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Pretreatment with H_2O_2 Decreases the Ca^{2+} Sensitivity of the Exocytosis of Glutamate in Cerebrocortical Synaptosomes

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The treatment of cerebrocortical synaptosomes with low concentrations of H_2O_2 induces a long-lasting inhibition of the Ca^{2+}-dependent release of glutamate induced by KCl or ionomycin, without interfering with the cytosolic calcium and without damaging the synaptosomes (Zoccarato, F., Valente, M., and Alexandre, A. (1995) *J. Neurochem.* **64,** 2552–2558). We report now that the inhibition exerted by H_2O_2 decreases (from 50 ± 9% to 25 ± 11%) if exocytosis is triggered by high (80 mM) rather than by low (30 mM) KCl. Similarly the inhibition decreases when glutamate release is triggered by high rather than by low ionomycin. The decreased inhibition by H_2O_2 on increasing KCl is accompanied by an increase of $[Ca^{2+}]_i$. We conclude that the treatment with H_2O_2 decreases the Ca^{2+} sensitivity of the synaptosomal exocytotic apparatus.

Aerobic cells are constantly exposed to reactive oxygen intermediates (ROIs). Exposure to H_2O_2 activates the transcription factors *c-jun* [1,2],*c-fos* [1,2] and *NF-kB* [3,4] and stimulates mitogen-activated protein kinase activity [5] and human immunodeficiency virus transcription [6]. Reactive oxygen intermediates appear to function as natural second messengers in some signaling pathways. In fact, N-acetylcysteine, a glutathione precursor and anti-oxidant, prevents activation of *NF-kB* by a number of stimuli that act at the plasma membrane [1,3,6] and inhibits the mammalian UV response [7].

H_2O_2 can induce rapid tyrosine phosphorylation of multiple cellular proteins [4,7–11]. In the CNS H_2O_2 induces the production of NGF by astrocytes [12].

These observations show that, besides being the cause of oxidative injury [13–17] ROIs may act as physiological messengers.

In the course of previous research we established that the synaptosomes are the site of H_2O_2 synthesis in apparently physiological conditions [18–21]. We later showed that the treatment of synaptosomes with small amounts of H_2O_2 for limited periods of time induces a long lasting inhibition of the exocytosis of glutamate, without affecting the ATP/ADP ratio or the creatine phosphate content and in the absence of lipid peroxidation. The effect of H_2O_2 was not on ion channels and it was visible also when glutamate release was activated with a Ca^{2+}-ionophore. It was concluded that H_2O_2 interferes directly with the exocytotic process [22].

The present research further characterizes the effect of H_2O_2 on exocytosis. It is shown that H_2O_2 modulates the Ca^{2+} sensitivity of the exocytosis of glutamate.

MATERIALS AND METHODS

Materials. Bovine serum albumin (BSA; essentially fatty acid free), catalase from bovine liver (thymol free) (EC 1.11.1.6), superoxide dismutase from bovine erythrocytes (EC 1.15.1.1), glutamate dehydrogenase (EC 1.4.1.3), indo 1 pentaacetoxymethyl ester (indo 1-AM), and ionomycin were supplied by Sigma. All other reagents were of analytical grade.

Synaptosomal preparation. Synaptosomes from rat cerebral cortex were obtained and stored as described previously [23] with minor modifications [21]. Protein was determined by the biuret method. Synaptosomes were finally suspended at 32°C

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Abbreviations used: BSA, bovine serum albumin; $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; ROI, reactive oxygen intermediates; TES, 2-([2-hydroxy-1, 1- bis(hydroxymethyl)ethyl]amino)ethane-sulforin acid.

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in a buffered medium containing 122 mM NaCl, 3.1 mM KCl, 0.4 mM KH_2PO_4, 12 mM glucose, 1.2 mM $MgCl_2$,5 mM $NaHCO_3$, 2.5 μg/ml superoxide dismutase, 500μ g/ml BSA, 20 mM TES (pH 7.3) adjusted with NaOH.

