Biochemical Pharmacology, Vol. 38, No. 7, pp. 1109–1113, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

RUTHENIUM RED INHIBITS THE VOLTAGE-DEPENDENT INCREASE IN CYTOSOLIC FREE CALCIUM IN CORTICAL SYNAPTOSOMES FROM GUINEA-PIG

HANNU T. TAIPALE,* RISTO A. KAUPPINEN†‡ and HANNU KOMULAINEN*

*National Public Health Institute, Department of Environmental Hygiene and Toxicology, Kuopio, Finland, and †Department of Clinical Neurophysiology, University Central Hospital of Kuopio, Finland

(*Received* 21 *July* 1988; *accepted* 20 *October* 1988)

Abstract—Effects of ruthenium red (RuR) on adenylates, plasma membrane potential ($\Delta\Psi_p$) and cytosolic free calcium concentration ($[Ca^{2+}]_c$) in cortical synaptosomes from guinea-pig were investigated. Ten micromoles of RuR did not affect either energy levels as indicated by ATP/ADP ratio or the basal $\Delta\Psi_p$. The resting $[Ca^{2+}]_c$ in the presence of RuR was unchanged, but above 5 μM it inhibited by more than 50% of the voltage-activated increase in $[Ca^{2+}]_c$ by K^+-depolarization. In another experiment the potencies of 10 μM RuR and 100 μM verapamil to inhibit high K^+-induced increase in $[Ca^{2+}]_c$ were compared. It was found that either produced 59% inhibition and this inhibition was not potentiated by the substances together (65% inhibition). The extent of depolarisation of $\Delta\Psi_p$ by high external K^+ was independent of the presence of RuR. RuR blocked only 20% of the increase in $[Ca^{2+}]_c$ by veratridine treatment, indicating that Ca^{2+} accumulation into synaptosomal cytoplasm by veratridine involves some additional mechanisms other than depolarisation of $\Delta\psi_p$. The mechanism of inhibition of evoked release of neurotransmitters by RuR is discussed.

Ruthenium red (RuR), a hexavalent cationic dye, has been observed to exert numerous effects both intracellularly and extracellularly in preparations from nervous system. It has been observed to inhibit at micromolar concentration the evoked release of labelled acetylcholine from myenteric-plexus [1] and release of endogenous transmitter from either the presynaptic terminals of neuromuscular junctions [2, 3] or neuronal synapses [4]. RuR blocks as well the release of accumulated [^{3}H]-γ-aminobutyrate from nerve terminals isolated from cerebral cortices [5]. However, the mechanism by which this inhibition occurs is not fully understood.

The neurotransmitter release mechanism that is activated upon depolarization of synaptosomal plasma membrane is dependent on the presence of Ca^{2+} [6]. Apart from Ca^{2+}-dependency the same pathway requires the presence of ATP [7, 8]. We have previously reported that the release of endogenous transmitter glutamate from metabolically inhibited nerve terminals is effectively blocked in the absence of chemical energy [8]. It has been suggested that RuR has properties of a Ca^{2+} entry blocker in synaptosomes [5]. In addition, the RuR at micromolar concentrations has been observed to reduce the activity of pyruvate dehydrogenase complex of synaptosomes [9] and thus can be expected to disturb synaptosomal bioenergetics.

The aim of the present study was to investigate the effects of RuR on the synaptosomal energy levels (judged from ATP and ADP) and cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$) after K^+-depolarization. These two play an ultimate role in the transmitter release from synaptosomes [1–5, 7, 8]. The results show that RuR does not primarily affect synaptosomal ATP concentrations but it blocks the voltage-dependent increase in $[Ca^{2+}]_c$ as effectively as verapamil.

‡ To whom correspondence should be addressed. Present address: Department of Physics, Hunterian Institute, The Royal College of Surgeons of England, 35–43 Lincoln's Inn Fields, London WC2A 3PN.

