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Depolarization-induced changes in cellular energy production

(synaptosome/regulation of respiration/calcium/energetics)

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ABSTRACT **Addition of high concentrations of KCl to preparations of rat brain synaptosomes incubated with either glucose or pyruvate caused a transient stimulation of oxygen uptake. This increased respiration was insensitive to 1 mM ouabain and 10 μM ruthenium red but was dependent upon the presence of calcium. With 40 mM KCl in the incubation medium, the levels of high-energy phosphate compounds in the synaptosomes were unaltered, whereas pyridine nucleotides underwent a rapid, albeit small and temporary, oxidation. It is postulated that there is a calcium-dependent mechanism in synaptosomes through which the function of the mitochondrial respiratory chain or of oxidative phosphorylation is stimulated directly without the involvement of either adenine nucleotides or mitochondrial dehydrogenases.**

In the course of studies on the relationship between the activity of the neuronal Na/K pump and energy production (1), it was noticed that addition of high concentrations of KCl to a synaptosomal suspension containing physiological concentrations of potassium (3–5 mM) caused a transient increase in oxygen uptake. This rise in respiration rate was unusual in two respects: it was insensitive to 1 mM ouabain, an inhibitor of the Na/K-ATPase, and it showed an absolute requirement for external calcium.

A rise in extracellular potassium concentration is an inevitable result of enhanced neuronal activity (2) and is accompanied by an augmentation of oxygen consumption, which is usually attributed to stimulation of the Na/K pump activity (3–5). However, the insensitivity to ouabain of the high K^+ concentration-induced changes in respiration in isolated nerve endings (1) indicates that the Na/K-ATPase may not be involved and that there must be some other mechanism(s) that stimulates ATP synthesis. In view of the importance of maintaining a proper energy supply to actively firing neurons, we have investigated the effects of high concentrations of KCl on synaptosomes from rat brain in some detail. This preparation is well-suited for such studies because it contains a large number of ion channels and a high activity of the Na/K pump and because it is not susceptible to interference from secondary changes that arise in the intact brain from spreading depression.

MATERIALS AND METHODS

Preparation of Synaptosomes. Male Sprague–Dawley rats (200–250 g) were used throughout. Synaptosomes were isolated according to the method of Booth and Clark (6). In most experiments the synaptosomal pellet was washed and suspended in a modified Krebs–Henseleit buffer (140 mM NaCl/5 mM KCl/1.3 mM $MgSO_4$/1 mM Na_2HPO_4) containing 10 mM Hepes neutralized to pH 7.4 with Tris base.

Incubations. Synaptosomes were suspended at either 3–4 mg of protein per ml (measurement of oxygen uptake, pyridine nucleotide fluorescence, and metabolite levels) or 7–9 mg of protein per ml [measurement of 2-deoxy-D-glucose (2-DG) uptake] and incubated for 10 min at 30°C in a shaking water bath. In most experiments, the suspensions were supplemented with either 10 mM glucose or 5 mM pyruvate and 1.27 mM $CaCl_2$. The concentration of the former substrate was lowered to 0.5 mM for determinations of 2-DG uptake because high concentrations of glucose markedly reduce uptake of the tracer by competing for its transport. When the effect of calcium elimination was studied, $CaCl_2$ was omitted and replaced with 10 mM $MgSO_4$. All samples taken after a 10-min preincubation are referred to as "time 0" samples.

Oxygen Uptake and Metabolite Levels. Oxygen uptake was assayed with a Clark-type electrode in a stirred chamber thermostated at 30 ± 1°C. For measurements of ATP, creatine phosphate (Cr*P*), and creatine (Cr), samples were incubated for either 1 or 15 min and then rapidly quenched by addition of cold perchloric acid (0.6 M final concentration). After centrifugation to remove precipitated protein, the perchloric acid extracts were neutralized with 2.5 M $KHCO_3$ and centrifuged again; the clear supernatants were used for the determination of metabolites by standard enzymatic techniques (7).

