Mechanism of depolarization of rat cortical synaptosomes at submicromolar external Ca^{2+} activity

The use of Ca^{2+} buffers to control the synaptosomal membrane potential

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Rat cortical synaptosomes responded to a reduction of external Ca^{2+} from pCa 3.5 to pCa 4.8 in the absence of MgCl_2 with a slight decrease of internal K^+ and an increase of Na^+ . The effects were prevented by tetrodotoxin or millimolar concentrations of MgCl_2 . Further lowering of external pCa to 7.7 with *N*-hydroxyethylethylenediaminetriacetate evoked a rapid fall of internal K^+ , which was specifically blocked by Ruthenium Red; tetrodotoxin and nifedipine were ineffective. A linear relationship was established between K^+ and methyltriphenylphosphonium cation distribution ratios by varying external pCa between 4.8 and 7.7, indicating that K^+ efflux resulted from a depolarization of the plasma membrane. An increase of Na^+ permeability was suggested by the synaptosomes' gain of Na^+ and the disappearance of the depolarization in an Na^+ -free sucrose medium. According to the constant field equation, the permeability ratio $P_{\text{Na}}/P_{\text{K}}$ increased from 0.029 at pCa 4.8 to 0.090 at pCa 7.7 with plasma membrane potentials of -74 mV and -47 mV , respectively. Since the plasma membrane responded to variation of external Ca^{2+} activities in the micromolar range with a graded and sustained depolarization, the use of Ca^{2+} buffers to control membrane potentials is suggested.

Neuronal membranes are endowed with different types of ion channels and carriers providing the molecular basis of excitability. Within this system, the role of Ca^{2+} as modulator and second messenger has attracted considerable attention. At the external side of the axonal membrane, a reduction of the Ca^{2+} concentration is associated with a marked increase of excitability (Brink, 1954) caused by the action of Ca^{2+} on systems controlling Na^+ and K^+ permeability (Frankenhaeuser & Hodgkin, 1957). At the inner surface of the membrane, the increase of Ca^{2+} concentration evoked

by the influx during depolarization activates K^+ channels in several neuronal tissues (Meech, 1978). The influx of Ca^{2+} during depolarization of the presynaptic membrane is an essential prerequisite for the liberation of transmitters (Katz & Miledi, 1967). Moreover, Ca^{2+} was found to accelerate the uptake of neurotransmitters into brain synaptosomes (White, 1975) by an unknown mechanism.

The present study was initiated during an investigation of the mechanism of action of Ca^{2+} on the rate of neurotransmitter uptake into synaptosomes. Lowering of the external Ca^{2+} activity to submicromolar levels depolarized the synaptosomal plasma membrane, probably by increasing Na^+ permeability. The Na^+ channel blocker tetrodotoxin and the Ca^{2+} channel blocker nifedipine failed to prevent depolarization, but low concentrations of the glycoprotein stain Ruthenium Red mimicked the effect of external Ca^{2+} . Parts of these results have been included in a short communication (Schmalzing, 1983).

Abbreviations used: $\Delta\psi_p$, membrane potential across the plasma membrane; TPMP^+ , methyltriphenylphosphonium cation; TPB^- , tetraphenylboron anion; CCCP, carbonyl cyanide *m*-chlorophenylhydrazine; HEDTA, *N*-hydroxyethylethylenediaminetriacetate; $\text{K}_{i/o}^+$, $\text{TPMP}_{i/o}^+$, indices *i/o* indicate accumulation ratios of the respective substances with the signification $[\text{K}^+]_i/[\text{K}^+]_o$ and $[\text{TPMP}^+]_i/[\text{TPMP}^+]_o$; P_{Na} , P_{K} , permeability coefficients of the respective cations.

Materials and methods

Chemicals

[³H]TPMP⁺ iodide (44.6 Ci/mmol) and Protosol, a tissue solubilizer, were obtained from New England Nuclear Chemicals (Dreieich, Germany). [¹⁴C]sucrose (10 mCi/mmol) and ³H₂O were from Amersham-Buchler (Braunschweig, Germany). Unisolve I (Zinsser, Frankfurt, Germany) was used as a scintillator for aqueous samples. Ficoll 400 was obtained from Pharmacia (Uppsala, Sweden). HEDTA, Ruthenium Red, tetrodotoxin, valinomycin, CCCP, Tes, TPB⁻ and TPMP⁺ were purchased from Sigma (München, Germany). Ethylenephénylethylenglycol (Nonidet) was from Fluka (Neu-Ulm, Germany) and the silicone oils AR 20, AR 200, and AP 200 were from Wacker Chemie (München, Germany). All other substances were obtained through Merck (Darmstadt, Germany) in analytical or Suprapur grade.