MATERIALS AND METHODS

Reagents. ^{86}RbCl was from Amersham International (Bucks, U.K.). RuR (Sigma R-2751) was obtained from Sigma (Poole, Dorset, U.K.) and was purified according to the method of Luft [10]. The purified dye consisted of 90% RuR as indicated by the absorbance at 532 nm [10]. Ficoll was from Pharmacia (Uppsala, Sweden) and was dialyzed exhaustively prior to use. Fura-2/AM was purchased from Molecular Probes Inc. (Junction City, OR). ATP-Monitoring Kit was obtained from Wallac Inc. (Turku, Finland). All other reagents were at least of analytical grade.

Preparations of synaptosomes and incubations. Synaptosomes from cerebral cortices of Dunkin–Hartley strain guinea-pigs were prepared in a discontinuous Ficoll gradient according to reference [11]. The incubation medium contained 122 mM NaCl, 3.1 mM KCl, 0.4 mM KH_2PO_4, 5 mM $NaHCO_3$, 20 mM Na-2-{[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino}ethanesulphonate, 1.2 mM $MgSO_4$, 16 μM bovine serum albumin, 10 mM D-glucose, pH 7.4 at 37°. $CaCl_2$ (1.2 mM) was added after 5 min preincubation to allow synaptosomal membranes to become polarised [12].

Measurements of cytosolic free calcium. The cytosolic free calcium concentration ($[Ca^{2+}]_c$) was determined using intrasynaptosomally-trapped fura-2 as an indicator essentially as described by Komulainen and Bondy [13]. Briefly, synaptosomes (2 mg of protein/ml) were incubated in the presence of 5 μM

fura-2/AM for 35 min. Synaptosomes loaded with fura-2 were spun in a bench-top centrifuge (Hereaus-Christ, Biofuge A) at 10,000 rpm for 2 min and the supernatant was carefully discarded. Pellets were suspended into Ca^{2+}-free incubation medium to give a protein concentration of 1 mg/ml. Fluorescence of intrasynaptosomal fura-2 was recorded in the excitation beam of a Hitachi F-4000 fluorometer (Tokyo, Japan) scanned from 340 nm to 380 nm with emission at 510 nm. For control experiments $[Ca^{2+}]_c$ was determined 15 min after addition of 1.2 mM $CaCl_2$. In experiments with RuR it was added 9 min after addition of Ca^{2+} and $[Ca^{2+}]_c$ assayed 6 min later. KCl or veratridine were added in both cases 16 min after addition of Ca^{2+}. Values of $[Ca^{2+}]_c$ were corrected for extrasynaptosomal fura-2 by Mn^{2+} quenching according to [14].

Plasma membrane potential. Plasma membrane potential ($\Delta\Psi_p$) was estimated from the diffusion potential of $^{86}Rb^+$ (6 μM, 0.33 μCi/ml) according to Scott and Nicholls [14] and calculated using the Nernst equation.

Measurement of ATP and ADP. ATP and ADP were measured from neutralized perchloric acid (7% in 25 mM EDTA) extracts of synaptosomes with the luciferin assay as in Ref. 15. Each assay was standardised with an internal standard of 80 nM ATP.

RESULTS

The synaptosomal ATP concentrations in the absence and presence of 10 μM RuR were 1.8 ± 0.17 and 1.7 ± 0.05 (mean ± SEM, N = 3) nmol/mg protein, respectively. ATP/ADP ratios were 8.7 ± 0.5 and 9.7 ± 0.2, accordingly. A drop in synaptosomal ATP/ADP ratio has been shown to be a good indicator of either inhibition of energy metabolism or increase in the energy demand [7, 8]. Thus the unchanged ATP/ADP ratio in the presence of RuR indicates that it affected neither synaptosomal energy production nor energy consumption. The latter is supported by our previous observation that RuR does not influence synaptosomal respiration.*

