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Na^+-Ca^{2+} Exchange Activity in Central Nerve Endings. II. Relationship Between Pharmacological Blockade by Amiloride Analogues and Dopamine Release from Tuberoinfundibular Hypothalamic Neurons

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SUMMARY

The aim of the present study was to investigate the possible role played by the Na^+-Ca^{2+} exchange system in the modulation of [^{3}H]dopamine ([^{3}H]DA) release from tuberoinfundibular hypothalamic (TIDA) neurons. 2′,4′-Dimethylbenzamil (DMB) dose-dependently (10–100 μM) inhibited Na^+-dependent $^{45}Ca^{2+}$ efflux from brain synaptosomes. This compound (30–300 μM), as well as α-phenylbenzamil amiloride (30–100 μM), another inhibitor of the Na^+-Ca^{2+} antiporter, was also able to stimulate basal release of [^{3}H]DA from superfused TIDA neurons. This stimulation was completely prevented by the removal of extracellular Ca^{2+} ions, in the presence of 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid. In addition, DMB-induced [^{3}H]DA release was unaffected by the dopamine transport inhibitor nomifensine (10 μM). On the other hand, 5-[*N*-methyl-*N*-guanidinocarbonylmethyl]amiloride (MGCMA) (100–300 μM), which lacks inhibitory properties on the Na^+-Ca^{2+} exchanger but behaves as an inhibitor of the Na^+-H^+ antiporter, failed to modify basal [^{3}H]DA release from TIDA neurons. When the Na^+-Ca^{2+} antiporter operates as a Ca^{2+} influx pathway, as occurs upon the removal of extracellular Na^+ ions, Na^+-dependent $^{45}Ca^{2+}$ uptake in brain synaptosomes was dose-dependently (10–300 μM) inhibited by DMB, whereas DMB itself was unable to prevent 55 mM K^+-induced $^{45}Ca^{2+}$ uptake, which mainly reflects the activation of voltage-operated Ca^{2+} channels. In keeping with these results, ouabain (500 μM)-induced [^{3}H]DA release, which depends on the activation of the Na^+-Ca^{2+} exchanger due to inhibition of the Na^+-K^+-ATPase pump, was prevented by superfusion of TIDA neurons with DMB (50 μM). By contrast, MGCMA (100 μM) failed to modify either Na^+-dependent $^{45}Ca^{2+}$ influx or ouabain-induced [^{3}H]DA release. In conclusion, the results of the present study appear to suggest that the pharmacological inhibition of the Na^+-Ca^{2+} antiporter by amiloride analogues may affect DA release from central neurons. Opposite effects are observed, depending on the direction of operation of the exchanger. In fact, when the Na^+-Ca^{2+} exchanger operates as a Ca^{2+} efflux pathway, its pharmacological blockade can produce a stimulation of DA release. In contrast, when this antiporter operates as a Ca^{2+} influx pathway, as occurs as a consequence of the inhibition of the Na^+-K^+-ATPase pump by ouabain, its pharmacological blockade can prevent ouabain-induced DA release from TIDA neurons.

It is now generally established that, among the membrane systems that regulate Ca^{2+} transport in neuronal cells, the Na^+-Ca^{2+} exchanger can mediate Ca^{2+} fluxes across the synaptic plasma membrane in a bidirectional way (1, 2). In resting excitable cells, this transport mechanism couples the uphill extrusion of Ca^{2+} to the entrance of Na^+ ions into the cells down their electrochemical gradient. This occurs in order to maintain the 10^4-fold difference in Ca^{2+} concentration that exists between the intracellular and extracellular spaces (3). In contrast, when the transmembrane Na^+ electrochemical gradient is reduced (i.e., increased intracellular Na^+ levels or reduced extracellular Na^+ concentration), the Na^+-Ca^{2+} exchanger, in a reverse manner, operates as a Ca^{2+} influx pathway (4, 5).