Preparation of synaptosomes

Cerebral cortices of Wistar rats (200–250 g) of either sex were homogenized in isolation medium (0.32 M-sucrose/5 mM-sodium phosphate, pH 7.4) containing 1 mM-Na₃EDTA. In the following steps, EDTA was omitted. Synaptosomes were purified according to the method of Booth & Clark (1978), using a discontinuous Ficoll gradient. The purified synaptosomes were washed in isolation medium and were stored as pellets at 4°C. Experiments in Na⁺-free media required a second washing in 0.32 M-sucrose/5 mM-Tris, pH 7.4, at 4°C, to remove contaminating Na⁺. The protein concentration was determined by the Coomassie method of Sedmak & Grossberg (1977) after dissolution of synaptosomes in NaOH (final concn. 0.5 M), using bovine serum albumin as a standard.

Composition of media

The standard suspension medium consisted of 120 mM-NaCl, 10 mM-glucose and 30 mM-Tes, adjusted to pH 7.4 with NaOH at 37°C. The Cl⁻-free medium was similar to the standard medium except that NaCl was replaced by 120 mM-sodium gluconate. The two Na⁺-free media contained 30 mM-Tris, 10 mM-glucose, and either 120 mM-choline chloride or 280 mM-sucrose, adjusted to pH 7.4 with HCl at 37°C. K⁺ and divalent cations were added as indicated in the legends to Figures.

Adjustment and determination of Ca²⁺ activities

Pilot tests had shown that it was desirable to buffer Ca²⁺ in the range of 30 nM–30 μM. Therefore, HEDTA (anhydrous free acid) was preferred as the complexing agent, since it has a lower Ca²⁺ affinity than EGTA. The Ca²⁺ buffers were adjusted to pH 7.4 with NaOH and added to the

test tubes as described below at a final concn. of 1 mM-HEDTA, if not indicated otherwise. The chloride-free Ca²⁺ buffer was prepared with calcium gluconate. Ca²⁺ activities were determined with a liquid membrane electrode (Orion Research, Cambridge, MA, U.S.A.) at 37°C.

Measurement of Na⁺, K⁺ and TPMP⁺ distribution

Incubations were carried out in 0.4 ml micro-sediment tubes (Sarstedt, Nümbrecht, Germany). Just before use, the synaptosomal pellet was suspended in one of the media described above, equilibrated with 100% O₂ at room temperature. Aliquots of 100 μl were added to the test tubes containing drugs, isotopes and cations to alter the external milieu in the desired manner (total volume 200 μl). The addition of synaptosomes to 56 tubes was completed within 1 min by using a Multipette (Eppendorf, Germany) for repetitive pipetting. Separate samples for determination of internal Na⁺ and K⁺ or [³H]TPMP⁺ were incubated in parallel. The distribution of [³H]TPMP⁺ (0.2 μM-TPMP⁺; 0.05 μCi/sample) was assayed in the presence of TPB⁻ (Scott & Nicholls, 1980). Water-insoluble drugs were added from stock solutions in dimethyl sulphoxide. Control experiments confirmed that the final concn. of 0.5% dimethyl sulphoxide influenced neither TPMP⁺ nor K⁺ distribution.

At the predetermined times, the synaptosomes were centrifuged through a layer of 5 μl of a mixture of silicone oils by using a Beckman Microfuge (10000 g for 1 min), equipped with water cooling. In experiments designed for measuring steady-state distributions of cations, silicone oil centrifugation was performed in a Beckman J-21B centrifuge at 37°C (45000 g for 5 min) after 10 min of incubation. The fixed angle rotor (Beckman JA 20) was prewarmed in a water bath to achieve a temperature of 37°C during centrifugation.

Measurement of pellet spaces

Internal concentrations were calculated by referring to pellet spaces determined in triplicate with each experiment. The test tubes received 0.2 μCi of ³H₂O to measure the total pellet volume or 0.1 μCi of [¹⁴C]sucrose to measure the external volume.

Sample preparation for liquid-scintillation counting

The tips of the tubes were cut off within the silicone oil layer. The remaining tube including the entire supernatant was added to 3 ml of Unisolve I. The sediment was removed from the tip by short centrifugation, solubilized in 50 μl of Protosol by shaking overnight, and then added to 4 ml of toluene scintillator. Radioactivity was estimated by liquid-scintillation spectrometry in a Packard

Tricarb 2660 using the channels-ratio method for automatic quenching correction.

Sample preparation for flame emission or atomic absorption spectroscopy

The tip of the test tube containing the synaptosomal pellet was placed inverted into a 2 ml tube to remove the sediment by a short centrifugation. The empty tip was discarded. After resuspension of the pellet in 150 µl of 1% (w/v) Nonidet by shaking overnight, the sample was diluted with 1.55 ml of 0.1% CsCl (Suprapur, Merck) in bidistilled water. CsCl was added to suppress ionization. Nonidet was chromatographically purified by a weak cation exchange resin.

Cation concentrations were determined in a Philips SP9 spectrophotometer by flame emission (Na, K) or atomic absorption (Ca) spectrometry. For Ca measurements, a nitrous oxide/fuel flame was used, in which Ca can be determined virtually free of chemical interferences.