Fura-2 has been proven to be a specific and sensitive indicator of ionized Ca^{2+} within cells [16]. The fura-2/Ca^{2+} complex emits light with a maximum of 505 nm when excited at 340/380 nm [17]. Thus RuR can be expected to quench the fluorescence of intrasynaptosomal fura-2, because RuR has an intense absorbance at 532 nm (extinction coefficient of 68 $mM^{-1}\,cm^{-1}$ [15]). The reduction of the intrasynaptosomal fura-2/Ca^{2+} signal intensity using emission at 510 nm was, however, similar for excitation at 340 nm (19.2 ± 0.4%, N = 8, ±SEM) and at 380 nm (23.2 ± 0.3%, N = 8, ±SEM). The ratios of fura-2/Ca^{2+} at the saturating Ca^{2+} concentration to fura-2 in the absence of Ca^{2+}, i.e. R_{max}/R_{min} [12, 16], were the same in the absence and presence of 10 μM RuR (6.8 ± 0.3 and 7.3 ± 0.3, N = 12, ±SEM, respectively) making it possible to calibrate the signal and thus to quantify $[Ca^{2+}]_c$.

The resting synaptosomal $[Ca^{2+}]_c$ in the absence and presence of 10 μM RuR was 254 ± 29 (N = 6, ±SEM) and 241 ± 22 (N = 6, ±SEM) nM, respectively. After K^+-depolarization there is an initial overshooting in increase in $[Ca^{2+}]_c$, which stabilizes after 40–60 sec [8]. Five minutes after addition of 36 mM K^+, $[Ca^{2+}]_c$ was 365 ± 13 and 298 ± 5 nM (N = 5, ±SEM) in the absence and presence of 10 μM RuR, respectively. Thus RuR inhibited 50% of the rise in $[Ca^{2+}]_c$ (Fig. 1). The inhibiting effect of RuR on K^+-depolarization-activated increase in $[Ca^{2+}]_c$ showed concentration dependence (Fig. 2), with the maximal response obtained by 10 μM. Assuming that the effect of RuR on the voltage-dependent increase in $[Ca^{2+}]_c$ was due to optical quenching of intrasynaptosomal fura-2/Ca^{2+} signal, one would expect to see higher inhibition at high concentrations of RuR. The effect of RuR, however, attained a plateau at some 5 μM confirming that the optical effect of RuR did not significantly interfere with the determination of $[Ca^{2+}]_c$.

The rise in $[Ca^{2+}]_c$ in the presence of veratridine was greater than by K^+-depolarization (Fig. 1). This finding is consistent with the report by Hansford and Castro [9] and with our previous work with rat cortical synaptosomes [13]. Ten micromoles of RuR inhibited only 20% of the veratridine-induced increase in $[Ca^{2+}]_c$ (Fig. 1) and $[Ca^{2+}]_c$ was 545 ± 21 and 479 ± 14 (N = 3, ±SEM) in the absence and presence of the dye, respectively.

K^+-depolarization collapses $\Delta\Psi_p$ and activates Ca^{2+} entry through the voltage-dependent Ca^{2+}-channel [6, 12, 13]. Submillimolar concentrations of verapamil have been shown to block some 70% of the high K^+-induced increase in $[Ca^{2+}]_c$ in rat cortical synaptosomes [13]. We compared potencies of verapamil and RuR in a paired experiment, where synaptosomes from the same preparation were used. In the control experiment 36 mM KCl increased $[Ca^{2+}]_c$ from 240 nM to 348 nM. One hundred micromoles of verapamil and 10 μM of RuR blocked the increase in $[Ca^{2+}]_c$ by KCl-depolarization equally effectively (59% each). Addition of verapamil (100 μM) and RuR (10 μM) in combination did not potentiate the inhibition of $[Ca^{2+}]_c$ increase produced by high K^+, which was blocked by 65%. This might indicate that RuR and verapamil are acting at the same locus of Ca^{2+} entry in nerve terminals.

