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# $Na^+$-$Ca^{2+}$ Exchange Activity in Central Nerve Endings. I. Ionic Conditions That Discriminate $^{45}Ca^{2+}$ Uptake Through the Exchanger from That Occurring Through Voltage-Operated $Ca^{2+}$ Channels

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**SUMMARY**

$Ca^{2+}$ entrance in central nerve endings can occur through voltage-operated $Ca^{2+}$ channels and/or through the $Na^+$-$Ca^{2+}$ antiporter. The aim of the present study was to evaluate, in brain synaptosomes, the possible contribution of these two $Ca^{2+}$ entrance pathways in the process of $^{45}Ca^{2+}$ uptake elicited by different extracellular ionic conditions. The decrease in extracellular $Na^+$ concentration from 145 mM to 95 mM and its concomitant substitution with complemental concentration of $K^+$ (5–55 mM) caused an increase in $^{45}Ca^{2+}$ uptake, whereas an equimolar concentration of choline (50 mM), although in the presence of the same $Na^+$ concentration (95 mM), failed to stimulate $^{45}Ca^{2+}$ uptake. Only when the extracellular $Na^+$ concentration was further lowered from 95 mM to 0 mM and substituted with equivalent amounts of choline (50–145 mM) did a dose-dependent stimulation of $^{45}Ca^{2+}$ uptake occur. In addition, when the lowering of the extracellular $Na^+$ concentration from 95 mM to 0 mM was compensated for by $K^+$ concentrations higher than 55 mM (55–150 mM), $^{45}Ca^{2+}$ uptake was higher than that elicited by $Na^+$ ion substitution with equimolar amounts (50–145 mM) of choline. The amount of $^{45}Ca^{2+}$ uptake induced by 55 mM $K^+$ did not differ either in $Na^+$-preincubated or in $Na^+$-depleted synaptosomes. Synaptosomal membrane potential, monitored with the potential-sensitive fluorescent dye bis-(1,3-diethyltiobarbiturate)trimethineoxonol, showed a progressive depolarization when extracellular $K^+$ concentrations were raised from 5 to 150 mM, reaching a plateau at 55 mM extracellular $K^+$ concentration, whereas when choline (145 mM) completely substituted for extracellular $Na^+$ ions, synaptosomal membrane potential did not show any depolarization. Collectively, these results demonstrate that $^{45}Ca^{2+}$ uptake induced by 55 mM $K^+$ ions occurs selectively through voltage-operated $Ca^{2+}$ channels, whereas, in choline-substituted media, starting from 70 mM choline, $Ca^{2+}$ ions seemed to utilize the $Na^+$-$Ca^{2+}$ antiporter to penetrate into synaptosomes. In contrast, when extracellular $K^+$ concentrations are raised above 55 mM, $^{45}Ca^{2+}$ entrance may occur through two cumulative mechanisms, the opening of $Ca^{2+}$ channels that are activated by high $K^+$-induced depolarization and the activation of the $Na^+$-$Ca^{2+}$ antiporter, which follows the reduction of the transmembrane $Na^+$ electrochemical gradient. Furthermore, studies on the kinetics of $^{45}Ca^{2+}$ uptake induced by 145 mM choline or 55 mM $K^+$ showed significant differences in both $K_m$ and $V_{max}$ values, suggesting that $^{45}Ca^{2+}$ uptake in brain synaptosomes induced by 145 mM choline or 55 mM $K^+$ may occur through pathways characterized by different kinetic parameters. In conclusion, the results of the present experiments suggest that it is possible to selectively promote $Ca^{2+}$ entrance into brain synaptosomes through voltage-operated $Ca^{2+}$ channels and/or through the $Na^+$-$Ca^{2+}$ antiporter, depending on the ionic environment of the extracellular fluid.

The crucial role of $Ca^{2+}$ ions in nerve cell activity and neurotransmitter release has now been clearly established (1). $Ca^{2+}$ ions can enter into the nerve cells using two different pathways, the bidirectional $Na^+$-$Ca^{2+}$ exchanger, which couples $Ca^{2+}$ influx to $Na^+$ extrusion (2), and $Ca^{2+}$ channels, which are gated by the electrical potential across the membrane (VOCCs) (3, 4).

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When $Ca^{2+}$ uptake is elicited by depolarizing extracellular $K^+$ concentrations, it is difficult to discriminate the relative contribution on these two $Ca^{2+}$ influx pathways (5). In fact, when the effects on $^{45}Ca^{2+}$ uptake of elevated extracellular concentrations on $K^+$ ions are investigated, equimolar amounts of $Na^+$ ions are removed from the extracellular medium, in order to keep constant the medium osmolarity (6). This ionic substitution causes an alteration in the transmembrane electrochemical gradient for $Na^+$ ions, which could result in a concomitant activation of the $Na^+$-$Ca^{2+}$ exchanger, operating

**ABBREVIATIONS:** VOCCs, voltage-operated $Ca^{2+}$ channels; bisoxonol, bis-(1,3-diethyltio-barbiturate)trimethineoxonol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

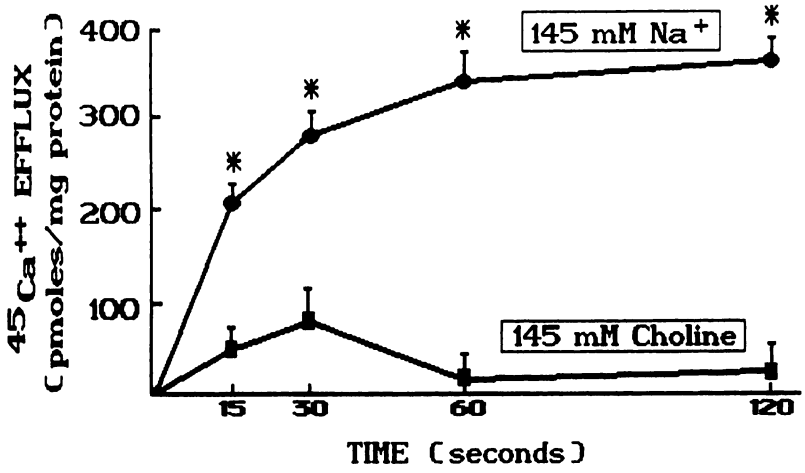


**Fig. 1.** Extracellular $Na^+$ dependence of $^{45}Ca^{2+}$ efflux from striatal synaptosomes. After the preincubation period in the presence of 145 mM extracellular $Na^+$ ions, synaptosomes were loaded for 15 sec with $^{45}Ca^{2+}$ (10 $\mu$M) in a medium containing (in mM) 75 NaCl, 75 KCl, 1.2 $MgCl_2$, 10 HEPES, and 10 glucose, pH 7.4, at 37° (final incubation volume, 200 $\mu$l). After this period, $^{45}Ca^{2+}$ uptake was terminated and $^{45}Ca^{2+}$ efflux was initiated simultaneously by dilution of the uptake medium 22.5 times with 4.5 ml of a $^{45}Ca^{2+}$-free prewarmed (37°) medium containing either 145 mM choline chloride or 145 mM $Na^+$. Data are the mean ± standard error of at least three separate experiments. In each experiment, at least three separate determinations were carried out. *, Values statistically different ($p < 0.01$) from both the corresponding values in the 145 mM choline group and the value at time 0. Intrasynaptosomal $^{45}Ca^{2+}$ taken up before the beginning of the efflux experiment (time 0) was 483 ± 30 pmol of $^{45}Ca^{2+}$/mg of protein.