Determination of Ruthenium Red

Ruthenium Red was determined at 532 nm, assuming an ε of 61 500 litre·mol⁻¹·cm⁻¹ in water (Fletcher *et al.*, 1961). According to spectroscopy, the commercial product (Sigma) contained 43% Ruthenium Red.

Results

External and cytoplasmic pellet spaces

The pellet spaces depended upon the composition of the incubation medium and the centrifugal force, as shown in Table 1. Qualitatively similar effects were observed in the synthetic media. The omission of Na⁺ caused pellet spaces, including the internal volumes, to decrease to 2.9 ± 0.3 (s.d.) µl/mg of protein (choline⁺ medium) or 3.5 ± 0.2 µl/mg (sucrose medium), respectively. The ³H₂O and [¹⁴C]sucrose volumes were about 20% larger at 10 000 g (Microfuge) than at 45 000 g. Internal volumes were not appreciably influenced

by centrifugal force. Since the experimental conditions significantly affected the volumes of pellet spaces, it was essential to calculate cytoplasmic concentrations on the basis of volume measurements carried out in parallel, especially in the case of [Na⁺]_i determinations.

Effect of external divalent cations on internal K⁺ and Na⁺ levels

A survey of the effects of divalent cations is given in Table 2. The sole omission of MgCl₂ induced a slight increase of [Na⁺]_i, but was ineffective in altering [K⁺]_i. When CaCl₂ was additionally omitted, [K⁺]_i fell to a variable extent depending upon the contaminating Ca²⁺ in the nominally Ca²⁺-free medium. The Ca²⁺ of the medium amounted to 2–3 µM only, but during incubation contaminating Ca²⁺ increased to 25–35 µM, as measured by atomic absorption spectroscopy. Therefore, external Ca²⁺ activities were controlled with Ca²⁺ buffers. Two Ca²⁺-dependent effects were observed. (i) Lowering external Ca²⁺ from pCa 3.5 to pCa 4.8 resulted in a 10% decrease of internal K⁺ and a slight increase of Na⁺ (Table 2). (ii) Activities below pCa 4.8 induced a steep decline of internal K⁺ (Fig. 1). A maximal (half-maximal) loss was observed at pCa 7.7 (5.7).

The effect of Mg²⁺ on internal K⁺ at low Ca²⁺ was investigated in the presence of 2 mM-EGTA (Fig. 1). On a molar basis Mg²⁺ was 56 times less effective than Ca²⁺ in preventing the loss of internal K⁺, assuming that about 30% of the added MgCl₂ was thermodynamically active.

Concomitant with the K⁺ loss, synaptosomes gained Na⁺ (Table 2). The large entrapment of external Na⁺ within the pellet required a substantial correction to calculate [Na⁺]_i depending critically on the volume of the [¹⁴C]sucrose space. Moreover, Åkerman & Nicholls (1981) have brought up the question of additional Na⁺ binding to the external surface of synaptosomes. Therefore, in several experiments contaminating external Na⁺ was reduced by using a rapid washing procedure. Similar

Table 1. Influence of divalent cation concentrations in the standard medium on pellet spaces

The ³H₂O and [¹⁴C]sucrose spaces of the synaptosomal pellets were determined in the standard medium at 37°C in the presence of 3 mM-KCl and divalent cations as indicated. The Beckman J-21B centrifuge was used to spin the synaptosomes through a layer of silicone oil at 37°C (45 000 g for 5 min). Values given are means ± s.d. for *n* independent experiments.

Divalent cations	<i>n</i>	Volume (µl/mg of protein)		
		Total	External	Internal
pCa 7.7	10	13.3 ± 0.3	9.3 ± 0.3	4.0 ± 0.2
pCa 4.8	10	13.0 ± 0.2	8.9 ± 0.2	4.1 ± 0.2
1 mM-CaCl ₂ (pCa 3.5)	6	12.7 ± 0.2	8.6 ± 0.2	4.1 ± 0.2
1 mM-CaCl ₂ + 1 mM-MgCl ₂	5	12.8 ± 0.3	8.6 ± 0.3	4.2 ± 0.1

Table 2 *Influence of external divalent cations on intrasynaptosomal Na⁺ and K⁺ concentrations*

Synaptosomes were incubated at 37°C in the standard medium in the presence of 3 mM-KCl and divalent cations as indicated. Method A was the routinely used silicone oil centrifugation (37°C, 5 min, 45000g) of 200 µl samples of synaptosomes (0.8 mg of protein/ml). Pellet spaces were determined in parallel. The cation concentrations of the pellets were referred to internal volumes after correcting for entrapment of external Na⁺ or K⁺ within the pellet. External Na⁺ amounted to 143 mM. In method B, aliquots (20 µl) of a concentrated synaptosomal suspension (about 5 mg of protein/ml) were incubated in test tubes. [Na⁺]_i was determined after a 16-fold dilution of the external medium with ice-cold 100 mM-MgCl₂ or 152 mM-choline chloride + 2 mM-MgCl₂, added just before silicone oil centrifugation at 4°C (20000g for 10 min). Both washing solutions yielded similar results. Results are the means ± S.D. of three independent experiments per method.