Recently, it has been reported that the organic Ca^{2+} entry blocker verapamil (6), the antineoplastic antibiotic doxorubicin (7), the acridine derivative quinacrine (8) and the K^+-sparing diuretic amiloride (9, 10) posses a certain degree of effectiveness in inhibiting the Na^+-Ca^{2+} antiporter. However, the physiolog-

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ABBREVIATIONS: DA, dopamine; TIDA neurons, tuberoinfundibular dopaminergic neurons; KRB buffer, Krebs-Ringer bicarbonate buffer; EGTA, ethylene glycol bis(β-aminoethyl ether)*N,N,N′,N′*-tetraacetic acid; DMSO, dimethyl sulfoxide; DMB, 2′,4′-dimethylbenzamil amiloride; MGCMA, 5-[*N*-methyl-N-(guanidinocarbonylmethyl)]amiloride; α-PB, α-phenylbenzamil amiloride; MAO, monoamine oxidase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

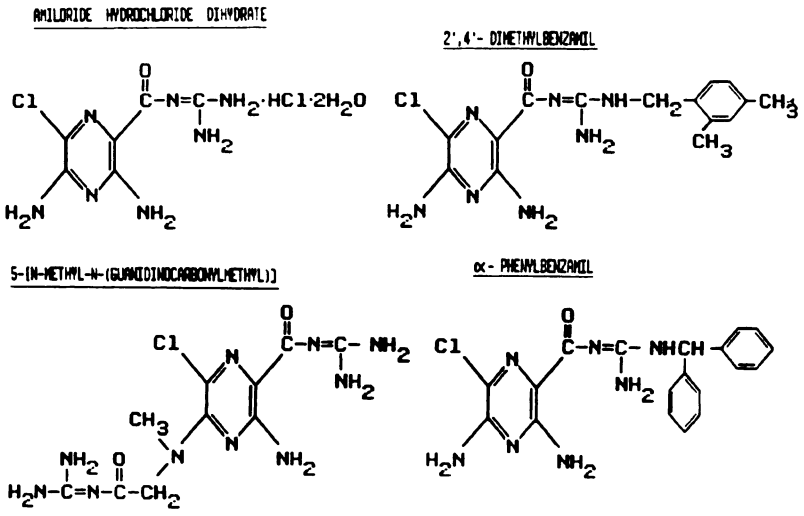

Fig. 1. Structures of amiloride and its analogues used in the present study.

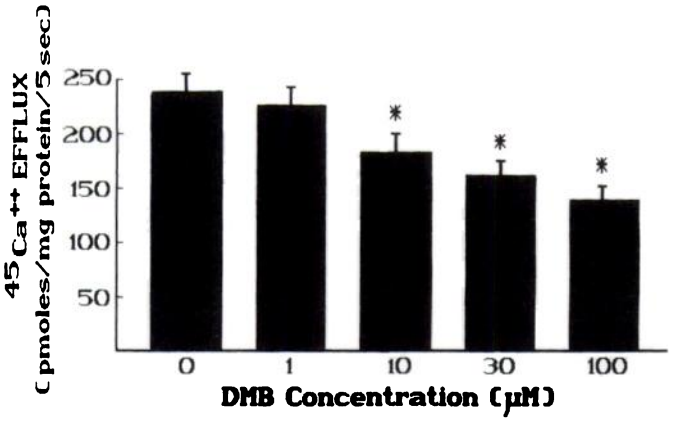

Fig. 2. Effect of different concentrations of DMB on $^{45}Ca^{2+}$ efflux from whole brain synaptosomes into a 145 mM Na^+-containing solution. After a 60-min preincubation period at 37° in a standard medium containing 145 mM Na^+, synaptosomes were loaded with 50 μM $^{45}Ca^{2+}$ in a solution containing (in mM) 95 NaCl, 55 KCl, 1.2 $MgCl_2$, 10 glucose, 10 HEPES, pH 7.4 (100 μl final incubation volume, 37°). After 30 sec, $^{45}Ca^{2+}$ uptake was terminated and $^{45}Ca^{2+}$ efflux was measured for 5 sec by dilution of $^{45}Ca^{2+}$-loaded synaptosomes with 2.5 ml of prewarmed medium containing either 145 mM Na^+ or 145 mM choline. $^{45}Ca^{2+}$ efflux in the control group represents the difference between $^{45}Ca^{2+}$ efflux in the presence of 145 mM Na^+ and 145 mM choline. DMB (or vehicle) was added during the last 30 min of the preincubation phase, the 30 sec of the uptake phase, and the 5 sec of the efflux phase. DMB did not interfere with $^{45}Ca^{2+}$ uptake during the 30 sec of the loading phase (see data of Fig. 8). Data are the mean ± standard error of three separate experiments. In each experiment, at least three samples for each DMB concentration were evaluated. *, Values statistically different ($p < 0.01$) versus their respective control group ($^{45}Ca^{2+}$ efflux in the absence of DMB).

ical role played by this membrane ionic exchanger in the process of neurotransmitter release in the central nervous system has been difficult to evaluate, because of the lack of pharmacological tools that potently and specifically inhibit this Ca^{2+}-transporting system.