Glutamate release. Glutamate release was determined by continuous fluorometric assay as described previously [24] using a Shimadzu RL-5000 spectrofluorometer at 340 nm (excitation) and 460 nm (emission) with a stirred, thermostatted cuvette attachment. The synaptosomal pellets (1.2–1.8 mg protein) were resuspended in 2 ml of incubation medium containing 200 μM EGTA. NADP (1 mM) and glutamate dehydrogenase (20 U/ml) were added at 25 min. Glutamate release was initiated (in the presence or absence of $CaCl_2$) by the addition of KCl or ionomycin as detailed in the legends to the figures. H_2O_2 was added as three pulses of 90, 60, 60 μM at 2-min intervals starting at 14 min of the incubation, followed by catalase (22 U/ml) 2 min after the last pulse. Catalase alone was added in the controls. The second and third pulses of H_2O_2 allow compensation for the consumption of H_2O_2 by the synaptosomes [22].

Cytoplasmic free Ca^{2+}. The cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured using the calcium fluorophore indo 1-AM [25,26]. Synaptosomes (0.7 mg/ml) were preincubated in incubation medium containing 4 μM indo 1-AM for 30 min at 32°C. After centrifugation of a 0.5 ml aliquot of the suspension, the pellet (0.35 mg protein) was resuspended in 2 ml of fresh medium, in the presence of 100 μM sulfinpyrazone [27]. Incubations were performed as above. Where present, H_2O_2 was supplied as three pulses of 70, 25 and 20 μM at 2 min intervals, followed by catalase 2 min after the last pulse, starting at 6 min of the incubation. Fluorescence ratios were determined at 410/480 nm emission, after excitation at 340 nm in a Shimadzu RL-5000 spectrofluorometer capable of calculating ratios at 2 s intervals. A K_D of 250 nM for the Ca^{2+}-indo 1 complex was used [25].

All the experiments were repeated with five to nine independent synaptosomal preparations.

RESULTS

The treatment of synaptosomes with 50-150 μM H_2O_2 for few minutes determines a long lasting inhibition of the Ca^{2+}-dependent release of glutamate induced by KCl [22]. We now report that the H_2O_2 inhibition varies with the concentration of KCl employed to induce exocytosis. This is shown in Fig. 1 where glutamate release was triggered in untreated and H_2O_2-treated synaptosomes with two different KCl concentrations (30 and 80 mM). In the experiments where exocytosis was triggered with 30 mM KCl, NaCl (50 mM) was also included in order to maintain a comparable osmolarity. In separate experiments it was also observed that the inclusion of NaCl was uninfluential on the KCl (30 mM) induced exocytosis, an indication that changes in osmolarity and Na^+