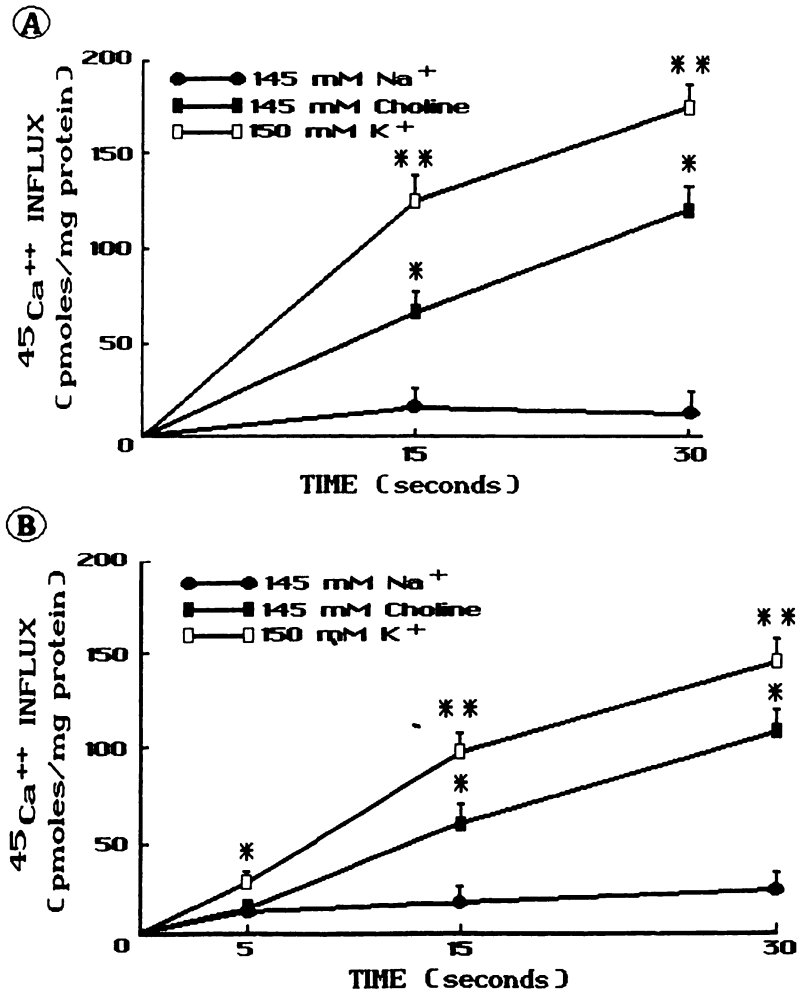


**Fig. 2.** Time-course of 150 mM $K^+$- and 145 mM choline-stimulated $^{45}Ca^{2+}$ uptake in whole brain and striatal synaptosomes. After preincubation for 60 min in a 145 mM $Na^+$-containing medium, whole brain (A) or striatal (B) synaptosomes were exposed to 2 $\mu$M $^{45}Ca^{2+}$ (0.2 $\mu$Ci/tube) in the presence of either 145 mM $Na^+$, 150 mM $K^+$, or 145 mM choline. Data are the mean ± standard error of at least three separate experiments. In each experiment, at least three determinations were carried out. *, Values statistically different ($p < 0.01$) from the value of $^{45}Ca^{2+}$ uptake, at the corresponding time, of 145 mM $Na^+$-exposed synaptosomes. **, Values significantly different ($p < 0.01$) versus both the corresponding value of $^{45}Ca^{2+}$ uptake in the 145 mM $Na^+$ group and that in the 145 mM choline group.

as a $Ca^{2+}$ influx pathway (2). On the other hand, when studies are performed on the activity of the $Na^+$-$Ca^{2+}$ exchanger, extracellular $Na^+$ ions are omitted from the medium and the osmotic balance is kept constant with the addition of equimolar amounts of $K^+$ ions (7–9). With these elevated concentrations of $K^+$ ions, $Ca^{2+}$ uptake can occur not only through the $Na^+$-$Ca^{2+}$ antiporter but also through VOCCs.

The aim of the present study was to characterize the appropriate extracellular ionic conditions that selectively promote the entrance of $Ca^{2+}$ ions either through VOCCs or through the $Na^+$-$Ca^{2+}$ exchange mechanism in Percoll-purified brain synaptosomes. For this purpose, $^{45}Ca^{2+}$ uptake was stimulated by an increase in the extracellular concentration of $K^+$ ions or by $Na^+$ substitution with augmenting amounts of choline in synaptosomes, which either were preincubated in the presence of $Na^+$ ions or were not allowed to accumulate $Na^+$ ions by a preincubation in a sucrose-containing medium.

In addition, in order to correlate synaptosomal membrane potential variations with $Ca^{2+}$ entrance through the two pathways, the potential-sensitive fluorescent dye bisoxonol (10, 11) was used.