Uptake of 2-DG. Synaptosomes preincubated with 0.5 mM glucose were diluted 5-fold into medium containing no glucose and 20 nM 2-[1,2-^{3}H]DG (0.5 μCi/ml, 42 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear). When appropriate, the incubation mixture was supplemented with either 1 mM ouabain, 40 mM KCl, or both. Aliquots were withdrawn after 0.5, 2, and 5 min and rapidly centrifuged through a layer of silicone oil (specific gravity = 1.03; General Electric). Radioactivity was then determined in both the pellets and the total suspensions in a Searle Delta 300 liquid scintillation counter (TM Analytic, Elk Grove Village, IL) using Liquiscint 121 (National Diagnostics, Sommerville, NJ). Rates of 2-DG uptake were calculated from the increase of radioactivity in the pellets.

Measurements of Cytosolic Free Calcium Using Quin-2. The levels of free calcium were measured with the fluorescent probe quin-2 (8) as adapted for synaptosomes by Hansford and Castro (9) with the modifications described by Erecińska and Dagani (1).

Fluorescence of Pyridine Nucleotides. Pyridine nucleotide fluorescence was determined by using fiber-optic surface fluorimetry (10). Preincubated synaptosomes (4–5 mg of protein per ml) were placed in a spectrophotometric cuvette equipped with a magnetic stirrer. Reflectance (366 nm), fluorescence (450 nm), and corrected fluorescence (450 nm minus 366 nm) were monitored continuously via a fiber-optic

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Abbreviations: 2-DG, 2-deoxy-D-glucose; Cr, creatine; Cr*P*, creatine phosphate; $[Ca^{2+}]_i$, internal Ca^{2+} concentration.

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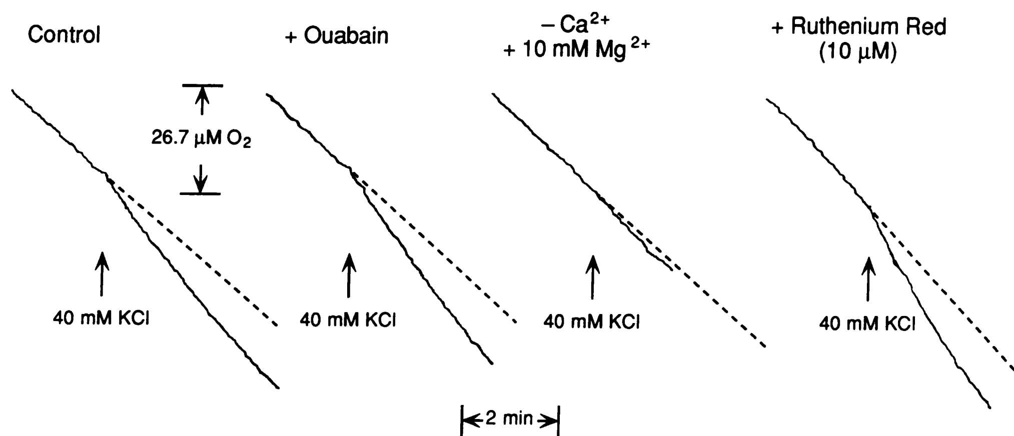

FIG. 1. Effect of a high KCl concentration on synaptosomes respiring with 10 mM glucose and with or without ouabain, calcium, and ruthenium red. Oxygen uptake was measured as described in *Materials and Methods*. The protein concentration was 3.22 mg/ml.

light guide placed against the side of the cuvette below the surface of the liquid.

Other Procedures. Protein content was measured by the biuret reaction (11) with bovine serum albumin as the standard. Statistical analysis was performed by the *t* test or multifactor analysis of variance.

RESULTS

Addition of KCl to synaptosomes suspended in Krebs–Henseleit/Hepes buffer (5 mM KCl) caused a concentration-dependent increase in respiration. The effect was seen irrespective of whether 10 mM glucose or 5 mM pyruvate was used as the substrate; the characteristics of the changes are illustrated in Figs. 1 and 2. It can be seen that stimulation of oxygen consumption was rapid and was followed by a gradual return to the baseline (see the marked curvature in the tracings), within 15 min. The increase in respiration was specific for potassium chloride as neither 10–60 mM choline chloride nor sodium chloride had any influence. By drawing a tangent to the very initial portion of the trace, it could be calculated that with 40 mM KCl the rate was enhanced by 69% ± 10% in the presence of glucose and 63% ± 8% with pyruvate as substrate (mean ± SD for five experiments, $P < 0.001$). The respiratory activity was inhibited by >90% with 10 μM rotenone and under such conditions was not stimulated by addition of high concentrations of KCl.