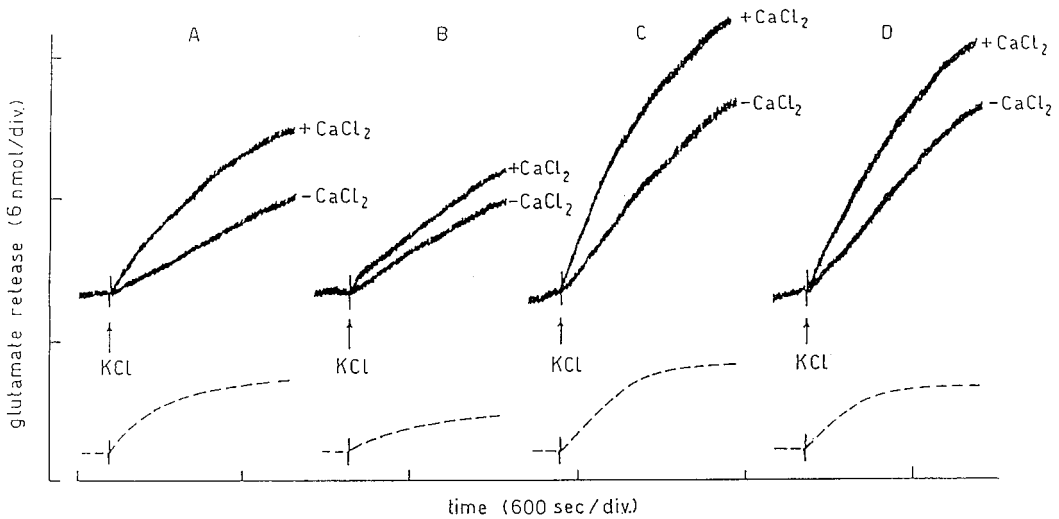

FIG. 1. Glutamate release by low and high KCl in untreated and H_2O_2-treated synaptosomes. Synaptosomes (0.7 mg protein/ml) were incubated as described in Materials and Methods. KCl was added 28 min after catalase. Where present, $CaCl_2$ (1.2 mM) was added 15 min before KCl. A: 30 mM KCl, untreated; B: 30 mM KCl, H_2O_2-treated; C: 80 mM KCl, untreated; D: 80 mM KCl, H_2O_2-treated. NaCl (50 mM) was added together with KCl in A and B to maintain comparable osmolarity. The Ca^{2+}-dependent release of glutamate (i.e. the difference between the curves with and without $CaCl_2$) is reported below each couple of traces. Typical traces are reported representative of duplicate experiments from at least five independent preparations.

concentration were not a problem in our conditions (data not reported). As it may be expected, the rate of the Ca^{2+}-independent glutamate release, which represents an efflux of glutamate from the cytosol via reversal of the electrogenic import system [28,29], increases with increasing KCl (an indication of a more extensive deporalization). H_2O_2 is without effect on this Ca^{2+}-independent release at both KCl concentrations. The inclusion of $CaCl_2$ in the suspending medium increases the KCl-induced glutamate output relative to the control without $CaCl_2$. The Ca^{2+}-induced increment (calculated by subtracting the Ca^{2+}-free trace from that with Ca^{2+}) represents the true exocytotic release. In untreated synaptosomes, such a Ca^{2+}-dependent release is only slightly increased by increasing KCl. On the contrary in H_2O_2-treated synaptosomes, the Ca^{2+}-dependent glutamate release is about 50% inhibited when KCl is 30 mM, in accordance with previously published results [22]. However, the inhibition decreases strongly when KCl is increased to 80 mM. Thus, increasing KCl decreases the degree of inhibition induced by H_2O_2.

In a previous study [22] we reported that H_2O_2 inhibits the exocytosis of glutamate without altering the KCl-induced increase of cytosolic Ca^{2+}; furthermore, that the inhibition by H_2O_2 is observed also on the glutamate release induced by the Ca^{2+} ionophore ionomycin. As shown in Fig. 2 also the inhibition by H_2O_2 of the ionomycin-induced release of glutamate decreases if the concentration of the ionophore is increased (from 4 to 10 μM). Again, increasing ionomycin potentiates the release of glutamate in H_2O_2-treated samples, but is without significant effect on the controls.

These observations are consistent with the idea that the inhibition exerted by the treatment with H_2O_2 on the exocytosis of glutamate decreases if the concentration of Ca^{2+} at the release sites is increased. Accordingly, as shown in Fig. 3 the average concentration of cytosolic Ca^{2+}, as determined with the fluorescent dye indo-1, is higher after pulsing with 80 mM KCl than with 30 mM KCl, both in H_2O_2-treated and untreated synaptosomes (the values of $[Ca^{2+}]_i$ tend to be slightly higher in the treated samples). In the control exocytosis, the higher $[Ca^{2+}]_i$ elicited by 80 mM KCl has only a marginally potentiating effect on glutamate release (Fig. 1), indicating that a quasi-maximally efficient Ca^{2+} concentration is attained at the site of exocytosis with the lower KCl concentration (30 mM). On the contrary, the potentiating effect observable in the H_2O_2-treated experiments upon increasing KCl or ionomycin shows that the Ca^{2+} sensitivity of the exocytotic machinery is decreased by the treatment.