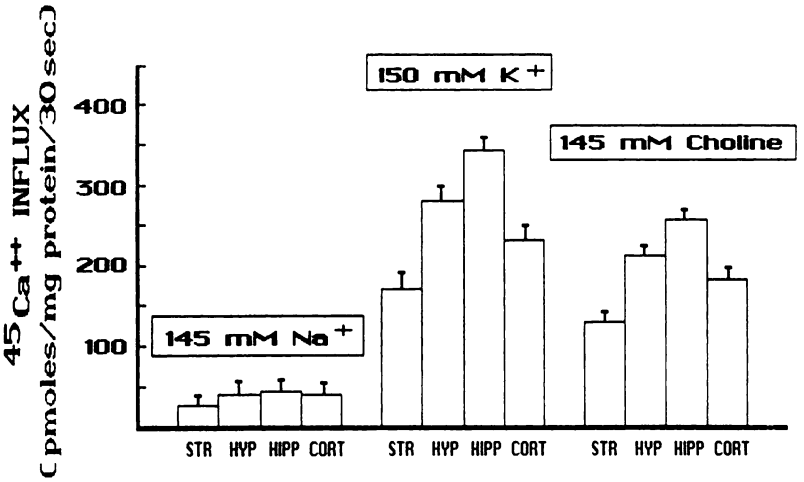


**Fig. 3.** $^{45}Ca^{2+}$ uptake stimulated by an extracellular medium containing either 150 mM $K^+$ or 145 mM choline in different brain areas. After 60 min of preincubation with a 145 mM $Na^+$-containing solution, synaptosomes obtained from corpus striatum (*STR*), hypothalamus (*HYP*), hippocampus (*HIPP*), and frontal cortex (*CORT*) were exposed to 2 $\mu$M $^{45}Ca^{2+}$ in the presence of either 145 mM $Na^+$ ions, 150 mM $K^+$ ions, or 145 mM choline ions. Data are the mean ± standard error of at least three separate experiments. In each experiment, at least three separate determinations were carried out. $^{45}Ca^{2+}$ uptake induced by 150 mM $K^+$ or 145 mM choline in the hippocampus was statistically different ($p < 0.01$) from that occurring in all the other brain regions examined. $^{45}Ca^{2+}$ uptake in hypothalamic synaptosomes evoked by 150 mM $K^+$ or 145 mM choline was statistically different ($p < 0.01$) from that occurring in the striatum. Choline (145 mM)-stimulated $^{45}Ca^{2+}$ uptake in the frontal cortex was significantly higher ($p < 0.05$) than that occurring in the striatum.

## Materials and Methods

**Synaptosomal preparation.** Synaptosomal fractions from brains of adult (150–200-g body weight) male Wistar rats were prepared according to the procedure of Dunkley *et al.* (12), using a discontinuous Percoll gradient. Briefly, rats were killed by decapitation and the brains were removed and kept on ice. The cerebellum, brainstem, and most of the white matter were discarded. Whole brains or selected brain regions (striatum, hippocampus, frontal cortex, and hypothalamus) were homogenized using a Teflon-glass homogenizer in 9 ml/g of tissue of cold sucrose medium, whose composition was (in mM) 320 sucrose, 1 EDTA, 0.25 dithiothreitol, adjusted to pH 7.4 with 1 N NaOH. The homogenate was centrifuged for 10 min at 1000 × *g* at 4°. The supernatant ($S_1$) was diluted to 14 ml/g of starting tissue and 2 ml of $S_1$ were layered on the top of a tube containing four different Percoll concentrations (from the bottom, in v/v, 23%, 15%, 10%, and 3% Percoll-sucrose media). The gradients were prepared using a peristaltic pump, in order to achieve flat interfaces between the solutions at different Percoll concentrations. These gradients were placed in a Sorvall RC5B superspeed centrifuge and spun at 4° for 5 min at 32,000 × *g*, in a SS-34 rotor.

After centrifugation, the fraction at the interface between the 23% and the 15% Percoll layers [fractions 4 as described by Dunkley *et al.* (12)], which is highly enriched in viable synaptosomes (13), was removed and diluted 5 times in a solution containing (in mM) 125 NaCl, 2.5 KCl, 1.2 $KH_2PO_4$, 1.2 $MgSO_4$, 5 $NaHCO_3$, 25 HEPES, and 6 glucose, pH 7.4. Diluted synaptosomes were centrifuged two times at 15,000 × *g* for 15 min (4°), in order to remove Percoll. The final pellet was resuspended (5–10 mg of protein/ml) in a medium containing (in mM) 145 NaCl, 5 KCl, 1.2 $MgCl_2$, 10 glucose, and 10 HEPES, pH 7.4 (145 mM $Na^+$-containing medium) and was immediately used for the following experimental procedures.

**$^{45}Ca^{2+}$ uptake studies.** Resuspended synaptosomes were preincubated in a 145 mM $Na^+$ medium for 60 min at 37°, to allow $Na^+$ accumulation into the nerve terminals (5). After this period, 30–50 $\mu$g of synaptosomal protein (3–5 $\mu$l) were diluted in 200 $\mu$l of the incubation medium, containing 2 $\mu$M $^{45}Ca^{2+}$ (specific activity, 2 mCi/mmol); The Radiochemical Centre, Amersham). $^{45}Ca^{2+}$ uptake was terminated by addition to the tubes of 4.5 ml of ice-cold quench solution, whose composition was (in mM) 145 choline·HCl, 5 KCl, 1.2 $MgCl_2$, 10 HEPES, adjusted to pH 7.4 with 1 M Tris. Synaptosomes were separated from the extracellular medium by rapid filtration on an Hoefler SH 224 V manifold filtration apparatus, using Whatman GF/C glass fiber filters (25-mm diameter). Synaptosomes retained on the filters were then washed three times with 4.5 ml of ice-cold wash solution of the following composition (in mM): 145 NaCl, 5 KCl, 1.2 $MgCl_2$, 2 $LaCl_3$, 10 HEPES, buffered to pH 7.4 with 1 M Tris. $LaCl_3$ (2 mM) was added to the washing medium to remove surface-bound $^{45}Ca^{2+}$, as reported by Coutinho *et al.* (5). Filters were then placed into scintillation vials with 7 ml of scintillation cocktail (Dynagel, Baker), and the trapped radioactivity was determined by liquid scintillation counting.

**$^{45}Ca^{2+}$ efflux studies.** After a 60-min preincubation period in a 145 mM $Na^+$-containing solution, synaptosomes were loaded for 15 sec with 10 $\mu$M $^{45}Ca^{2+}$ in a medium containing (in mM): 75 NaCl, 75 KCl, 1.2 $MgCl_2$, 10 HEPES, and 10 glucose, pH 7.4, at 37°, in a final incubation volume of 200 $\mu$l, according to a previously published procedure (14). $^{45}Ca^{2+}$ uptake was terminated and $^{45}Ca^{2+}$ efflux was initiated simultaneously by addition to the tubes of 4.5 ml of a $^{45}Ca^{2+}$-free prewarmed (37°) medium containing either 145 mM choline chloride or 145 mM NaCl. At different times, synaptosomes were filtered and filters were washed and counted as described in the previous paragraph. $^{45}Ca^{2+}$ efflux was expressed as the difference between $^{45}Ca^{2+}$ content retained in synaptosomes after different efflux times and that at time 0.