Figs. 1 and 2 also show that activation of oxygen uptake occurred at an undiminished rate when 1 mM ouabain was present in the medium. By contrast, omission of $CaCl_2$ and its replacement with 10 mM $MgSO_4$ almost eliminated the stimulatory response. Ruthenium red at 10 μM did not prevent the increase in respiration caused by 40 mM KCl with either glucose or pyruvate as a fuel.

It is well known that depolarization leads to an influx of calcium into nerve endings by opening voltage-sensitive channels. To allow correlations between stimulation of ATP synthesis and the internal Ca^{2+} concentration ($[Ca^{2+}]_i$), the latter was measured with the fluorescent indicator quin-2 under the same conditions as were used to examine the O_2 uptake. As determined in three independent experiments and displayed in Fig. 3, the addition of 40 mM KCl to the incubation medium raised the intrasynaptosomal Ca^{2+} concentration by, on average, 144 ± 27 nM—i.e., from a baseline concentration of 254 ± 20 nM to 398 ± 25 nM ($P < 0.002$). When $CaCl_2$ was omitted from the incubation mixture and replaced by 10 mM $MgSO_4$, the intrasynaptosomal calcium concentration declined to 81 ± 17 nM and was no longer affected by addition of 40 mM KCl (84 ± 21 nM). The rise in $[Ca^{2+}]_i$ (in the presence of external Ca^{2+}) was dependent on the concentration of KCl added to the medium (smaller at

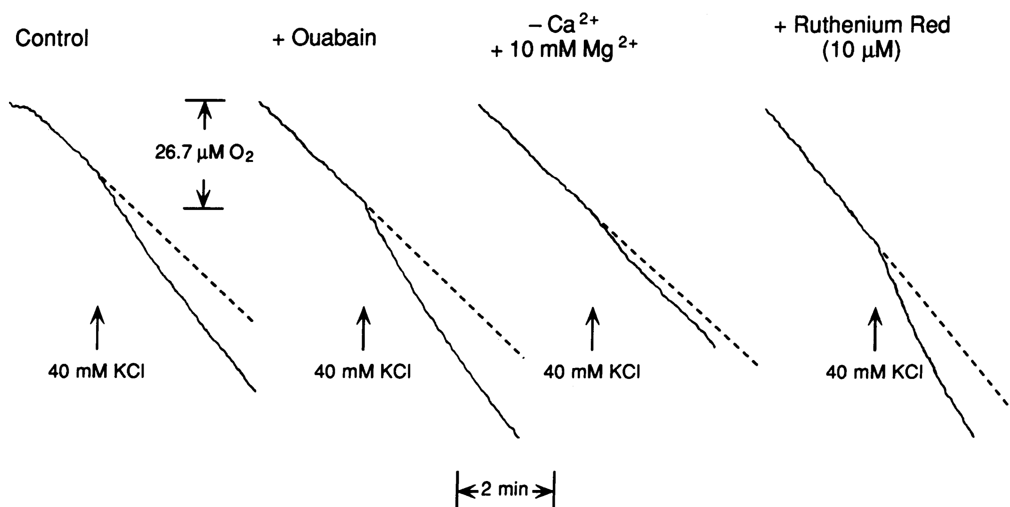

FIG. 2. Effect of a high KCl concentration on synaptosomes respiring with 5 mM pyruvate and with or without ouabain, calcium, and ruthenium red. Oxygen uptake was measured as described in *Materials and Methods*. The protein concentration was 2.61 mg/ml.