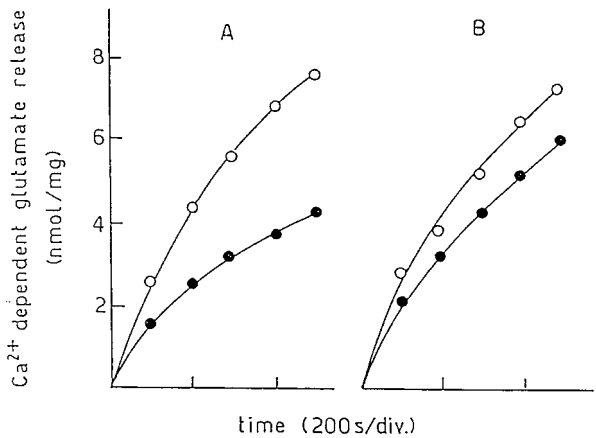

FIG. 2. Glutamate release by low and high ionomycin in untreated and H_2O_2-treated synaptosomes. Conditions as in Fig. 1. Ionomycin was added 28 min after catalase and $CaCl_2$ (1.2 mM) was added 15 min before ionomycin. The points represent the difference between glutamate release in the presence vs absence of $CaCl_2$ (or alternatively vs presence of $CaCl_2$ and absence of ionomycin). Ionomycin was 4 μM in A and 10 μM in B. (○), untreated; (●), H_2O_2-treated. Typical traces are reported representative of duplicate experiments from seven independent preparations.

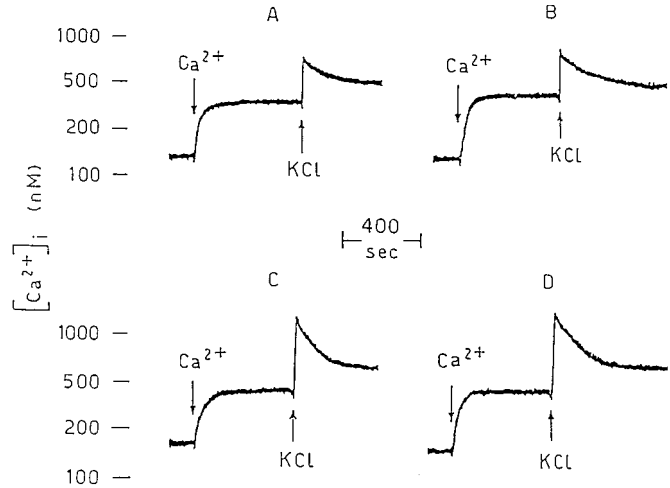

FIG. 3. The KCl-induced increase of $[Ca^{2+}]_i$ is unaffected by treatment with H_2O_2. Incubation as in Materials and Methods. KCl was added 18 min after catalase and $CaCl_2$ (1.2 mM) was added 9 min before KCl. A: KCl 30 mM, untreated; B: KCl 30 mM, H_2O_2-treated; C: KCl 80 mM, untreated; D: KCl 80, H_2O_2-treated. NaCl (50 mM) was added together with KCl in A and B to maintain comparable osmolarity. Typical traces are reported representative of at least seven experiments.

Fig. 4 shows that the increase of $[Ca^{2+}]_i$ induced by ionomycin is unaffected by the treatment with H_2O_2. The concentration of ionomycin used in this experiment was low (0.7 μM) in order to obtain reliable measurements of $[Ca^{2+}]_i$. However, the concentrations of ionomycin required to induce a sizeable release of glutamate (e.g. those used in Fig. 2) increase $[Ca^{2+}]_i$ beyond the measuring capacity of Indo 1 (and much above the concentrations obtained with KCl). This confirms that much higher average $[Ca^{2+}]_i$ needs to be attained with the ionophore than with KCl in order to activate exocytosis and is in accordance with the idea that the opening of Ca^{2+} channels by the KCl-induced depolarization specifically increases the Ca^{2+} concentration at locations adjacent to the exocytotic apparatus, affecting much less the average Ca^{2+} concentration in the overall cytosolic space. On the other hand, Ca^{2+} influx operated by the ionophore being not localized at the exocytotic sites, higher average concentrations have to be attained in order to activate exocytosis [30,31].