**Determination of synaptosomal membrane potential variations with the potential-sensitive fluorescent dye bisoxonol.** The lipophilic anion bisoxonol has been widely used to monitor membrane potential changes (10), because its fluorescence emission increases when it moves from polar solutions (i.e., extracellular medium) to nonpolar environments (i.e., lipids or proteins). Therefore, depolarization of the cells results in a net movement of the dye anions from the external solution to cellular binding sites, causing a detectable increase in the fluorescent signal (11). In the present experiment, the following procedure was used: bisoxonol (300 nM), from a stock solution of 150 $\mu$M in dimethyl sulfoxide, was added to 2 ml of prewarmed medium, whose ionic composition was varied according to the experimental protocol, in a thermostated quartz cuvette of a Perkin-Elmer LS-5 spectrofluorometer equipped with a magnetic ministirrer (DBS Strumentazione Scientifica, Albignasego, Padova, Italy). Fluorescence intensity of the dye was recorded at excitation and emission wavelengths of 540 and 580 nm, respectively (5-nm slits for both excitation and emission wavelengths). About 1 min after the addition of bisoxonol, 40–50 $\mu$g of synaptosomal proteins (4–5 $\mu$l) were pipetted into the cuvette. All subsequent additions of KCl were made from a concentrated stock solution of 1 M. Bisoxonol fluorescence intensity variations were recorded in a Perkin-Elmer computer connected to the spectofluorometer and were stored for further graphical and statistical analysis. The valinomycin-null point method, which converts fluorescence signals into absolute membrane potential values, could not be applied, because of the formation of complexes between bisoxonol and valinomycin (10).

**Protein determination.** Synaptosomal protein concentration was determined by the method described by Bradford (15).

**Statistics.** Statistical analysis of the data was performed by means of analysis of variance, followed by the Neuman-Keul's test.

**Materials.** All the reagents used in this study were from commercial sources and were of the highest purity available. Bisoxonol was a kind gift from Prof. Tullio Pozzan (University of Padova, Italy).

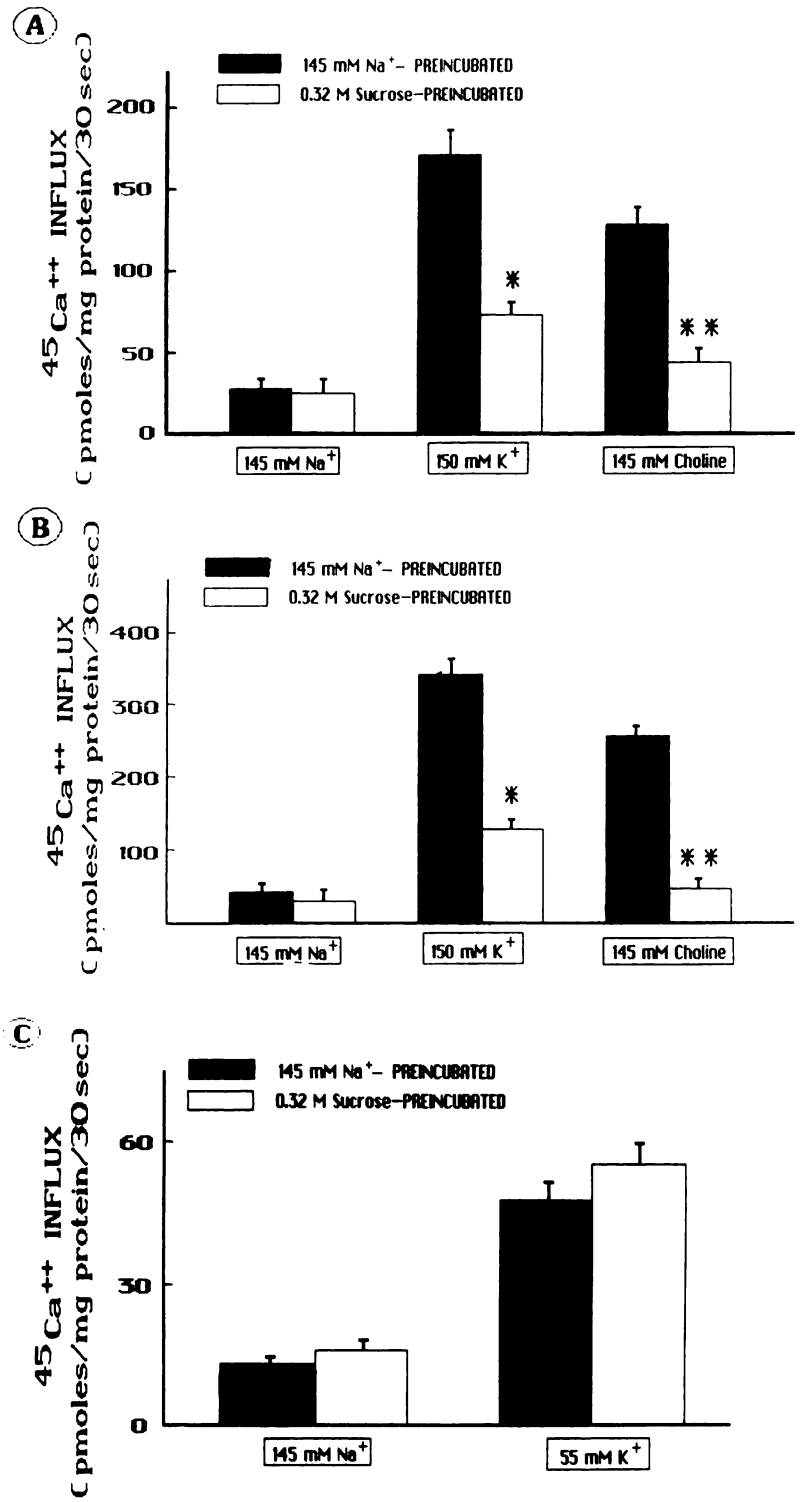


**Fig. 4.** Effect of preincubation of synaptosomes in the presence or in the absence of extracellular $Na^+$ ions on $^{45}Ca^{2+}$ uptake stimulated by 55 mM $K^+$, 150 mM $K^+$ or 145 mM choline. Synaptosomes from the corpus striatum (A), the hippocampus (B), and whole brain (C) were preincubated for 60 min at 37° either in a 145 mM $Na^+$-containing solution or in a medium in which 0.32 M sucrose substituted for extracellular NaCl. After this preincubation period, $^{45}Ca^{2+}$ uptake was evaluated in both groups of striatal and hippocampal synaptosomes in extracellular media containing either 145 mM $Na^+$, 150 mM $K^+$ or 145 mM choline. In addition, $^{45}Ca^{2+}$ uptake was determined in whole brain synaptosomes preincubated either in 145 mM $Na^+$ or 0.32 M sucrose and then exposed to 145 mM $Na^+$- or 55 mM $K^+$-containing media. Data are the mean ± standard error of at least three separate experiments. In each experiment, at least three separate determinations were carried out. *, Values statistically different ($p < 0.01$) both from $^{45}Ca^{2+}$ uptake occurring upon stimulation with 150 mM $K^+$ in $Na^+$-preincubated synaptosomes and from $^{45}Ca^{2+}$ uptake occurring in sucrose-preincubated synaptosomes exposed to 145 mM $Na^+$ during the uptake phase. **, Values statistically different ($p < 0.01$) from $^{45}Ca^{2+}$ uptake in $Na^+$-preincubated synaptosomes exposed to 145 mM choline but not from $^{45}Ca^{2+}$ uptake occurring in sucrose-preincubated synaptosomes exposed to 145 mM $Na^+$ ions during the uptake phase.