The resting $\Delta\Psi_p$ from the diffusion potential of $^{86}Rb^+$ is 52 ± 3 and 53 ± 3 mV (N = 3) in the absence and presence of 10 μM RuR, respectively. RuR does not prevent the depolarization of $\Delta\Psi_p$ by high K^+ (Fig. 3).

DISCUSSION

In this paper we show that RuR acts as an effective blocker of the voltage-activated increase in $[Ca^{2+}]_c$ in the cortical synaptosomes of the guinea-pig. In perfused heart, RuR has been shown to cause effects similar to blockers of slow Ca^{2+} inward current [18]. The Ca^{2+}-channel of nerve terminals is relatively insensitive to verapamil-type Ca^{2+}-entry blockers as indicated by the four orders of magnitude higher concentration of verapamil required to show an effect in synaptosomes [13] compared to heart [18].

* Kauppinen RA, Taipale HT and Komulainen H, submitted for publication.

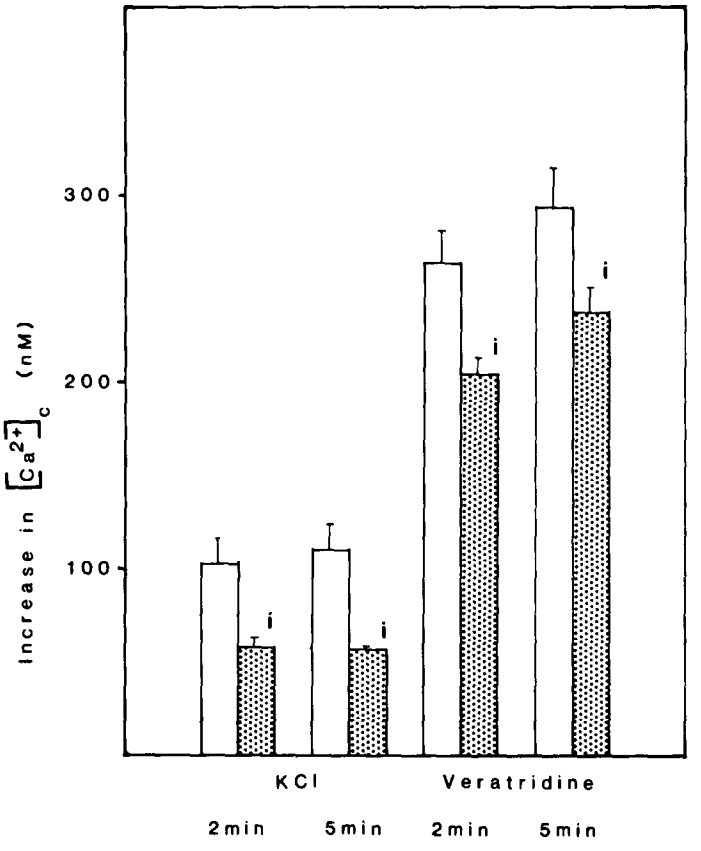

Fig. 1. Increase in $[Ca^{2+}]_c$ by KCl-depolarization and veratridine treatment in the absence and presence of RuR. $[Ca^{2+}]_c$ was determined as described in text. 10 μM RuR was added to experiment depicted with dotted histograms 5 min after 1.2 mM $CaCl_2$. 36 mM KCl or 75 μM veratridine were added 15 min after 1.2 mM $CaCl_2$. Histograms are representative of means of 3–5 independent experiments, SEM indicated by bars. Statistical difference relative to experiments free of RuR, (i) $P < 0.05$, Student's *t*-test.