Divalent cations	Method A		Method B
	[K ⁺] _i (mM)	[Na ⁺] _i (mM)	[Na ⁺] _i (mM)
pCa 7.7	17 ± 1	128 ± 10	118 ± 8
pCa 4.8	47 ± 1	89 ± 7	93 ± 5
pCa 4.8 + 5 µM-tetrodotoxin	55 ± 2	77 ± 7	
1 mM-CaCl ₂	52 ± 2	76 ± 6	86 ± 5
1 mM-CaCl ₂ + 1 mM-MgCl ₂	53 ± 2	71 ± 8	81 ± 5

results were obtained by the different methods, as compared in Table 2.

Effect of A23187

If external Ca²⁺ must gain access to an internal site of action, it might be expected that Ca²⁺ exerts its effect at lower activities in the presence of A23187. Results of a corresponding experiment are included in Fig. 1. A23187 caused an additional drop of [K⁺]_i already at pCa 7.7 instead of shifting the curve to the left.

Time course of K⁺ efflux subsequent to Ca²⁺ complexation

Synaptosomes were incubated in the presence of 20 µM-CaCl₂. During incubation, total Ca²⁺ rose to about 50 µM (HEDTA absent), sufficient to maintain high internal K⁺ of 45–50 mM. As shown in Fig. 2, the addition of 1.3 mM-HEDTA initiated an immediate K⁺ efflux (pH_o declined from 7.40 to 7.37). The effect was completely reversible if enough CaCl₂ was added within 30 s to restore a pCa above 4.8 (pH_o declined to 7.23). The later the addition of CaCl₂, the slower was the re-uptake of K⁺.

Relation between K⁺ and TPMP⁺ accumulation

When Ca²⁺ was varied between pCa 7.7 and 4.5, the accumulation ratios of TPMP⁺ and K⁺ were found to be linearly related (Fig. 3). A similar relationship was established by varying MgCl₂ between 10 µM and 1 mM in the presence of 2 mM-EGTA (pCa 7.9) with K_i_{TPMP}⁺ = 0.0301 TPMP_i⁺ + 0.4 (r = 0.998). Using the equations given by Scott & Nicholls (1980), it can be shown that the membrane potential of internal mitochondria remained

unaffected in the present range of concentrations of divalent cations.

Obviously, the efflux of K⁺ and TPMP⁺ at pCa 7.7 resulted from a depolarization of the synaptosomal plasma membrane. Abolishing the membrane potential of intrasynaptosomal mitochondria by CCCP or valinomycin (Scott & Nicholls, 1980) supported this interpretation. Results are included in Fig. 3. CCCP and valinomycin reduced TPMP⁺ accumulation by a factor of 4 and 7, respectively, whereas K⁺ accumulation declined by only 10–20%. Since valinomycin induces a high electrical permeability to K⁺, it can be concluded that Ca²⁺ affected internal K⁺ by a mechanism which is independent of K⁺ permeability.

Mechanism of depolarization at pCa 7.7

A depolarization in a Ca²⁺-free medium can theoretically be produced by a permeability increase of the membrane to Na⁺ or Cl⁻. Replacing external Na⁺ by choline⁺ failed to prevent K⁺ efflux at pCa 7.7, although the relative decrease of internal K⁺ was attenuated (results not shown). Since an electrogenic influx of choline⁺ at pCa 7.7 might have depolarized the membrane in the Na⁺-free medium, additional experiments were performed in the sucrose medium. As shown in Table 3, internal K⁺ in the Na⁺-free sucrose medium was independent of external Ca²⁺. The addition of 38 mM-Na⁺ induced depolarizations of the plasma membrane, which were much more pronounced at pCa 7.7 than at pCa 4.8.

Degree of alteration of Na⁺ permeability induced by Ca²⁺ complexation

The permeability ratio α (= P_{Na}/P_K) was as-

Fig. 1. Effect of external divalent cations on internal K^+ levels

Synaptosomes were incubated for 10 min at 37°C in the standard medium in the presence of 3 mM-KCl. The samples contained either CaCl_2 (●, low activities buffered with HEDTA) or 2 mM-EGTA plus increasing MgCl_2 concentrations (○). Several samples received A23187 (■, final concn. $5 \mu\text{M}$) in dimethyl sulphoxide and were handled in parallel. Silicone oil centrifugation was performed at 37°C ($45000g$ for 5 min). Intrasyntosomal K^+ levels were calculated on the basis of the $[^{14}\text{C}]$ sucrose-impermeable volumes determined in parallel incubations. Ca^{2+} activities given on the abscissa were determined with a Ca^{2+} -selective electrode. In the presence of 2 mM-EGTA plus MgCl_2 , pCa was 7.9. Mg^{2+} activities were calculated by assuming an activity coefficient of 0.3. Results are means $\pm$ S.D. for a representative experiment.

essed according to the procedure of Hodgkin & Horowitz (1959), who determined α of muscle fibres in an electrophysiological study. In the present experiments, the membrane potential was calculated from TPMP $^+$ distribution. A similar procedure for determining α of synaptosomes was employed by Blaustein & Goldring (1975), who used a voltage-sensitive fluorochrome to monitor $\Delta\psi_p$.