## Results

**$^{45}Ca^{2+}$ efflux through the $Na^+$-$Ca^{2+}$ antiporter in striatal synaptosomes.** When striatal synaptosomes are incubated in an extracellular medium containing 145 mM $Na^+$ ions, an inwardly directed $Na^+$ gradient exists, which favors the operation of the $Na^+$-$Ca^{2+}$ antiporter as a $Ca^{2+}$ efflux pathway. In fact, as shown in Fig. 1, after $^{45}Ca^{2+}$ loading the presence of 145 mM $Na^+$ ions in the extracellular medium caused a marked efflux of $^{45}Ca^{2+}$ from striatal synaptosomes. This efflux was faster in the first 15 sec, reaching a plateau at about 30–60 sec. In contrast, removal of $Na^+$ ions from the extracellular space (with choline substitution), a condition that determines an outwardly directed $Na^+$ electrochemical gradient, completely prevented the efflux of $^{45}Ca^{2+}$ ions from striatal synaptosomes.

**Effect of different extracellular ionic conditions on $^{45}Ca^{2+}$ influx in whole brain and striatal synaptosomes.** $^{45}Ca^{2+}$ uptake in whole brain (Fig. 2A) or striatal (Fig. 2B) synaptosomes preincubated for 60 min in a 145 mM $Na^+$-containing medium was studied in the presence of extracellular

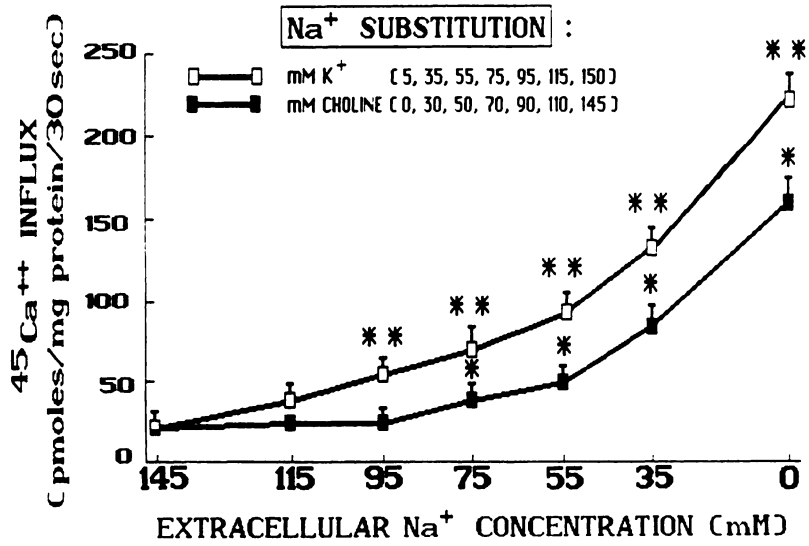


**Fig. 5.** Effect of decreasing extracellular $Na^+$ concentration and increasing extracellular $K^+$ or choline concentration on $^{45}Ca^{2+}$ uptake in whole brain synaptosomes. Synaptosomes, after the preincubation period in the presence of 145 mM extracellular $Na^+$ ions, were added to tubes containing decreasing concentrations of extracellular $Na^+$ ions (145–0 mM), substituted with complemental concentrations of $K^+$ (5–150 mM) or choline (0–145 mM). Data are the mean ± standard error of at least three separate experiments. In each experiment, at least three separate determinations were carried out. *, Values statistically different ($p < 0.01$) from the value of the 145 mM $Na^+$-exposed synaptosomes. **, Values significantly different ($p < 0.01$) from both $^{45}Ca^{2+}$ uptake occurring in synaptosomes incubated in the presence of 145 mM $Na^+$ and from $^{45}Ca^{2+}$ uptake occurring in synaptosomes exposed to corresponding values of choline-substituted medium.

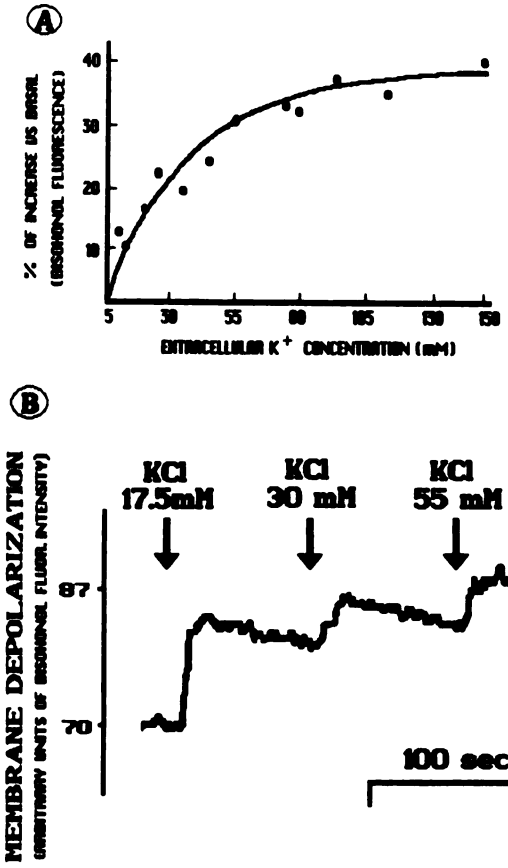


**Fig. 6.** Effect of incubation of whole brain synaptosomes in the presence of increasing concentrations of $K^+$ ions on synaptosomal membrane potential monitored with bisoxonol. A, Bisoxonol (300 nM) was added to 2 ml of prewarmed medium containing either 145 mM $Na^+$ ions or decreasing concentrations of $Na^+$ ions, compensated for by equimolar additions of $K^+$ ions. About 1 min after the addition of bisoxonol, 40–50 μg of synaptosomal proteins (4–5 μl) were pipetted into the cuvette. B, Synaptosomes were added in 2 ml of prewarmed medium containing 145 mM $Na^+$ ions and were subsequently exposed to increasing concentrations of $K^+$ ions by addition of small volumes of KCl from a concentrated stock of 1 M. For more experimental details, see Materials and Methods.