An overall picture of the effect of increasing KCl or ionomycin on the H_2O_2 inhibition of

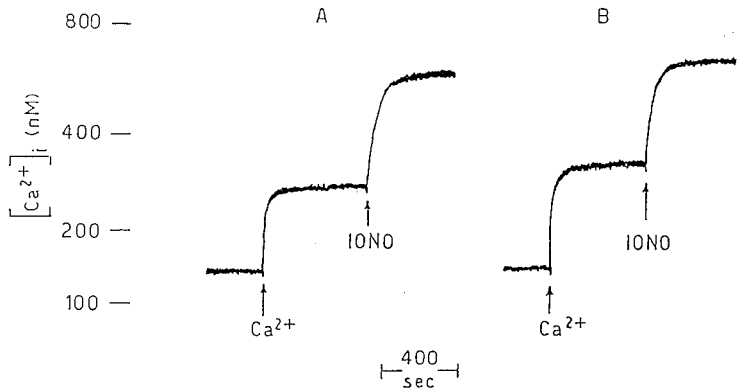

FIG. 4. The ionomycin-induced increase of $[Ca^{2+}]_i$ is unaffected by treatment with H_2O_2. Conditions as in Fig. 3. Ionomycin (0.7 μM) was added 18 min after catalase and $CaCl_2$ (1.2 mM) was added 9 min before ionomycin. A: untreated; B: H_2O_2-treated. Typical traces are reported representative of at least five experiments.

glutamate exocytosis, summarizing the results of over 20 independent preparations, is presented in Fig. 5.

DISCUSSION

Previous research has shown that synaptosomes are the site of a Ca^{2+}-dependent H_2O_2 production [18–20]. The oxidase activity is membrane-bound, utilizes NADH as the electron donor and requires chelated iron. Two sites were detected for the production of H_2O_2, intrasynaptosomal and superficial. The latter was tentatively assigned to the postsynapsis [21]. The effect of supplying controlled small amounts of H_2O_2 to the synaptosomal suspension for limited times was studied next [22]. It was found that, in the absence of damaging effects, H_2O_2 induces a long-lasting depression of the Ca^{2+}-dependent glutamate release. The inhibition appeared to be directly on the exocytotic system, since(i) the KCl-induced of $[Ca^{2+}]_i$ was not affected by H_2O_2, and (ii) the inhibition was observed also when glutamate release was activated with a Ca^{2+} ionophore.

The data presented in this study show that the degree of the H_2O_2-induced inhibition is variable and depends on the concentration of Ca^{2+} in the proximity of the exocytotic apparatus. Specifically, increasing Ca^{2+} at the exocytotic site decreases the inhibition by H_2O_2. The increase of $[Ca^{2+}]_i$ has