The experiments of Fig. 4 were performed in a medium in which Cl^- was replaced by the impermeant gluconate $^-$. In the absence of Cl^- , the simplified equation of Goldman (1943) and Hodgkin & Katz (1949) is given as:

$$\Delta\psi_p = \frac{RT}{F} \ln \frac{[\text{K}^+]_i + \alpha[\text{Na}^+]_i}{[\text{K}^+]_o + \alpha[\text{Na}^+]_o}$$

In most resting nerve cells, α is in the range of 0.01–0.05. For resting synaptosomes, α was reported to be 0.05 (Blaustein & Goldring, 1975). Therefore, the terms $\alpha[\text{Na}^+]_o$ and $\alpha[\text{Na}^+]_i$ can be neglected at

Fig. 2. Time course of K^+ distribution as affected on removal and readdition of external Ca^{2+}

Equally composed samples of synaptosomes ($190 \mu\text{l}/\text{tube}$) were incubated in the standard medium containing 3 mM-KCl and $47 \mu\text{M}$ - Ca^{2+} (total Ca^{2+} measured by atomic absorption spectroscopy). The measuring points refer to the time at which silicone oil centrifugation was initiated in a Beckman Microfuge equipped with water cooling ($10000g$ for 1 min). At the time indicated by the first arrow, $10 \mu\text{l}$ aliquots of HEDTA in saline were added to each test tube (final concn. 1.3 mM) with a semi-automatic pipette. The centrifugation of the first samples was initiated 10 s after the addition of HEDTA to monitor K^+ efflux (○). After 1 min of incubation in the presence of HEDTA, half of the remaining tubes additionally received $10 \mu\text{l}$ aliquots of CaCl_2 in saline (final concn. 1.5 mM) as marked with the second arrow. The ascending line (●) represents the synaptosomes' gain of internal K^+ after the restoration of a pCa above 4.8. Total and external volumes of pelleted synaptosomes were determined in parallel assays. The results are means of a representative experiment, giving S.D. values of triplicate determinations as vertical bars.

high $[\text{K}^+]_o$ and $[\text{K}^+]_i$. Assuming that these conditions are fulfilled at external $\text{K}^+ \geq 20 \text{ mM}$, the above equation reduces to the Nernst equation, i.e.

$$\Delta\psi_p = 27 \ln (\text{K}^+_{i/o})$$

Accordingly, the relationship between TPMP $^+$ and $\text{K}^+_{i/o}$ was established by varying external K^+ between 20 and 70 mM to calibrate the relation be-

Fig. 3. Effect of CCCP and valinomycin on the relationship between K^+ and TPMP $^+$ accumulation at pCa 7.7–4.5. Synaptosomes were incubated in the standard medium in the presence of 3 mM-KCl at pCa 7.7–4.5 adjusted with $CaCl_2$ /HEDTA buffers. Separate samples incubated in parallel served for measurements of K^+ and $[^3H]$ TPMP $^+$ distribution. Equilibration of TPMP $^+$ was accelerated by adding 6 μ M-TPB $^-$. CCCP ($\circ$, final concn. 0.5 μ M) and valinomycin ($\square$, 5 μ M) were added after a 3 min preincubation period. Controls ($\bullet$) were performed without further addition. After 5 min of incubation, centrifugation was initiated (45000g for 5 min at 37°C). Vertical bars indicate S.D. values for the mean of the triplicate K^+ determinations. The S.D. values of TPMP $^+$ distribution were too low to be depicted. The following lines were calculated by least squares regression: $K_{i/o}^+ = 0.0306 \text{ TPMP}_{i/o}^+ - 0.9$ (control; $r = 0.999$); $K_{i/o}^+ = 0.087 \text{ TPMP}_{i/o}^+ - 0.9$ (CCCP-treated; $r = 0.994$). Inset: $K_{i/o}^+ = 0.46 \text{ TPMP}_{i/o}^+ - 3.9$ (valinomycin-treated; $r = 0.990$). Results are from one representative experiment.

tween $TPMP_{i/o}^+$ and $\Delta\psi_p$ (inset of Fig. 4). The mean internal K^+ concentration in the present range of external K^+ was 92.8 ± 10.4 (S.D.) mM.