In fact, with regard to amiloride as a Na^+-Ca^{2+} exchanger inhibitor, its use is limited by two major inconveniences. First, millimolar concentrations are required for inhibition of the Na^+-Ca^{2+} exchanger (9). Second, it lacks specificity, because it inhibits the epithelial Na^+ channel at micromolar concentrations (11) and the Na^+-H^+ exchanger in the millimolar range (12). This latter system is a major regulator of cytoplasmic pH in neuronal cells (13). More recently, in order to overcome these problems, two classes of amiloride analogues have been developed (Fig. 1). The compounds of one type 1) bear substituents on the terminal guanidino nitrogen atom, 2) behave as specific inhibitors (K_i = 1–10 μM) of the epithelial Na^+ channel and the Na^+-Ca^{2+} antiporter (14–17), and 3) are devoid of any inhibitory effect on the Na^+-N^+ exchange system (14). In contrast, a second class of amiloride derivatives 1) are substituted on the 5-amino nitrogen atom of the pyrazine ring, 2) lack inhibitory properties on the epithelial Na^+ channel and the membrane Na^+-Ca^{2+} exchanger, and 3) display great effectiveness in inhibiting the Na^+-H^+ exchange system (12, 17).

In a previous study, we have shown that amiloride could modulate [^{3}H]DA release from hypothalamic TIDA neurons (18), a neuronal system that is primarily involved in the control of anterior pituitary hormonal secretion (19). The purpose of the present study was to investigate the possible involvement of the Na^+-Ca^{2+} exchange system in the process of [^{3}H]DA release from TIDA neurons. To attain this objective, the effect on [^{3}H]DA release from superfused TIDA neurons of amiloride analogues that selectively inhibited this ionic antiporter was evaluated.

Materials and Methods

Tissue preparation. Fragments of arcuate periventricular nucleus-median eminence complexes, containing, respectively, the cell bodies and the axonal projections of TIDA neurons, were obtained from adult female Wistar rats (200–250 g of body weight). Rats were decapitated and one fragment, weighing approximately 2 mg, was obtained from each brain by dissection of a triangular-shaped area 1-mm wide and 1-mm high, with the help of a stereomicroscope and fine scissors, according to a previously described method (20).

Release of [^{3}H]DA "previously taken-up" from TIDA neurons. After being dissected, the fragments were incubated at 37° in a Dubnoff metabolic shaker in flasks containing 890 μl of KRB buffer, whose composition was (in mM) 118 NaCl, 5 KCl, 1.15 $MgSO_4$, 1.15 NaH_2PO_4, 1.25 $CaCl_2$, 25 $NaHCO_3$, 11 glucose, 0.01 tyrosine, 1 ascorbic acid. The KRB medium was adjusted to pH 7.4 after equilibration with a mixture of 95% O_2/5% CO_2. After 7 min of incubation, 100 μl of KRB plus chlorimipramine (1 μM final concentration) were added to the flasks. This concentration of chlorimipramine is known to inhibit DA uptake into noradrenergic and serotoninergic nerve terminals (21). Five minutes later, the fragments were labeled for 25 min with 0.2 μM [*ethylamine*-1,2-^{3}H]DA hydrochloride (specific activity, 49 Ci/mmol.; The Radiochemical Centre, Amersham).