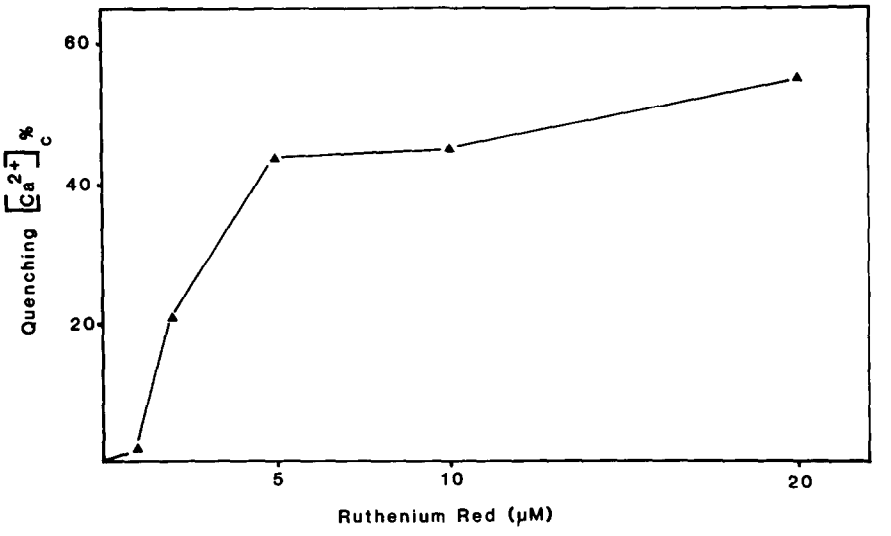

Fig. 2. Concentration dependency of inhibition of increase in $[Ca^{2+}]_c$ by RuR in KCl-depolarized synaptosomes. $[Ca^{2+}]_c$ was determined as described in text. 0, 1, 2, 5, 10 or 20 μM RuR was added 5 min after 1.2 mM $CaCl_2$. 36 mM KCl was added 10 min later and $[Ca^{2+}]_c$ redetermined after 5 min. Inhibition of increase in $[Ca^{2+}]_c$ is compared to the experiment with 0 external RuR.