Fig. 4 illustrates the effect of $[K^+]_o$ between 1 and 6 mM at pCa 7.7 and pCa 4.8 on $\Delta\psi_p$ in the absence of Cl^- . The straight line in Fig. 4 with a slope of 62 mV per unit increase of $\log([K^+]_o)$ was calculated from the Nernst equation, assuming that internal K^+ remained at a constant level of 92.8 mM. At external $K^+ \geq 20$ mM, $\Delta\psi_p$ calculated

Table 3. Effect of external Ca^{2+} on K^+ and TPMP $^+$ distribution in sucrose medium

Assays for internal K^+ and TPMP $^+$ concentrations were performed in the sucrose medium containing 3 mM- K^+ and external Na^+ as indicated. Silicone oil centrifugation (45000g for 5 min at 37°C) was initiated after 10 min of incubation of synaptosomes at 37°C. $[Na^+]_o$ of nominally Na^+ -free samples refers to the concentration in the supernatant at the end of the incubation, as determined by flame emission spectroscopy. $[Na^+]_o$ -dependent alterations of pellet spaces were considered in calculations. The Ca^{2+} activities of Ca^{2+} buffers were assumed to be similar to those in the Na^+ medium; the lower ionic strength in the sucrose medium was not considered. Results are means $\pm$ S.D. for triplicate determinations of a representative experiment.

pCa	$[Na^+]_o$ (mM)	$K_{i/o}^+$	TPMP $_{i/o}^+$
4.8	0.18	27.9 ± 0.2	496 ± 25
7.7	0.18	28.2 ± 1.3	493 ± 18
4.8	38	23.3 ± 0.1	834 ± 6
7.7	38	17.6 ± 0.3	599 ± 23

from TPMP $^+$ distribution diverged from this slope both at pCa 7.7 and 4.8 (Fig. 4) According to:

$$\alpha = \frac{92.8 \text{ mM} - \beta[K^+]_o}{\beta[Na^+]_o - [Na^+]_i}$$

with $\beta = (0.0206 \text{ TPMP}_{i/o}^+ - 0.57)$, α values of 0.029 ± 0.003 at pCa 4.8 and of 0.090 ± 0.005 at pCa 7.7 were calculated from the data of Fig. 4.

Effects of tetrodotoxin, nifedipine and Ruthenium Red

The effects of Ca^{2+} -chelating agents resembled those of veratridine, but tetrodotoxin (5 μ M) failed to prevent the depolarization produced by Ca^{2+} complexation. A constant tetrodotoxin-induced increase of internal K^+ of 7.5 mM both at pCa 7.7 and 4.8, but not at pCa 3.5, indicated that a fraction of synaptosomes' Na^+ channels was in the activated state at low external divalent cation concentration. TPMP $_{i/o}^+$ paralleled this increase of $K_{i/o}^+$ (results not shown).

Kostyuk & Krishtal (1977) suggested that Na^+ might pass through calcium channels at low external Ca^{2+} , since nifedipine abolished Na^+ inward currents elicited by EGTA. In synaptosomes, nifedipine partially prevented K^+ efflux at pCa 7.7, but failed to increase internal K^+ at pCa 7.7 in the presence of tetrodotoxin (Table 4). Hence nifedipine presumably mimicked the effects of tetrodotoxin on K^+ and TPMP $^+$ distribution at low external divalent metal ion activity described above by blocking sodium channels.

Fig. 4. Effect of varying $[K^+]_o$ on $\Delta\psi_p$ in a Cl^- -free medium at pCa 7.7 and 4.8

Synaptosomes were incubated in the Na^+ /gluconate medium in the presence of 1–6 mM- K^+ /gluconate as indicated (K^+ substituted for Na^+). The Cl^- -free Ca^{2+} buffer was prepared with Ca^{2+} /gluconate. After 10 min at 37°C, the incubation was terminated by silicone oil centrifugation (5 min, 37°C, 45 000g). The membrane potential $\Delta\psi_p$ at pCa 7.7 (○) and 4.8 (●) was derived from TPMP⁺ distribution ratios according to the theoretically founded relationship described below (inset). It should be mentioned, however, that at external K^+ of ≥ 3 mM similar values of $\Delta\psi_p$ were calculated from $\Delta\psi_p = 27 \ln(K^+_{i/o})$ by inserting internal K^+ levels determined in parallel. The straight line corresponds to $\Delta\psi_p = 27 \ln(92.8 \text{ mM}/[K^+]_o)$. The curves fitting the experimental data (circles) were computed from the Goldman–Hodgkin–Katz equation, using P_{Na}/P_K ratios of 0.029 at pCa 4.8 (lower curve) and 0.090 at pCa 7.7 (upper curve). Inset: samples were run for measuring K^+ and TPMP⁺ distribution at pCa 4.8 and external K^+ of 20–70 mM in the Cl^- -free medium to establish the relationship between $\Delta\psi_p$ and TPMP⁺_{i/o} as $\Delta\psi_p = 27 \ln(0.0206 \text{ TPMP}^+_{i/o} - 0.57)$ ($r = 0.997$). The rationale of the procedure is described in the text.