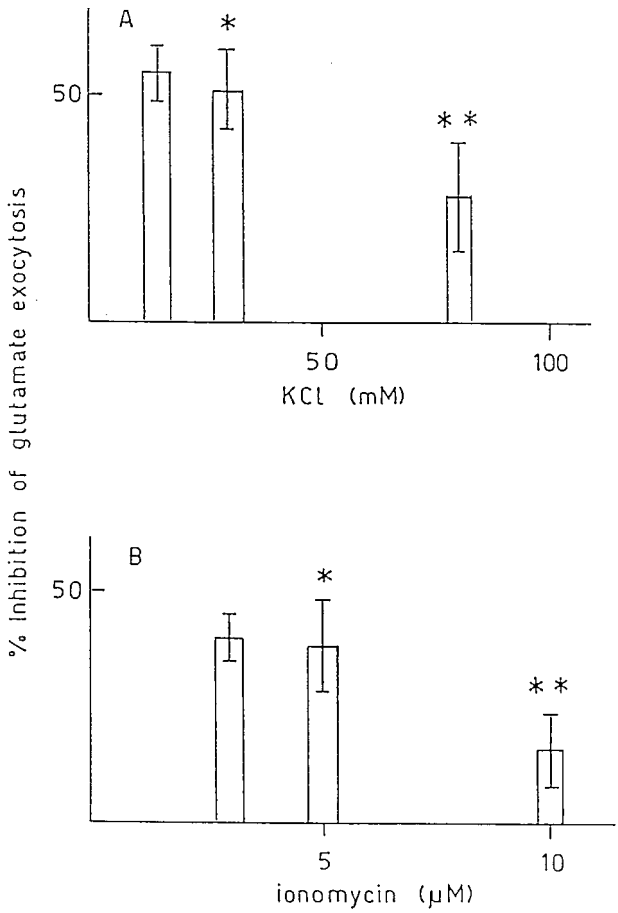

FIG. 5. The effect of increasing KCl or ionomycin on the H_2O_2 inhibition of glutamate exocytosis. The inhibition exerted by treatment with H_2O_2 on glutamate exocytosis is reported as a function of KCl (A) or of ionomycin (B) concentrations. The data are collected from 4–6 determinations at each KCl or ionomycin concentration. The experiments were performed in at least 20 synaptosomal preparations. * Significantly different from ** $p < 0.005$ by Student's t test.

been achieved in two different ways, by increasing the concentration of depolarizing KCl and by increasing the Ca^{2+}-ionophore ionomycin.

In the experiments where KCl was varied, we compensated with NaCl for the changes in osmolarity. In control experiments it was also shown that the inclusion of extra NaCl was without effect on the KCl (30 mM)-induced exocytosis. This rules out the possibility that increasing NaCl might *per se* act as an interfering factor, e.g. by stimulating the Na^+/Ca^{2+} exchanger. Indeed the activity of this exchanger was reported to be negligible in synaptosomes [32]. The higher KCl concentration potentiates the Ca^{2+}-independent efflux of glutamate (via reversal of the electrogenic uptake system) and increases the average $[Ca^{2+}]$. However, it potentiates only marginally the exocytotic (i.e. Ca^{2+}-dependent) glutamate release of untreated synaptosomes. Thus, a quasi-saturating concentration ofCa^{2+} is reached at the release sites at the lower (30 mM) KCl concentration, and increasing Ca^{2+} further has no major effect on exocytosis. The opposite is true in H_2O_2-treated synaptosomes, where the exocytotic Ca^{2+}-dependent release is higher (and approaches the control) at 80 than at 30 mM KCl. Since the treatment with H_2O_2 has no effect on the basal or the KCl-stimulated $[Ca^{2+}]_i$, it is concluded that H_2O_2 decreases the sensitivity to Ca^{2+} of the exocytotic system. The same conclusions are reached in the experiments with ionomycin.

The average concentrations $[Ca^{2+}]_i$ required to activate exocytosis are much higher with ionomycin than with KCl [30,31], in accordance with the idea that the Ca^{2+}-sensing element(s) (synaptotagmin, but possibly also other proteins [33–36]) have a relatively low affinity for Ca^{2+} and are localized in close proximity to the Ca^{2+} channels, where quite high Ca^{2+} concentrations can be reached for very short times.

Most of the known controls in fast transmitting synapses center on the modulation of the activity of ion channels. In some instances it has been proposed that the activation of presynaptic receptors sets in motion cellular events that suppress transmitter release independently of the control of membrane channels [37–42]. The H_2O_2 modulation of the Ca^{2+} sensitivity of glutamate release [22,this paper] is one such examples.

The mechanism by which H_2O_2 decreases the affinity for Ca^{2+} of the glutamate release system is at present not clear. We have observed [22] that after H_2O_2 the sum of ATP plus ADP decreases significantly, although the ATP/ADP ratio and the total phosphocreatine content are unchanged. This may indicate an accumulation of adenosine. However, adenosine was reported to decrease the activity of synaptosomal Ca^{2+} channels [43], but it does not appear to modulate the exocytosis directly, unlike H_2O_2 (unpublished observations). An alternative possibility is that H_2O_2 promotes the oxidation of some critical groups, which in turn may modulate the Ca^{2+} sensitivity of the exocytotic process. Experiments are under way to test this possibility. It is unlikely that H_2O_2 decreases the availability of releaseable transmitter, as this would not explain the potentiation of exocytosis by increasing Ca^{2+}.

To our knowledge, this is the first report showing that the affinity for Ca^{2+} of the exocytotic apparatus can be modulated. Such a modulation may represent a novel mechanism in synaptic plasticity.

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