At the end of the labeling period, one fragment was placed into each

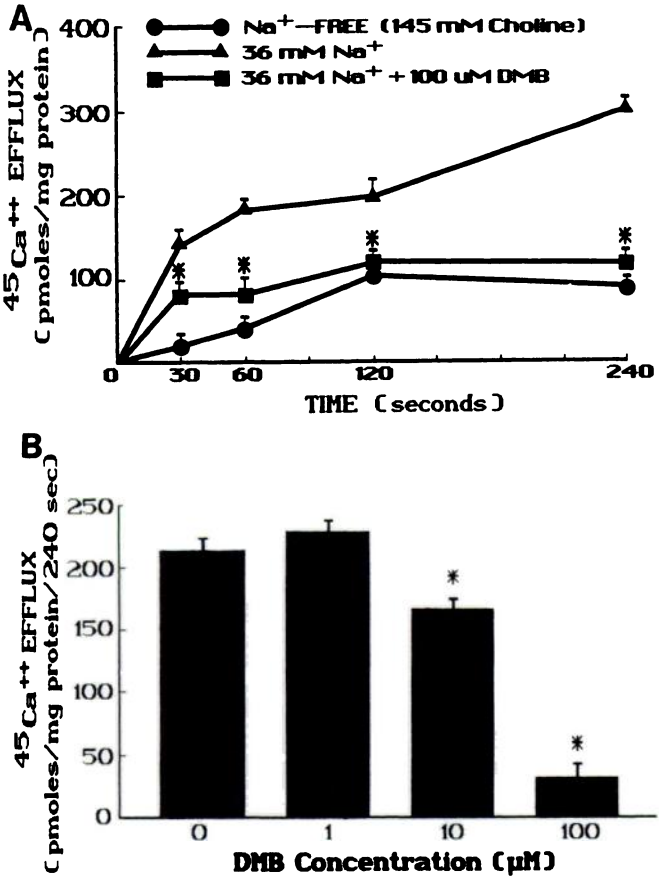

Fig. 3. Time-course and dose dependency of the effects of DMB on $^{45}Ca^{2+}$ efflux from whole brain synaptosomes into a 36 mM Na^+-containing solution. A, In this $^{45}Ca^{2+}$ efflux experiment, the procedure described by Schellenberg *et al.* (27) was followed. Briefly, after a 60-min preincubation period at 37° in a standard medium containing 145 mM Na^+, purified synaptosomes were loaded for 20 min with 2 μM $^{45}Ca^{2+}$ in a solution containing 150 KCl, 1.2 $MgCl_2$, 10 glucose, 10 HEPES, pH 7.4 (200 μl final incubation volume, 23°). $^{45}Ca^{2+}$ uptake was terminated by addition of 5 mM EGTA to the tubes, and $^{45}Ca^{2+}$ efflux was simultaneously initiated by addition (time 0) of 36 mM Na^+ or 36 mM choline to the tubes and determined at the indicated times. DMB (or vehicle) was added at time 0 to the respective group. B, Dose response of DMB obtained after 240 sec of the efflux phase, with the same experimental procedure described in A. Data are the mean ± standard error of three separate experiments. In each experiment, at least three samples for each DMB concentration were evaluated. *, Values statistically different ($p < 0.01$) versus their respective control group ($^{45}Ca^{2+}$ efflux in the absence of DMB).

parallel superfusion chamber (200-μl volume). These fragments were continuously superfused with KRB medium, at 37° and at a flow rate of 500 μl/min, and oxygenated for the whole duration of the experiment with a mixture of 95% O_2/5% CO_2, according to the originally described method (22). The preincubation time was at least 60 min. After the preincubation period, the fragments were superfused with standard KRB medium or with KRB medium containing the compound(s) under investigation. Because in our experimental conditions there is a lag period of 20 min before the medium reaches the superfusion chamber, superfusion media of different composition were changed taking into account the perfusion rate and the dead space. During the experiments, 4-min fractions of eluate were collected into tubes containing, for each 500 μl of eluate, 100 μl of a protective solution (40 mM EGTA, 56.8 mM ascorbic acid, 0.526 mM dopamine), in order to prevent [^{3}H]DA breakdown. The radioactivity present in a 200-μl aliquot of the eluate was counted by standard scintillation techniques for the determination of total ^{3}H radioactivity. The remaining volume of the eluate was subjected to ion exchange chromatography (Biorex-70 resin, 200–400 mesh), in order to separate [^{3}H]DA from its respective ^{3}H-metabolites (23). [^{3}H]DA, [^{3}H]DA metabolites, and total ^{3}H release were expressed as fractional rate/fraction, which represents [^{3}H]DA, [^{3}H]DA metabolites, and total ^{3}H released during each interval expressed as a percentage of [^{3}H]DA, [^{3}H]DA metabolites, and total ^{3}H content in the tissue at the onset of the respective interval. All the compounds were dissolved in DMSO at a concentration of 100 mM and then diluted with KRB to the final desired concentration. The maximal concentration of DMSO in the superfusion medium never exceeded 0.5%, a concentration that did not affect basal [^{3}H]DA release from TIDA neurons.[1]