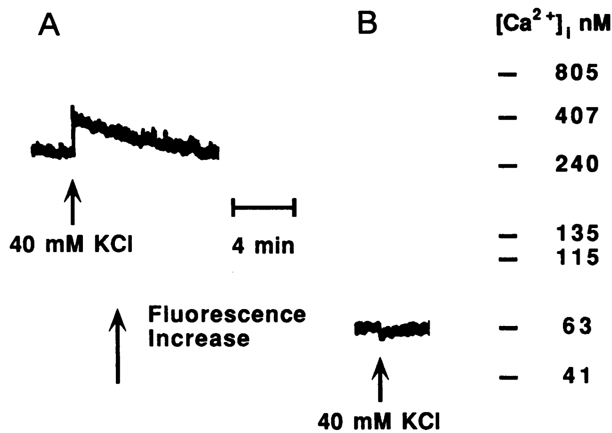

FIG. 3. Changes in $[Ca^{2+}]_i$ induced by addition of 40 mM KCl to synaptosomes incubated with (*A*) or without (*B*) calcium. The concentration of intrasynaptosomal calcium was measured with quin-2. Synaptosomes loaded with the fluorescent indicator (1 mg of protein per ml) were placed in a 3-ml cuvette thermostated at 30°C and stirred throughout the recording with a small magnetic bar. The fluorescence of the quin-2–calcium complex was recorded in a Perkin-Elmer model LS5 spectrofluorometer. The excitation wavelength was 399 nm (5-nm bandwidth), and the emission wavelength was 395 nm (10-nm bandwidth). The calibration of calcium concentration was carried out at the end of each recording as described by Tsien *et al.* (8) and Capponi *et al.* (12).

lower KCl concentrations) and was abrupt, but transient, returning to the baseline value in <10 min.

To determine whether or not the high KCl concentration caused changes in the energy state of synaptosomes, the latter was evaluated directly by measuring the levels of ATP, Cr*P*, and Cr and indirectly by determining the rates of 2-DG uptake. We have shown previously (1, 13) that the velocity of transport of this glucose analogue faithfully reflects the rate of glycolysis and is sensitive to alteration in concentrations of the regulators of phosphofructokinase (ATP, ADP, AMP, P_i, and Cr*P*), the rate-controlling enzyme of the pathway.

The data in Table 1 show that the levels of the high-energy phosphate compounds were not influenced greatly by 40 mM KCl. At the 1-min time point the Cr*P* concentration was slightly increased, although the rise was not statistically significant; after a 15-min incubation, it returned to the control value. Similarly, the rate of 2-DG uptake was not significantly affected at 5 min. To define the very initial changes, the increase in radioactivity in the pellets was measured during the first 1.5 min of incubation and compared to that during the next 3 min. Table 2 shows that perhaps there was a very small rise in the glycolytic rate immediately after KCl addition, but it was not sustained. The rate appeared to have been reduced in the 2- to 5-min interval.

Because a rise in $[Ca^{2+}]_i$ can stimulate mitochondrial dehydrogenases (14, 15) and hence increase the level of NADH (and thus respiration), fluorescence of synaptosomal pyridine nucleotides was measured to evaluate their redox states. In three independent experiments, in which multiple runs were made, changes in pyridine nucleotide fluorescence were generally found to be small. In the majority of experiments, the pattern displayed in Fig. 4 was obtained; an initial oxidation was followed by a return to the original level or by a small further reduction. These alterations were apparently insensitive to ouabain but were not seen in the absence of calcium.

Table 1. High-energy phosphate compounds in synaptosomes incubated with 40 mM KCl

Addition	Time, min	Concentration, nmol/mg of protein		
		ATP	Cr*P*	Cr
None (control)	0	4.13 ± 0.31	6.95 ± 0.58	14.53 ± 1.27
KCl	1	4.23 ± 0.46	7.51 ± 0.76	14.43 ± 1.08
KCl	15	3.87 ± 0.40	6.82 ± 0.50	15.47 ± 0.88

ATP, Cr*P*, and Cr were measured by standard enzymatic techniques (1) using synaptosomes incubated with KCl for 0, 1, or 15 min (after a 10-min preincubation) as described in *Materials and Methods*. Results represent means ± SD for three experiments.