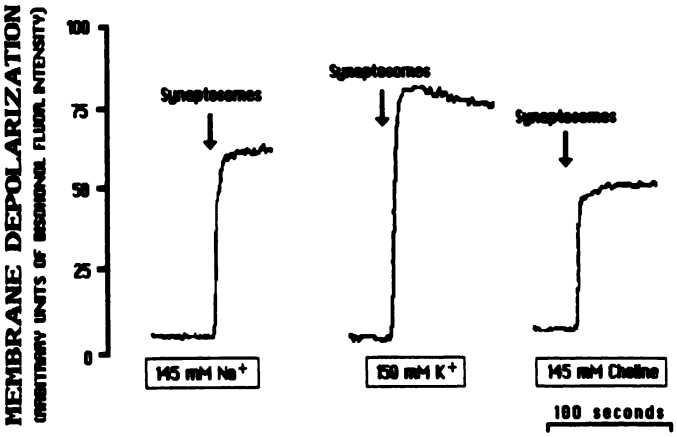


**Fig. 7.** Effect of incubation of whole brain synaptosomes in the presence of an extracellular medium containing either 145 mM $Na^+$ ions, 150 mM $K^+$ ions, or 145 mM choline ions on synaptosomal membrane potential monitored with bisoxonol. Synaptosomes, after the standard preincubation period, were added to the cuvette containing 300 nM bisoxonol, in the presence of either 2 ml of 145 mM $Na^+$-containing medium or in extracellular media where $Na^+$ ions were substituted with 150 mM $K^+$ or with 145 mM choline. For more experimental details, see Materials and Methods.

media containing either 145 mM $Na^+$, 150 mM $K^+$, or 145 mM choline. The complete removal of $Na^+$ ions from the medium and their substitution with $K^+$ or choline caused an increase in $^{45}Ca^{2+}$ uptake in both striatal and whole brain synaptosomes. Interestingly, at every time considered, $^{45}Ca^{2+}$ uptake elicited by $Na^+$ ion removal and their substitution with 150 mM $K^+$ ions was higher than that occurring upon substitution of $Na^+$ ions with an equimolar concentration of choline (145 mM).

**Effect of extracellular $Na^+$ ion removal and their substitution with 150 mM $K^+$ or 145 mM choline on $^{45}Ca^{2+}$ uptake in different brain areas.** In these experiments, we studied the regional distribution of 145 mM choline- or 150 mM $K^+$-stimulated $^{45}Ca^{2+}$ uptake in purified synaptosomal preparations obtained from the striatum, the frontal cortex, the hypothalamus, and the hippocampus of the rat brain (Fig. 3). In both experimental conditions, the greatest amount of $^{45}Ca^{2+}$ uptake was observed in synaptosomes purified from the hippocampus and the hypothalamus. In striatal and cortical syn-

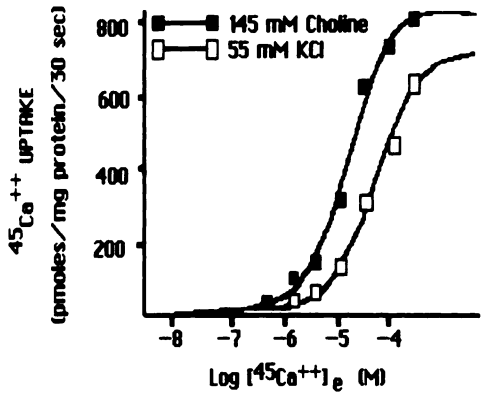


**Fig. 8.** Effect of increasing extrasynaptosomal concentrations of $^{45}Ca^{2+}$ on $^{45}Ca^{2+}$ uptake elicited by 55 mM $K^+$ or 145 mM choline in whole brain synaptosomes. After the standard preincubation phase in the presence of 145 mM extracellular $Na^+$ ions, 40–50 $\mu$g of synaptosomal proteins (4–5 $\mu$l) were exposed to increasing extrasynaptosomal $^{45}Ca^{2+}$ concentrations (0.3–300 $\mu$M) in an extracellular medium containing either 55 mM $K^+$ ions or 145 mM choline. After 30 sec, $^{45}Ca^{2+}$ uptake was terminated and $^{45}Ca^{2+}$ content in synaptosomes was determined, as described in Materials and Methods.

aptosomes, $^{45}Ca^{2+}$ uptake was lower than that observed in the hippocampus.

**Effect of preincubation of synaptosomes in the presence or absence of extracellular $Na^+$ ions on $^{45}Ca^{2+}$ uptake induced by 55 mM $K^+$, 150 mM $K^+$ or 145 mM choline.** Synaptosomes from the corpus striatum (Fig. 4A) from the hippocampus (Fig. 4B) and from whole brain (Fig. 4C) were preincubated for 60 min at 37° either in a 145 mM $Na^+$-containing medium or in a $Na^+$-free medium in which extracellular $Na^+$ ions were substituted with 0.32 M sucrose. This latter condition was chosen in order to prevent intrasynaptosomal $Na^+$ ion accumulation (5). After this period, synaptosomes from both groups were exposed to $^{45}Ca^{2+}$ in the presence of 145 mM $Na^+$, 55 mM $K^+$, 150 mM $K^+$, or 145 mM choline. The results obtained in both striatal and hippocampal synaptosomes showed that, when synaptosomes were "preincubated" in a $Na^+$-lacking sucrose-containing medium, the presence of 145 mM choline in the extracellular medium, a condition that in $Na^+$-preincubated synaptosomes elicited an increase in $^{45}Ca^{2+}$ uptake, failed to stimulate $^{45}Ca^{2+}$ influx. On the other hand, in sucrose-preincubated synaptosomes, 150 mM $K^+$ ions were still able to stimulate $^{45}Ca^{2+}$ uptake, although this increase was significantly lower than that occurring in synaptosomes that had been allowed to accumulate $Na^+$ by a previous preincubation in a $Na^+$-containing medium. Interestingly, the amount of $^{45}Ca^{2+}$ uptake elicited by 150 mM $K^+$ ions in synaptosomes that were allowed to accumulate $Na^+$ ions intracellularly during the preincubation period closely corresponded to the sum of $^{45}Ca^{2+}$ uptake induced by 150 mM $K^+$ ions in the sucrose-preincubated group plus $^{45}Ca^{2+}$ uptake elicited by 145 mM choline in synaptosomes that had been previously preincubated in the presence of extracellular $Na^+$ ions (Fig. 4A and B). Therefore, these results seem to suggest the conclusion that, in $Na^+$-preincubated synaptosomes, the complete removal of $Na^+$ ions from the extracellular space and their substitution with an equimolar concentration of $K^+$ ions stimulate $^{45}Ca^{2+}$ uptake by two cumulative mechanisms, 1) the opening of VOCCs, which are activated by high $K^+$-induced depolarization, and 2) the activation of the $Na^+$-$Ca^{2+}$ antiporter, which follows the reversal of the $Na^+$ electrochemical gradient across the synaptosomal membrane. In addition, the fact that the presence of 145 mM choline in the extracellular medium failed to enhance $^{45}Ca^{2+}$ uptake in sucrose-preincubated synaptosomes suggests that, under this experimental condition, the activity of the $Na^+$-$Ca^{2+}$ antiporter is blocked. On the other hand, in sucrose-preincubated synaptosomes in which the activity of the $Na^+$-$Ca^{2+}$ exchanger is blocked, $^{45}Ca^{2+}$ uptake elicited by 150 mM extracellular $K^+$ represents the amount of the bivalent cation that penetrates into the synaptosomes only via VOCCs. Furthermore, 55 mM $K^+$ elicited a stimulation of $^{45}Ca^{2+}$ uptake which was equivalent in both $Na^+$- and sucrose-preincubated synaptosomes (Fig. 4C).