$^{45}Ca^{2+}$ uptake studies. Synaptosomal fractions derived from whole brains of adult female Wistar rats were prepared according to the procedure described by Dunkley *et al.* (24). $^{45}Ca^{2+}$ uptake into synaptosomes exposed to extracellular media of different ionic compositions was studied according to the protocol of Sanchez-Armass and Blaustein (25), modified as described in the accompanying paper (26). Amiloride analogues were dissolved in DMSO (the final concentration of DMSO in the uptake media of both controls and amiloride analogue groups never exceeded 0.5%, v/v) and incubated for the last 20 min of the preincubation period in a 145 mM Na^+-containing solution. Maximal DMSO concentration (0.5%) did not affect $^{45}Ca^{2+}$ uptake in brain synaptosomes.[1]

$^{45}Ca^{2+}$ efflux studies. After a 60-min preincubation period in a 145 mM Na^+-containing medium, synaptosomes were loaded with $^{45}Ca^{2+}$, following the procedure of either Sanchez-Armass and Blaustein (25) or Schellenberg *et al.* (16), as indicated in the legends to Figs. 2 and 3, respectively. At different times, synaptosomes were filtered and filters were washed and counted as described in the accompanying paper (26).

Protein determination. Synaptosomal protein concentration was determined as described by Bradford (27).

Statistics. Statistical analysis in the experiment on [^{3}H]DA release was performed on the integrated areas under the [^{3}H]DA release curve for control and treated groups, during definite time intervals. These values were compared and analyzed by means of analysis of variance, followed by the Neuman-Keul's test. In the experiments on $^{45}Ca^{2+}$ transport in synaptosomes exposed to media of different ionic composition, $^{45}Ca^{2+}$ influx and efflux values for the different experimental groups were compared and analyzed by means of analysis of variance, followed by the Neuman-Keul's test.

Materials. The amiloride analogues were synthesized by the previously described method (28). Deprenyl was a kind gift of Prof. J. Knoll, (Budapest, Hungary). All other reagents were from commercial sources and of the highest purity available.

Results

Effect of DMB on Na^+-dependent $^{45}Ca^{2+}$ efflux from purified whole brain synaptosomes. DMB is an agent that belongs to the guanidino-substituted class of amiloride derivatives. This compound dose-dependently (10–100 μM) inhibited $^{45}Ca^{2+}$ efflux that occurred after 5 sec into a 145 mM Na^+-containing solution, which reflects the operation of the Na^+-Ca^{2+} antiporter when this system is operating as a Ca^{2+} efflux pathway (Fig. 2). This inhibitory effect of DMB on Na^+-dependent $^{45}Ca^{2+}$ efflux was still more evident when Na^+-dependent $^{45}Ca^{2+}$ efflux was studied at longer intervals (30, 60, 120, and 240 sec) and at increasing drug concentrations (10–100 μM) in an extracellular medium containing 36 mM Na^+ ions (Fig. 3).

Stimulatory effect of DMB on basal [^{3}H]DA release from TIDA neurons as a Ca^{2+}-dependent and DA car-

[1] Unpublished observations.