When added simultaneously with HEDTA, Ruthenium Red prevented K^+ efflux half-maximally at 3 μM (free Ruthenium Red 1 μM) corresponding to 3 nmol bound/mg of protein (Fig. 5). The effect of Ruthenium Red at pCa 7.7 per-

Table 4. Effect of nifedipine on K^+ and TPMP⁺ distribution

Synaptosomes were incubated in the Ca^{2+} -free standard medium (3 mM- KCl). Assays for K^+ and TPMP⁺ determinations were run in parallel. Prior to complexation of residual Ca^{2+} , 1 μl of nifedipine was added from stock solutions in dimethyl sulphoxide to 190 μl samples. At 2 min after commencing incubation at 37°C, the samples additionally received 10 μl of HEDTA in saline (final concn. 1 mM), and 8 min later the samples were subject to silicone oil centrifugation at 37°C (5 min, 45 000g). Results are means $\pm$ S.D. for triplicate determinations.

pCa	Nifedipine (mM)	Tetrodotoxin (μM)	$K^+_{i/o}$	TPMP ⁺ _{i/o}
7.7	None		5.3 ± 0.0	185 ± 8
	0.1		7.7 ± 0.3	251 ± 2
	0.3		10.3 ± 0.4	264 ± 11
	0.3	5	10.3 ± 0.2	
	0.6		9.6 ± 0.5	162 ± 3
4.8	None		14.8 ± 0.1	508 ± 8

sisted in the presence of 5 μM -valinomycin, excluding the possibility that Ruthenium Red reduced K^+ permeability. The behaviour of TPMP⁺ (Fig. 5b) supported this interpretation. Presumably, Ruthenium Red blocked the Na^+ influx at pCa 7.7, since $[Na^+]_i$ was 129 ± 5 (S.D.) mM in the absence and 81 ± 8 mM in the presence of 3 μM -Ruthenium Red. Addition of 30–100 μM -Ruthenium Red reduced internal Na^+ to 41 ± 4 mM. Determinations of $[Na^+]_i$ were complicated by the fact that high concentrations of Ruthenium Red reduced the external volume of the pellet.

Discussion

Determination of $\Delta\psi_p$ from TPMP⁺ or K^+ distribution ratios

Scott & Nicholls (1980) have demonstrated that the measurement of internal $^{86}\text{Rb}^+$ in conjunction with TPMP⁺ distribution permits to decide whether drugs affect potentials across the plasma membrane or the membrane of internal mitochondria. In the present study, parallel measurements of K^+ and TPMP⁺ were carried out in addition to determine the permeability ratio P_{Na}/P_K (see Fig. 4). It turned out that even under conditions of enhanced Na^+ permeability $\Delta\psi_p$ might be estimated from the K^+ distribution ratio. Additional experiments in the standard medium showed that K^+ and TPMP⁺ distribution ratios were linearly related in the absence and presence of tetrodotoxin or veratridine (results not shown). Apparently, any increase of Na^+ permeability induces a loss of internal K^+ , which quantitatively

Fig. 5. Inhibitory action of Ruthenium Red on K^+ efflux at pCa 7.7

(a) Synaptosomes were incubated in the standard medium containing 3 mM-KCl at pCa 7.7 (1 mM-HEDTA, $\circ$) and varying amounts of Ruthenium Red. The concentrations indicated on the abscissa were calculated assuming an M_r of 848 and a 43% dye content of the commercial Ruthenium Red (Sigma). Centrifugation was initiated after 10 min at 37°C. Internal K^+ of parallel samples at pCa 4.8 ($\bullet$) was 47.7 ± 1.2 mM. (b) Equally composed samples as described in (a) additionally received $0.2 \mu M$ - $[^3H]$ TPMP $^+$ plus $6 \mu M$ -TPB $^-$. In the absence of Ruthenium Red, TPMP $^+_{i/o}$ was 498 ± 12 at pCa 4.8. Results are the means $\pm$ S.D. for three independent experiments.

reflects the decrease of voltage. However, $\Delta\psi_p$ was overestimated when calculated from K^+ distribution ratios at external $K^+ \leq 2$ mM.

Increase of Na^+ permeability as the mechanism of depolarization at pCa 7.7

It is well known that a rise in $[Ca^{2+}]_i$ induces a hyperpolarization of the plasma membranes of many cells, including neurons, by increasing K^+ permeability (Meech, 1978). The Ca^{2+} -dependent K^+ efflux described in the present work was evoked at an apparently constant K^+ permeability by a decrease of $\Delta\psi_p$. The evidence suggesting an electrogenic influx of Na^+ comes from the increase of internal Na^+ by about 30 mM at pCa 7.7 (Table 2) and from the absence of the depolarization in the Na^+ -free sucrose medium (Table 3).

Similar to intact neurons, synaptosomes might be assumed to retain low internal Na^+ , but most experimental data reported failed to support this view (for references, see Li & White, 1977). The uncertainty results partially from the entrapment of Na^+ within the extrasynaptosomal space.

Åkerman & Nicholls (1981) supposed additional electrostatic binding to the outer surface of synaptosomes and used 125 mM-Li $^+$ to displace superficially bound Na^+ . However, the possibility has to be considered that the internal Na^+ of 25 mM they report for guinea-pig synaptosomes arose from a rapid Na^+/Li^+ exchange across the membrane (G. Schmalzing, unpublished results). The use of 100 mM-MgCl $_2$ as a washing solution failed to reduce pellet Na^+ (Table 2).