**Effect of decreasing extracellular $Na^+$ concentration and increasing extracellular $K^+$ or choline concentration on $^{45}Ca^{2+}$ uptake in whole brain synaptosomes.** When the extracellular $Na^+$ concentration was decreased from 145 mM to 95 mM and the medium osmolarity was kept constant with complementing concentrations of choline (0–50 mM), no stimulation of $^{45}Ca^{2+}$ uptake was observed (Fig. 5). In contrast, if this decrease in extracellular $Na^+$ ions was compensated for with increasing concentrations (5–55 mM) of $K^+$ ions, a stimulation of $^{45}Ca^{2+}$ uptake was observed. These results seem to suggest that the substitution of 55 mM extracellular $Na^+$ ions with an equimolar concentration of $K^+$ ions selectively activates $^{45}Ca^{2+}$ influx via VOCCs, without any concomitant activation of the $Na^+$-$Ca^{2+}$ antiporter. When the extracellular $Na^+$ concentration was further lowered from 95 mM to 0 mM, keeping the osmolarity constant with complemental substitutions of $Na^+$ with choline (50–145 mM), a dose-dependent stimulation of $^{45}Ca^{2+}$ uptake in whole brain synaptosomes occurred. On the other hand, an increase in extracellular $K^+$ concentrations from 55 mM to 150 mM caused a concentration-dependent increase in $^{45}Ca^{2+}$ uptake, which, at all the concentrations tested, was higher than that elicited by the substitution of extracellular $Na^+$ ions with equimolar amounts of choline. These results appear to suggest that reduction of the extracellular $Na^+$ concentration from 95 mM to 0 mM caused a progressive increase in $^{45}Ca^{2+}$ uptake, which was due to the activation of the $Na^+$-$Ca^{2+}$ antiporter when this exchange system operates as a $Ca^{2+}$ influx pathway. In addition, if the extracellular $Na^+$ ion concentration was decreased from 95 mM to 0 mM and the medium osmolarity was kept constant with complemental concentrations of $K^+$ ions (55–150 mM), the stimulation of $^{45}Ca^{2+}$ uptake was due to the activation of both VOCCs and the $Na^+$-$Ca^{2+}$ antiporter.

**Bisoxonol-monitored membrane potential in brain synaptosomes exposed to increasing concentrations of $K^+$ ions or to a $Na^+$-lacking choline-substituted medium.** When whole brain synaptosomes were incubated in the presence of the potential-sensitive fluorescent dye bisoxonol, synaptosomal membrane potential showed a progressive depolarization in the presence of increasing concentrations of extracellular $K^+$ ions (Fig. 6). $K^+$-induced depolarization showed a steep increase with $K^+$ concentrations ranging from 5 to 55 mM, whereas a further rise from 55 to 150 mM caused only a slightly raised heel in bisoxonol fluorescence, suggesting that depolarization of the synaptosomal plasma membrane reached a plateau of a concentration of about 55 mM extracellular $K^+$ ions.

On the other hand, when extracellular $Na^+$ ions were substi-

tuted with choline, synaptosomal membrane potential did not show any tendency to depolarization, but rather the bisoxonol fluorescence level was somewhat lower than that observed in a standard $Na^+$-containing solution, indicating that, in a medium where choline substituted for $Na^+$ ions, a slight tendency to hyperpolarization occurred (Fig. 7). Therefore, these results seem to further support the idea that, when extracellular $Na^+$ ions are completely substituted for by choline ions, $^{45}Ca^{2+}$ uptake occurs selectively through the $Na^+$-$Ca^{2+}$ antiporter, without any participation of VOCCs, because, under this experimental condition, synaptosomal membrane potential did not show any tendency to depolarization.

**Kinetic properties of $^{45}Ca^{2+}$ uptake elicited by 55 mM $K^+$ or 145 mM choline in the whole brain synaptosomes.** In order to determine the kinetic parameters of the processes involved in $^{45}Ca^{2+}$ uptake stimulated by 55 mM $K^+$ ions, a condition that appears to selectively activate VOCCs, or by the complete removal of $Na^+$ ions from the extracellular space (145 mM choline substitution), which reflects $^{45}Ca^{2+}$ uptake mediated by the $Na^+$-$Ca^{2+}$ antiporter, we measured the rates of $^{45}Ca^{2+}$ uptake, under both conditions, as a function of extrasynaptosomal $^{45}Ca^{2+}$ concentrations (Fig. 8).

Nonlinear regression analysis of the data (16),[1] using a model for saturable transport mechanisms, revealed for 55 mM $K^+$-stimulated $^{45}Ca^{2+}$ uptake an apparent $K_m$ of 50 ± 10 μM and a $V_{max}$ of 682 ± 43 pmols of $^{45}Ca^{2+}$ uptake/mg of synaptosomal protein/30 sec, whereas 145 mM choline-induced $^{45}Ca^{2+}$ uptake showed an apparent $K_m$ of 15 ± 2 μM and a $V_{max}$ of 809 ± 26 pmols of $^{45}Ca^{2+}$ uptake/mg of synaptosomal protein/30 sec. Statistical comparison of these two apparent $K_m$ values and the two $V_{max}$ values revealed significant differences at $p < 0.005$ and at $p < 0.05$, respectively, suggesting that $^{45}Ca^{2+}$ entrance into synaptosomes exposed to the two different experimental conditions (55 mM $K^+$ ions and 145 mM choline) occurred through pathways showing different kinetic properties.

## Discussion

The selective evaluation of the different pathways responsible for $Ca^{2+}$ influx in nerve cells is of crucial relevance for the study of the physiological role played by these membrane transport systems (17) and for the possibility of modulating their activity with pharmacological compounds that may selectively interfere with these systems.