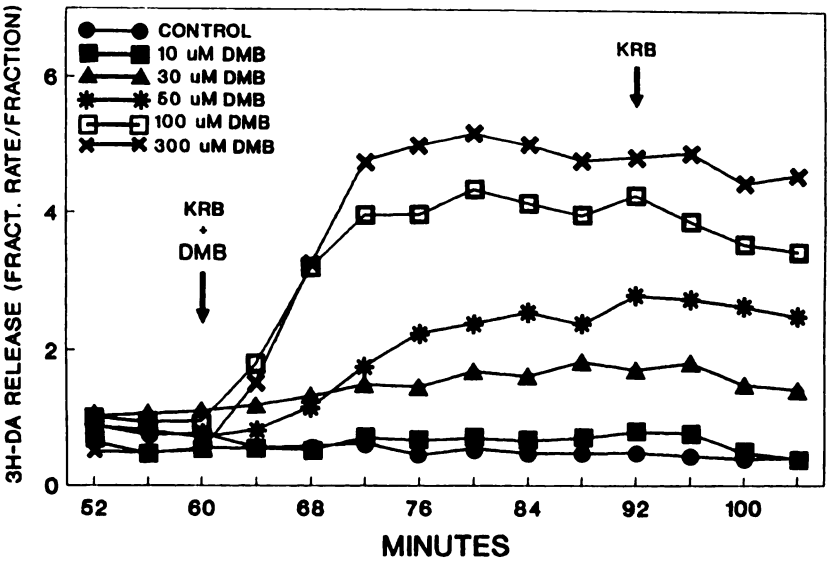

Fig. 4. Effect of DMB on basal [^{3}H]DA release from superfused TIDA neurons. After the labeling period, the hypothalamic fragment containing TIDA neurons was preincubated for 60 min in a standard KRB buffer. After the 60-min preincubation period, DMB (10–300 μM) was added to the superfusion medium, as indicated by the *first arrow*. At the 92nd minute from the beginning of the superfusion, the compound was removed from the KRB superfusing TIDA neurons, as indicated by the *second arrow*. The areas under the [^{3}H]DA release curve from TIDA neurons superfused with 30, 50, 100, and 300 μM DMB were statistically different from that of controls ($p < 0.01$), whereas that under the [^{3}H]DA release curve from TIDA neurons exposed to 10 μM DMB did not show any statistically significant difference versus that of the control group ($p < 0.05$). Each point of the curve represents the mean of four separate determinations. Standard errors (about 15% of the mean value) were omitted for clarity.

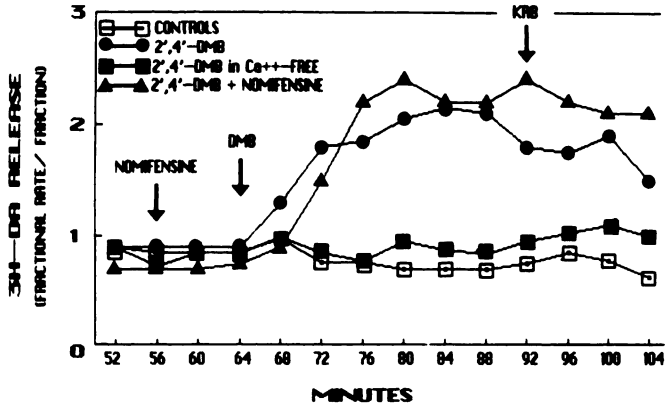

Fig. 5. Effect of a Ca^{2+}-free medium and nomifensine on DMB-induced [^{3}H]DA release from TIDA neurons. The experiment was performed as described in the legend to Fig. 4. In the DMB-Ca^{2+}-free group, the Ca^{2+}-free medium containing 1 mM EGTA was added from the beginning of the superfusion period. All the other drugs were added at the times indicated in the figure. At the 92nd minute from the beginning of the superfusion, all the compounds were removed and TIDA neurons were superfused with a standard KRB medium, as indicated by the *second arrow*. The area under the [^{3}H]DA release curve in the group treated with 100 μM DMB in a Ca^{2+}-free and EGTA-containing medium was statistically different from that of the DMB-treated group superfused in a standard Ca^{2+}-containing KRB ($p < 0.05$), whereas the area under the [^{3}H]DA release curve from TIDA neurons exposed to 100 μM DMB plus 10 μM nomifensine did not show any statistically significant difference versus that of 100 μM DMB treatment alone ($p > 0.05$). Each point of the curve represents the mean of four separate determinations. Standard errors (about 15% of the mean value) were omitted for clarity.

rier-independent phenomenon. When TIDA neurons were superfused with KRB under basal conditions in the presence of the Na^+-Ca^{2+} antiporter inhibitor DMB, a dose-dependent (30–300 μM) stimulation of [^{3}H]DA release occurred (Fig. 4). The time-course and the dose dependency of this effect of DMB were strictly related to the inhibition of Na^+-dependent $^{45}Ca^{2+}$ efflux (see the previous paragraph). In addition, the superfusion of TIDA neurons in the absence of extracellular Ca^{2+} ions, plus the addition of 1 mM levels of the Ca^{2+} chelator EGTA, completely prevented the stimulatory effect exerted by DMB (100 μM) on [^{3}H]DA release (Fig. 5), suggesting, therefore, that the entrance of extracellular Ca^{2+} ions into TIDA neurons is a crucial prerequisite for DMB-induced [^{3}H]DA release.