Table 2. Rate of 2-DG uptake by synaptosomes incubated with 40 mM KCl and/or 1 mM ouabain

Addition	Time interval, min		
	0.5–2.0	2.0–5.0	0.5–5.0
None (control)	187 ± 5 (4)	200 ± 7 (4)	195 ± 5 (4)
KCl	226 ± 7* (4)	172 ± 9* (4)	196 ± 7 (4)
Ouabain	169 ± 22 (3)	142 ± 12* (3)	151 ± 15* (3)
KCl + ouabain	218 ± 26 (3)	164 ± 9* (3)	181 ± 14 (3)

Synaptosomes incubated at 7–9 mg of protein per ml for 10 min with 0.5 mM glucose and 1.27 mM $CaCl_2$ were diluted 5-fold into a medium containing calcium, no glucose, and 20 nM 2-DG (final concentration). Samples were taken at 0.5, 2, and 5 min. The results represent the means ± SD (in fmol per mg of protein per min) for the number of experiments indicated in parentheses.

*$P < 0.005$ or lower when compared to the control; all other comparisons showed no statistically significant differences.

DISCUSSION

The results described in this paper demonstrate that the addition of high concentrations of KCl and subsequent depolarization of synaptosomes stimulate mitochondrial energy production. The effect is nontrivial: a 60–70% rise in oxygen consumption—i.e., a 60–70% augmentation in the amount of ATP generated by oxidative phosphorylation with 40 mM KCl. The enhancement of respiration does not result from increased activity of the Na/K-ATPase because it is insen-

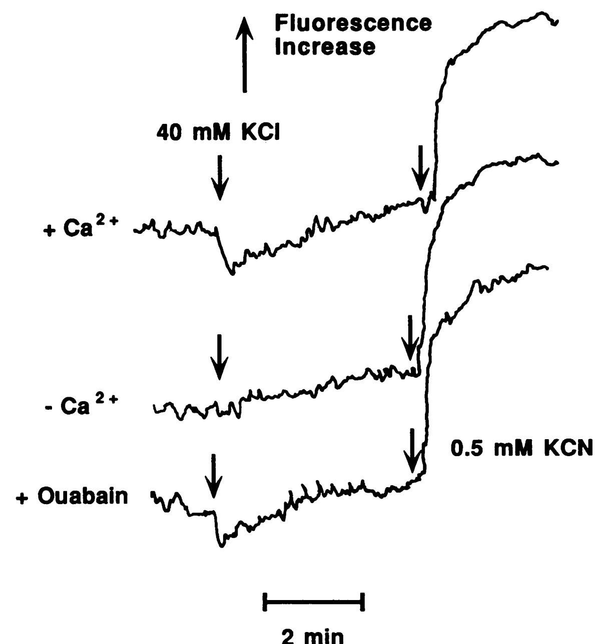

FIG. 4. Effect of a high KCl concentration on the fluorescence of synaptosomal pyridine nucleotides. Fluorescence of pyridine nucleotides was measured by the fiber-optic surface technique (10) as described in *Materials and Methods*. The traces represent corrected fluorescence (i.e., fluorescence at 450 nm minus reflectance at 366 nm). The protein content was 4.5 mg/ml.

sitive to 1 mM ouabain. This finding, although at first glance surprising, is nevertheless consistent with the observation that the K_m of the pump for K^+ is 1 mM or less (1, 16). The stimulation of oxidative phosphorylation by the high K^+ concentration (or depolarization) appears to be caused neither by a rise in ATP utilization, as evidenced by our failure to detect a decline in synaptosomal energy level or 2-DG uptake, even at the early time points, nor from direct activation of mitochondrial dehydrogenases by Ca^{2+}, because it is not sensitive to ruthenium red. The phenomenon does, however, require influx of calcium into the cytosol and does not occur when the cation is absent and replaced by magnesium. Before discussing the possible mechanisms that underlie our findings, the question has to be addressed whether the behavior observed is not merely a characteristic of the *in vitro* synaptosomal preparation.

There are two lines of evidence in the literature that indicate that a Ca^{2+}-dependent increase in respiration may be a general phenomenon. Ventral photoreceptors of *Limulus* respond to a pulse of light with a rise in O_2 uptake, which does not result from an augmentation of $[Na^+]_i$ (17) but requires the presence of external calcium; the increased respiration is inhibited by intracellular injection of rotenone and EGTA but not of ruthenium red (18). In another model system, the perifused rat neurohypophysis, decreases in external oxygen tension caused by either electrical stimulation or high concentrations of KCl are only marginally reduced by ouabain but are markedly diminished by calcium withdrawal (19). Thus, it can be concluded that the apparently unorthodox responses of synaptosomes may be a true reflection of a physiologically relevant phenomenon.