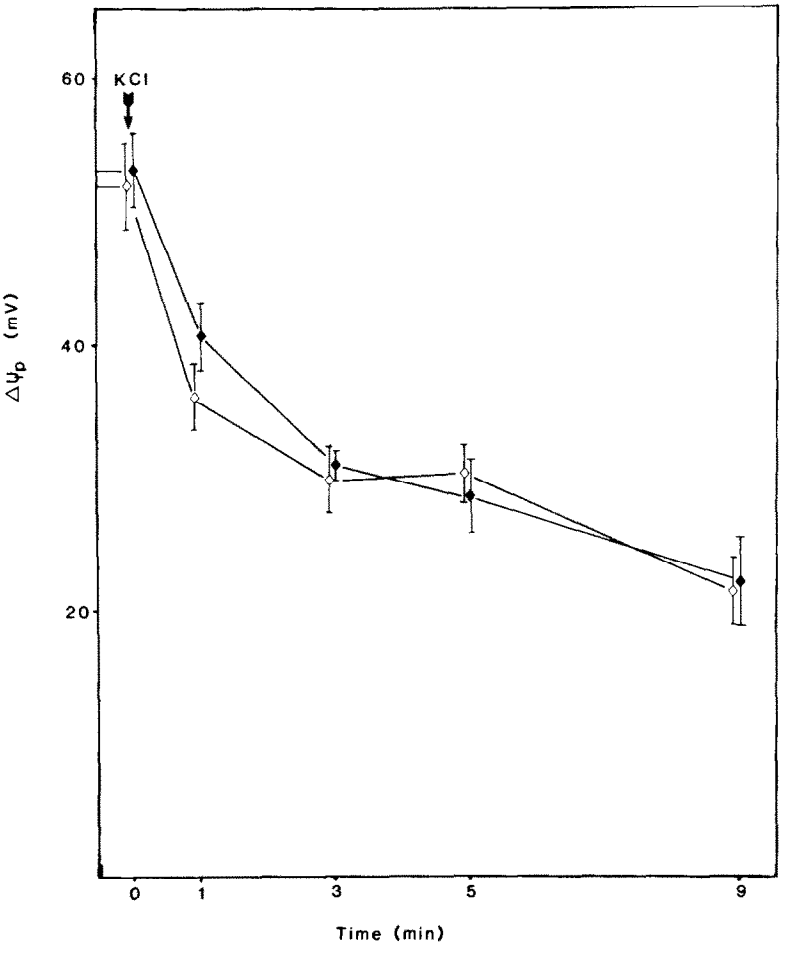

Fig. 3. Synaptosomal plasma membrane potential after KCl-depolarization. $\Delta\Psi_p$ was measured as described in text. Synaptosomes were incubated either in the absence (◇) or presence (◆) of 10 μM RuR for 34 min before addition of 36 mM KCl (KCl). Each symbol is a mean of three independent experiments, SD indicated by a bar.

On a molar basis RuR is at least as potent a blocker of the synaptosomal voltage-dependent Ca^{2+} channel as is verapamil [13].

RuR had much less effect on veratridine-induced increase in $[Ca^{2+}]_c$. Veratridine opens the voltage-dependent and tetrodotoxin-sensitive Na^+-channel thus allowing continuous influx of Na^+ and drop of $\Delta\Psi_p$ [19]. Simultaneously, it has been shown to increase synaptosomal energy consumption and decrease ATP/ADP ratio [7]. It has been suggested that Ca^{2+} entry into synaptosomes during exposure to veratridine is not only due to opening of the voltage-dependent Ca^{2+}-channel but might also be coupled to influx of Na^+ [19]. We have reported previously that RuR acts on tetrodotoxin-sensitive Na^+-permeability of synaptosomes [20]. Thus the larger increase in $[Ca^{2+}]_c$ by veratridine and the less potent effect of RuR on this increase can be understood as an involvement of tetrodotoxin-insensitive ionic permeability of the synaptosomal plasma membrane together with a drop of synaptosomal ATP/ADP ratio.

We have previously reported that RuR can also inhibit the increased Na^+-permeability which is activated upon omission of external divalent cations in isolated nerve terminals [20]. In the soma of snail neurons, RuR has been observed to prolong opening of the Na^+-channel at nanomolar concentrations and in the micromolar range can inhibit the Ca^{2+} inward current [21]. According to the present results, RuR does not increase synaptosomal energy demand which suggests that it does not increase plasma membrane Na^+ permeability which it has been shown to do in snail neurons. This is further supported by our previous work, where RuR was able to substitute for divalent cations in preventing influx of Na^+ into synaptosomes [20]. RuR, which bears six positive charges, is by no means specific in affecting either Ca^{2+} or Na^+ influx in neurons and nerve terminals.

RuR prevents the evoked release of transmitter from presynaptic terminals of various sources [1–5]. It has been suggested that the effects of RuR might be due to block of Ca^{2+} entry [5]. Another explanation has been that RuR might elevate resting $[Ca^{2+}]_c$ by inhibiting the Ca^{2+} uniporter of mitochondria [6]. The prolonged elevation of $[Ca^{2+}]_c$ has

been suggested to desensitise the release mechanism of transmitters [1, 2]. According to our results block of the voltage-dependent increase in $[Ca^{2+}]_c$ should be considered as a reason for inhibition of the transmitter release from nerve terminals.

Acknowledgements—Supported by a grant (No. 07/006) from the Finnish Academy of Sciences. Our thanks to Mrs. Leena Heikkinen for expert technical assistance.

