native Na^+ permeability, and is not increased by omitting Mg^{2+} (compare Fig. 4A). However, addition of ouabain and EGTA in the absence of Mg^{2+} (Fig. 4C) induces a rapid and extensive efflux of TPP, which was only partially inhibited by TTX (Fig. 4D).

The accumulation of labelled GABA by synaptosomes is a function of the Na^+ electrochemical potential [4]. It would therefore be predicted that both the TTX-sensitive and insensitive permeabilities would decrease the gradient of GABA maintained across the plasma membrane. Fig. 5A shows that, in the presence of Mg^{2+}, chelation of Ca^{2+} causes a 45% decrease in the gradient of GABA across the plasma membrane, which is not seen when the chelator is added to TTX-inhibited synaptosomes. In the absence of Mg^{2+} (Fig. 5B) the addition of EGTA induces a much more extensive efflux of GABA, which in this case is largely insensitive to TTX.

High concentrations of the hexavalent cation

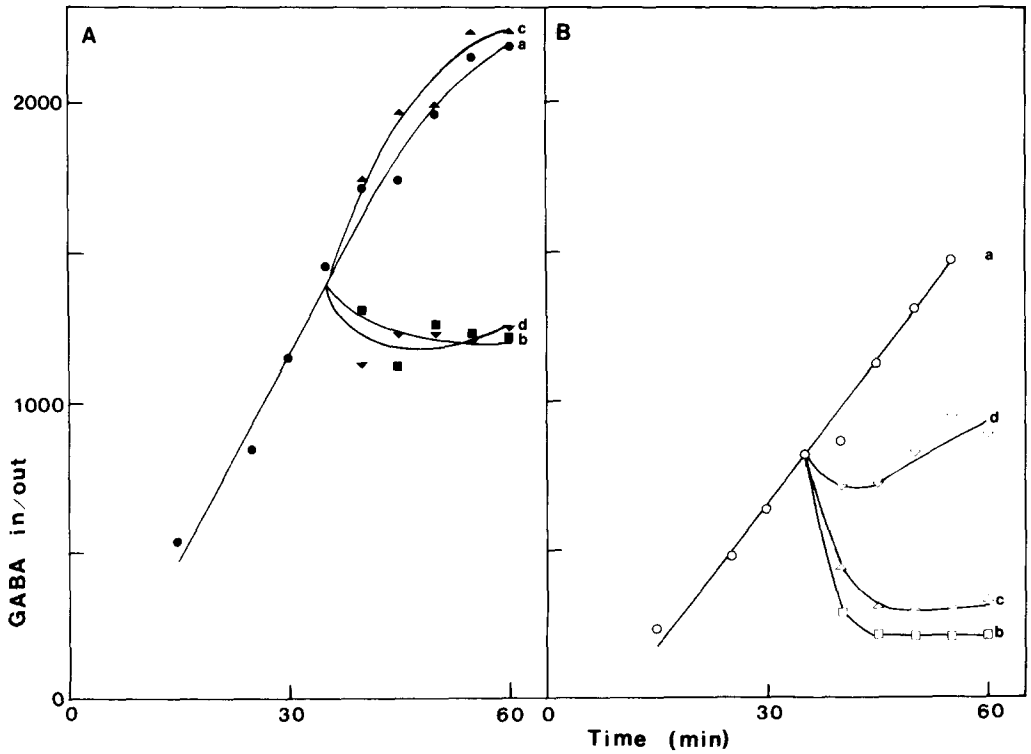

Fig. 5. γ-Aminobutyrate (GABA) accumulation ratios; influence of Ca^{2+}, Mg^{2+}, TTX and Ruthenium red. ^{14}C-GABA uptake was determined as described in Methods. 1.3 mM $CaCl_2$ was present in both experiments (A and B) and in (A) 1.2 mM $MgSO_4$ was additional present. (a) controls; (b) 2.6 mM EGTA added at 31 min; (c) 1 μg/ml TTX added at 28 min followed by 2.6 mM EGTA at 31 min; (d) 20 μM Ruthenium red added at 28 min followed by 2.6 mM EGTA at 31 min.

ruthenium red were reported by Schmalzing [6] to inhibit the TTX-insensitive permeability. Fig. 5 shows that 20 μM Ruthenium red does not prevent EGTA from lowering the GABA gradient in the presence of Mg^{2+} (Fig. 5A), but does inhibit the additional efflux seen in the absence of both cations (Fig. 5B).

The efflux of ions from divalent cation-depleted synaptosomes is not due to disruption of the plasma membrane. Under the conditions of Fig. 5B, 20 min of divalent cation depletion released only 4.4% of lactate dehydrogenase, compared with 3.1% in the presence of Ca^{2+}.

Discussion

Our results indicate that two distinct plasma membrane ion permeabilities are seen on removing divalent cations from synaptosomal incubations. The first is specifically activated by chelating Ca^{2+}, is not affected by other multivalent cations, is sensitive to TTX, and is evidently due to a partial activation of the voltage-dependent Na^+ channel. The second permeability is only seen in media completely lacking divalent cations, can be inhibited non-specifically by a variety of multivalent cations including ruthenium red, is TTX-insensitive but has qualitatively similar effects upon respiration, membrane potentials and Na^+ electrochemical gradients.

Both permeabilities are fully reversible upon re-addition of the relevant cations, and are evidently not associated with non-specific damage to the terminals, since lactic dehydrogenase is retained and the intra-synaptosomal mitochondria maintain high membrane potentials and good respiratory control.

A plasma membrane depolarization could be caused either by an increased Na^+ permeability or a decreased K^+ permeability. Ca^{2+}-activated K^+ channels are well characterized in a variety of cells [14] including synaptic membranes [15]. However, a decreased K^+ conductance due to Ca^{2+} depletion from the cytosol would not be associated with an energy dissipation (see Table I), an increased rate of ouabain-induced depolarization (Fig. 4) or an efflux of ^{86}Rb from the terminal (Fig. 3).

Schmalzing [6] first reported a TTX-insensitive loss of synaptosomal K^+ and gain of Na^+, which was dependent upon lowering the extra-synaptosomal Mg^{2+} concentration below 100 μM, and the Ca^{2+} concentration below 1 μM, and was blocked by 10 μM Ruthenium red.

The rise in $\Delta\psi_m$ upon chelation of extrasynaptosomal Ca^{2+} is maximal only after about 10 min. This is consistent with the time needed to deplete Ca^{2+} from the synaptosome [2,3]. The membrane potential of isolated mitochondria in the presence of limiting permeant anion is highly dependent on the Ca^{2+} accumulated [8] since entry of anions, particularly phosphate, by proton symport prevents the build up of a transmembrane pH gradient at the expense of $\Delta\psi_m$. It is not technically feasible to determine ΔpH across the intra-synaptosomal mitochondrial membrane. However, the total phosphate of our preparation is about 13 $nmol \cdot mg^{-1}$ and is independent of Ca^{2+} content [16]. It is therefore likely that matrix phosphate may be limiting in the Ca^{2+}-replete synaptosome, so that removal of the cation increases $\Delta\psi_m$ at the expense of ΔpH.

Acknowledgements

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