The nature of the mechanism(s) that supports Ca^{2+}-dependent stimulation of respiration is not clear at present; however, a discussion of the possibilities appears appropriate. Fein and Tsacopoulos (17, 18) postulated that increased oxygen uptake by photoreceptors upon illumination is caused by activation of α-glycerophosphate dehydrogenase, a calcium-requiring protein (20) that is exposed on the external surface of the inner mitochondrial membrane (21). This, however, is an unconvincing explanation because this enzyme is a flavoprotein, which is not sensitive to the action of rotenone (20), whereas the phenomenon described by the authors themselves and by us is. Shibuki (19) has argued that in neurohypophysis increased exocytosis triggered by the entry of Ca^{2+} during electrical stimulation or addition of high concentrations of KCl leads to ATP breakdown and a rise in ADP and P_i; the latter, in turn, stimulates the mitochondrial respiratory chain. Although we do not know if there is a change in the energy state of the neurohypophysis under such conditions, the argument cannot apply to synaptosomes where a fall in the energy-rich phosphate compounds could not be detected (Tables 1 and 2).

A completely different mechanism has been proposed by Halestrap (22). Based on several observations from his own and other laboratories, he postulated that the mitochondrial respiratory chain (or oxidative phosphorylation) can be activated directly by an increase in mitochondrial volume. Swelling of the mitochondria can be brought about by, for example, enhanced permeability of the inner membrane to K^+. In hepatocytes treated with calcium-mobilizing hormones it was suggested that stimulation of potassium entry resulted from a rise in pyrophosphate content following inhibition of mitochondrial pyrophosphatase by increased matrix Ca^{2+} concentrations. This mechanism relies on a rise of calcium inside the organelles and thus is sensitive to inhibition by ruthenium red. It is possible, however, that there may be other, as yet unrecognized, changes in mitochondrial structure that are dependent on an increase in the external (i.e., cytosolic) concentration of the cation and that cause stimulation of respiration.

In support of the contention that activity of the mitochondrial respiratory chain (or oxidative phosphorylation) is increased by addition of high concentrations of KCl is our finding that pyridine nucleotides become oxidized. Similar decreases in pyridine nucleotide fluorescence are observed in intact brain during electrical stimulation, K^+-induced depolarization, and seizures (23–26). Moreover, Landowne and Ritchie (27, 28) have noted that in nonmyelinated nerve fibers electrical stimulation also causes a prompt oxidation of pyridine nucleotides, which persists in the presence of ouabain but is eliminated by calcium withdrawal or addition of KCN.

A direct stimulation of the synthesis of ATP in the absence of an increase in the rate of its utilization means that the ATP concentration should rise. Our finding that at 1 min after addition of 40 mM KCl the concentration of Cr*P* appears to be somewhat larger than at time 0 is consistent with this supposition. However, the small size of the effect that was observed suggests that there may be a second, concurrent reaction that requires energy (e.g., neurotransmitter release or Ca^{2+} pumping) that is activated simultaneously. Such processes in isolated synaptosomes do not appear to use much energy; therefore, any increased ATP concentration $\{[ATP]/[ADP]$ or $[ATP]/([ADP][P_i])\}$ that is generated should act to inhibit oxidative phosphorylation, and thus the rate of respiration would gradually return to normal. This prediction is supported by the data in Figs. 1 and 2.

Our final comment concerns the physiological role of increased energy synthesis requiring neither a decline in the ATP concentration (or equivalent) nor direct stimulation by calcium of the cation-sensitive dehydrogenases in the tricarboxylic acid cycle. The occurrence of this phenomenon means that processes that utilize ATP as their substrate can continue to operate at undiminished velocities. This may be of special importance for those reactions that exhibit high K_m values for ATP, such as the neuronal Na/K pump (29, 30) and vesicular transmitter release (31).

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