REFERENCES

1. Bernath S and Vizi S, Inhibitory effect of ionized free intracellular calcium enhanced by ruthenium red and m-chlorocarbonylcyanide phenyl hydrazone on the evoked release of acetylcholine. *Biochem Pharmacol* **36**: 3683–3687, 1987.
2. Adams DJ, Takeda K and Umbach JA, Inhibitors of calcium buffering depress evoked transmitter release at the squid giant synapse. *J Physiol (Lond)* **369**: 145–159, 1985.
3. Alnaes E and Rahamimoff R, On the role of mitochondria in transmitter release from motor nerve terminals. *J Physiol (Lond)* **248**: 285–306, 1975.
4. Baux G, Simonneau M and Tauc L, Transmitter release: ruthenium red used to demonstrate a possible role of sialic acid containing substrates. *J Physiol (Lond)* **291**: 161–178, 1979.
5. Tapia R and Meza-Ruiz G, Inhibition by ruthenium red of the calcium-dependent release of [^{3}H]GABA in synaptosomal fractions. *Brain Res* **126**: 160–166, 1977.
6. Nicholls DG and Åkerman KEO, Ca^{2+} transport and regulation of transmitter release in isolated nerve endings. *Trends Biochem Sci* **8**: 63–64, 1983.
7. Sanchez-Prieto J, Sihra TS and Nicholls DG, Characterization of the exocytotic release of glutamate from guinea pig cerebral cortical synaptosomes. *J Neurochem* **49**: 58–64, 1987.
8. Kauppinen RA, McMahon H and Nicholls DG, Ca^{2+}-dependent and Ca^{2+}-independent glutamate release, energy status and cytosolic free Ca^{2+} concentration in isolated nerve terminals following metabolic inhibition: possible relevance to hypoglycaemia and anoxia. *Neuroscience* **27**: 175–182, 1988.
9. Hansford RG and Castro F, Role of pyruvate dehydrogenase in brain mitochondria and synaptosomes. *Biochem J* **227**: 129–136, 1985.
10. Luft JH, Ruthenium red and violet. Chemistry, purification, methods of use for electron microscopy and mechanism of action. *Anat Rec* **171**: 347–368, 1972.
11. Nicholls DG, Calcium transport and proton electrochemical potential in mitochondria from guinea-pig cerebral cortex and heart. *Biochem J* **170**: 511–522, 1978.
12. Heinonen E, Akerman KEO, Kaila K and Scott IG, Dependence of cytoplasmic calcium transients on membrane potential in isolated nerve endings of guinea pig. *Biochim Biophys Acta* **815**: 203–208, 1985.
13. Komulainen H and Bondy SC, The estimation of free calcium within synaptosomes and mitochondria with fura-2; comparison fo Quin-2. *Neurochem Int* **10**: 55–64, 1987.
14. Scott ID and Nicholls DG, Energy transduction in intact synaptosomes. Influence of plasma membrane depolarization on the respiration and membrane potential of internal mitochondria determined *in situ*. *Biochem J* **186**: 21–33, 1980.
15. Kauppinen RA and Nicholls DG, Synaptosomal bioenergetics. The role of glycolysis, pyruvate oxidation and responses to hypoglycaemia. *Eur J Biochem* **158**: 159–165, 1986.
16. Cobbold PH and Rink TJ, Fluorescence and bioluminescence measurement of cytoplasmic free calcium. *Biochem J* **248**: 313–328, 1987.
17. Grynkiewicz G, Poenie M and Tsien RY, A new generation of Ca^{2+} indicators with greatly improved fluorescent properties. *J Biol Chem* **260**: 3440–3450, 1985.
18. McCormack JG and England PJ, Ruthenium red inhibits the activation of pyruvate dehydrogenase caused by positive inotropic agents in the perfused rat heart. *Biochem J* **214**: 581–585, 1983.
19. Nicholls DG and Akerman KEO, Biochemical approach to study of cytosolic calcium regulation in nerve endings. *Phil Trans Soc Lond B* **296**: 115–122, 1981.
20. Kauppinen RA, Sihra TS and Nicholls DG, Divalent cation modulation of the ionic permeability of synaptosomal plasma membrane. *Biochim Biophys Acta* **860**: 178–184, 1986.
21. Stimmers JR and Byerly L, Slowing of sodium current inactivation by ruthenium red in snail neurons. *J Gen Physiol* **80**: 485–497, 1982.