The results of the present study seem to suggest that, in brain synaptosomes, it is possible to selectively activate VOCCs when the extracellular $K^+$ concentration is raised from 5 to 55 mM and $Na^+$ concentration is lowered from 145 mM to 95 mM. In these conditions, no concomitant activation of the $Na^+$-$Ca^{2+}$ exchanger occurs, because the substitution of equimolar amounts of $Na^+$ ions with choline failed to stimulate $^{45}Ca^{2+}$ uptake. This observation suggests that the reduction of the $Na^+$ electrochemical gradient that follows the reduction of the extracellular $Na^+$ concentration from 145 mM to 95 mM does not reach the activation threshold to trigger $Ca^{2+}$ entrance mediated by the $Na^+$-$Ca^{2+}$ exchanger.

The fact that the $^{45}Ca^{2+}$ uptake that occurs upon exposure of synaptosomes to 55 mM extracellular $K^+$ ions is due to the selective activation of VOCCs is also supported by the evidence that this $K^+$ concentration caused an intense depolarization of the synaptosomal membrane, as detected by the increased fluorescence emission of the membrane potential-sensitive dye bisoxonol. On the other hand, when extracellular $Na^+$ concentration is further lowered from 95 mM to 0 mM and substituted with equimolar amounts of choline, $Ca^{2+}$ ions selectively utilize the $Na^+$-$Ca^{2+}$ antiporter to penetrate into synaptosomes, whereas VOCCs do not seem to participate in this entrance. In fact, under these ionic conditions, synaptosomal membrane potential was not depolarized but rather showed a slight tendency to hyperpolarization, according to previous results obtained in mouse lymphocytes (10) and in sheep brain synaptosomes (5).

On the basis of these considerations, it seems reasonable to assume that, when the extracellular $Na^+$ concentration is lowered from 95 mM to 0 mM and substituted with complemental additions of $K^+$ ions, the stimulation of $^{45}Ca^{2+}$ uptake appears to be dependent on both activation of VOCCs, due to the depolarization of the synaptosomal plasma membrane, and the concomitant activation of the $Na^+$-$Ca^{2+}$ exchanger, which follows the reversal of the $Na^+$ electrochemical gradient.

The activity of the $Na^+$-$Ca^{2+}$ exchanger in central synaptosomes appears to be critically dependent on the internal $Na^+$ concentration. In fact, if synaptosomes were preincubated, in a $Na^+$-lacking medium (sucrose substitution), in order to prevent intrasynaptosomal $Na^+$ accumulation, and then exposed to 145 mM choline, the stimulation of $^{45}Ca^{2+}$ uptake was completely prevented, suggesting that, if the $Na^+$ electrochemical gradient is almost nullified, the activity of the $Na^+$-$Ca^{2+}$ exchanger is blocked. In contrast, the exposure of these $Na^+$-depleted synaptosomes to 150 mM extracellular $K^+$ ions was still able to stimulate $^{45}Ca^{2+}$ uptake, although $^{45}Ca^{2+}$ uptake was lower than that observed in synaptosomes that had been previously exposed to $Na^+$ ions in the preincubation phase.

Furthermore, the hypothesis that 55 mM $K^+$ stimulates $^{45}Ca^{2+}$ uptake mainly through VOCCs seems to be validated by the observation that the amount of $^{45}Ca^{2+}$ uptake elicited by 55 mM $K^+$ did not differ either in normal or $Na^+$-depleted synaptosomes.

Other evidence that supports the selective activation of VOCCs by 55 mM $K^+$ ions and of the $Na^+$-$Ca^{2+}$ exchanger by extracellular $Na^+$ replacement with choline derives from the kinetic analysis of $^{45}Ca^{2+}$ uptake studied in these two extracellular ionic conditions. The existence of a significant different between the $K_m$ and the $V_{max}$ values for $^{45}Ca^{2+}$ uptake stimulated by 55 mM $K^+$ or 145 mM choline seems to indicate that $^{45}Ca^{2+}$ ions enter into synaptosomes through pathways characterized by different kinetic parameters.

Furthermore, 2′,4′-dimethylbenzamil amiloride, which shares with other amiloride derivatives the property of inhibiting the $Na^+$-$Ca^{2+}$ exchanger in neuronal (18) as well as in other cell types (19, 20), dose-dependently prevented $^{45}Ca^{2+}$ uptake induced by the substitution of extracellular $Na^+$ ions with choline, whereas it failed to affect 55 mM $K^+$-stimulated $^{45}Ca^{2+}$ uptake (21). This result further reinforces the idea that the $Na^+$-$Ca^{2+}$ exchanger is selectively activated under conditions of complete substitution of extracellular $Na^+$ ions with equimolar amounts of choline.

On the other hand, it was not possible to demonstrate that the blockade of VOCCs could prevent $^{45}Ca^{2+}$ entrance evoked

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by 55 mM extracellular $K^+$ ions, because all the $Ca^{2+}$ entry blockers belonging to different chemical classes have been shown to be ineffective in blocking VOCCs in the central nervous system (22, 23). In addition, under the same experimental conditions as in the present study, ω-conotoxin GVIA, a presynaptic neurotoxin that has been recently reported to selectively block the neuron-specific N-type as well as the L-type VOCCs (24, 25), failed to inhibit $^{45}Ca^{2+}$ uptake elicited by 55 mM extracellular $K^+$ ions.[2]

Finally, it should be emphasized that compounds like the organic $Ca^{2+}$ entry-blocker verapamil (26, 27) and the inorganic cation cadmium (28) do not seem to be selective blockers of VOCCs, because they also inhibit the $Na^+$-$Ca^{2+}$ antiporter in brain synaptosomes.[3] On the other hand, since in our study $^{45}Ca^{2+}$ uptake was investigated for 15 sec or longer, the possibility should be considered that, after this period, all three types of VOCCs are inactivated (29), and this could account for the insensitivity to $Ca^{2+}$ channel blockers. However, it should be emphasized that even at very short $^{45}Ca^{2+}$ uptake intervals (1–10 sec), synaptosomal VOCCs are insensitive to organic $Ca^{2+}$-channel blockers (29, 30).

Collectively, the results of the present study show that $^{45}Ca^{2+}$ entrance into brain synaptosomes can occur selectively through VOCCs and/or the $Na^+$-$Ca^{2+}$ antiporter, according to the ionic environment of the extracellular fluid. Furthermore, discrimination of the relative contributions of these two membrane $Ca^{2+}$-transporting systems may be of crucial relevance for the study of their specific physiological roles and pharmacological modulation.

[2] L. Annunziato *et al.*, manuscript in preparation.

[3] L. Annunziato *et al.*, manuscript in preparation.

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