Inhibition of K^+ accumulation of brain cortex slices in the presence of EDTA was already shown by Gárdos (1960). Morrill *et al.* (1964) provided evidence that many plant and animal cells gained Na^+ and lost K^+ in Ca^{2+} -free EDTA media. In electrophysiological studies the effect of varying external Ca^{2+} in the millimolar range was found to be relatively small, in agreement with the lack of a marked response of synaptosomes to reducing external Ca^{2+} to pCa 4.8 (Fig. 1). For instance, the resting potential of the squid axon was displaced maximally by 5 mV from its normal value upon a 25-fold change of Ca^{2+} concentration (Franken-

haeuser & Hodgkin, 1957). More pronounced effects were observed in nominally Ca^{2+} -free bathing media. Myelinated frog nerves displayed depolarizations of 4–20 mV which disappeared in Na^{+} -free solutions (Stämpfli & Nishie, 1956). Neurons of *Helix pomatia* depolarized by up to 30 mV due to an increase of Cl^{-} conductance when Ca^{2+} was removed and EDTA was added (Christofferson, 1973). Kostyuk & Krishtal (1977) observed a large Na^{+} inward current depolarizing mollusc neurons on addition of EGTA to the bath in the absence of divalent cations. Similar increases in Na^{+} conductance causing marked depolarizations are known to occur when muscle fibres (Kimizuka & Koketsu, 1963; Reuben *et al.*, 1967; Almers *et al.*, 1982), heart muscle cells (Hohl *et al.*, 1983) or crystalline lenses (Delamere & Paterson, 1978) were challenged with Ca^{2+} -free EDTA media. The failure to detect this effect in other tissues as well is potentially due to residual Ca^{2+} in nominally Ca^{2+} -free media.

Possible mechanisms of action of Ca^{2+} on Na^{+} permeability systems

Although the view of an external site of action of Ca^{2+} is favoured in the following because of the rapid onset of K^{+} efflux in response to Ca^{2+} complexation (Fig. 2), a more indirect effect on Na^{+} permeability cannot be ruled out. Micromolar external Ca^{2+} activities might be necessary to maintain sufficiently high internal Ca^{2+} levels to close Na^{+} channels. In this case, Ruthenium Red might inhibit K^{+} efflux and Na^{+} influx by preventing the loss of internal Ca^{2+} . In fact, the efflux of ^{45}Ca from $^{45}\text{CaCl}_2$ -loaded synaptosomes into a Ca^{2+} -free HEDTA medium was considerably delayed in the presence of Ruthenium Red (G. Schmalzing, unpublished results).

For several years, the hypothesis was followed that Ca^{2+} ions are involved in channel gating (Hodgkin & Huxley, 1952). At present, the effects of divalent cations on neuronal excitability are characterized in terms of a modulation of the negative surface potential, as initially suggested by A. F. Huxley (in Frankenhaeuser & Hodgkin, 1957). The concept was successfully applied by Hille *et al.* (1975) to explain that Ca^{2+} deprivation lowers the firing threshold in nerve fibres (Frankenhaeuser & Hodgkin, 1957). Hille *et al.* (1975) have questioned the hypothesis that Ca^{2+} ions are voltage-dependent gates, not least because excitability was maintained in the presence of EGTA.

The effective Ca^{2+} activities in the present work were low as compared with Ca^{2+} dissociation constants of 1.2 or 28 M attributed to surface charges (Hille *et al.*, 1975). Therefore, it seems unlikely that the increase of tetrodotoxin-insensitive Na^{+} permeability was mediated by an alteration of sur-

face potentials. Apparently, environmental Ca^{2+} affects Na^{+} permeability systems by at least two different mechanisms of action. (1) The influence of varying Ca^{2+} in the millimolar range on the threshold of activation of tetrodotoxin-sensitive Na^{+} channels fits the surface potential theory (Hille *et al.*, 1975). In resting synaptosomes, the increase of open tetrodotoxin-sensitive Na^{+} channels both at pCa 7.7 and 4.8 might be due to such a shift of the threshold of activation, since the response to tetrodotoxin disappeared in the presence of $\geq 1 \text{ mM-CaCl}_2$. (2) Lowering of Ca^{2+} activities to the submicromolar range discloses tetrodotoxin-insensitive cation channels.

The nature of the present channel and its physiological significance remain obscure, since both tetrodotoxin and nifedipine were ineffective as inhibitors. In this respect, the channels appear to differ from those described by Kostyuk & Krishtal (1977) and Almers *et al.* (1983). Nicholson *et al.* (1977) observed low external Ca^{2+} in rat cerebellum during repetitive stimulation, but the lowest concentrations reported were still too high for being considered to evoke depolarizations. In analogy to the concepts of Hodgkin & Huxley (1952), it might be speculated that Ca^{2+} -chelating agents chemically provoke displacement currents which under physiological conditions are triggered by voltage, assuming that Ca^{2+} ions are the gating particles of tetrodotoxin-insensitive Na^{+} channels.

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