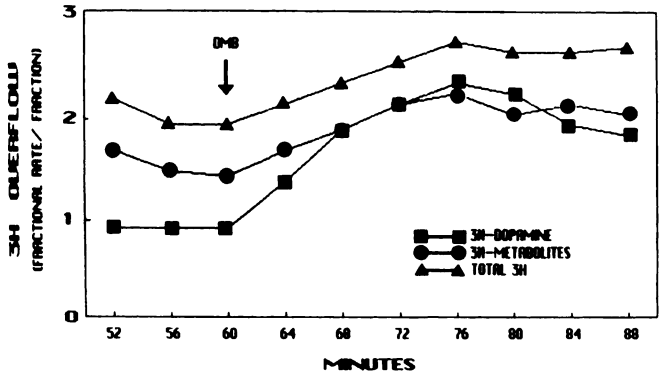

Fig. 6. Effect of DMB on the release of [^{3}H]DA, ^{3}H-metabolites of DA, and total ^{3}H from superfused TIDA neurons. The experiment was performed as described in the legend to Fig. 4. [^{3}H]DA, ^{3}H-metabolites of DA, and total ^{3}H were determined and expressed as reported in Materials and Methods. Each point of the curve represents the mean of four separate determinations. Standard errors (about 15% of the mean value) were omitted for clarity.

TABLE 1

Effects of different MAO inhibitors on [^{3}H]DA release from superfused TIDA neurons

[^{3}H]DA release is expressed as the integrated area under the curve of the fractional rate of the neurotransmitter efflux for the 32 min (60–92 minutes) of exposure of TIDA neurons to KRB (control group) or to KRB plus different MAO inhibitors. Each value is the mean ± standard error of three separate determinations.

Treatment	Dose	[^{3}H]DA release
	μM	
CONTROL		17 ± 1.6
PARGILYNE	10	22.6 ± 1.21
PARGILYNE	100	16.5 ± 3
NIALAMIDE	100	17.6 ± 5
DEPRENYL	100	13 ± 1.2

In contrast, the stimulatory effect exerted by DMB (100 μM) on [^{3}H]DA release was unaffected by the DA transport inhibitor nomifensine (10 μM) (Fig. 5), suggesting that the release of the neurotransmitter evoked by the amiloride analogue does not occur through the DA membrane carrier operating in a reverse manner.

Effect of DMB on the efflux of [^{3}H]DA metabolites and total ^{3}H and of the MAO inhibitors pargiline, nialamide,

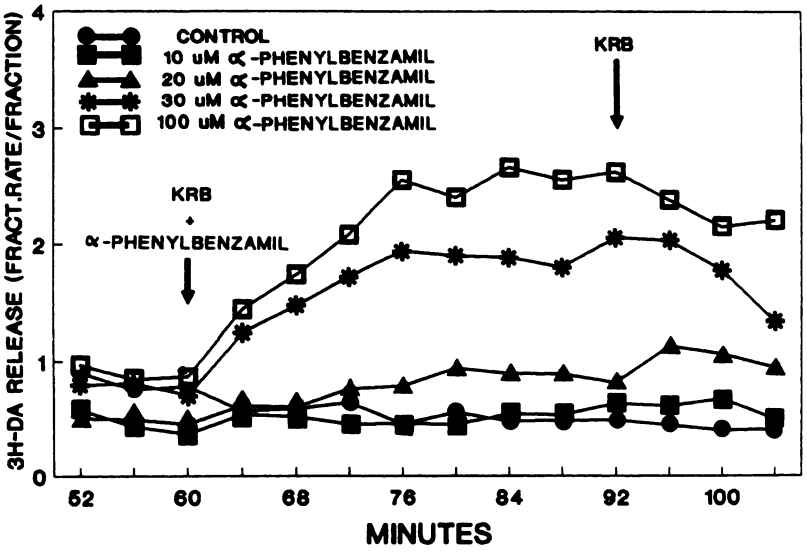

Fig. 7. Effect of α-PB on basal [³H]DA release from superfused TIDA neurons. After the 60-min preincubation period, α-PB (10–100 μM) was added to the superfusion medium, as indicated by the *first arrow*. At the 92nd minute from the beginning of the superfusion, the compound was removed from the KRB medium superfusing TIDA neurons, as indicated by the *second arrow*. The area under the [³H]DA release curve from TIDA neurons superfused with 30 and 100 μM α-PB was statistically different from that of controls ($p < 0.01$), whereas that under the [³H]DA release curve corresponding to 10 and 20 μM α-PB did not show any statistically significant difference versus that of the control group ($p > 0.05$). Each point of the curve represents the mean of four separate determinations. Standard errors (about 15% of the mean value) were omitted for clarity.

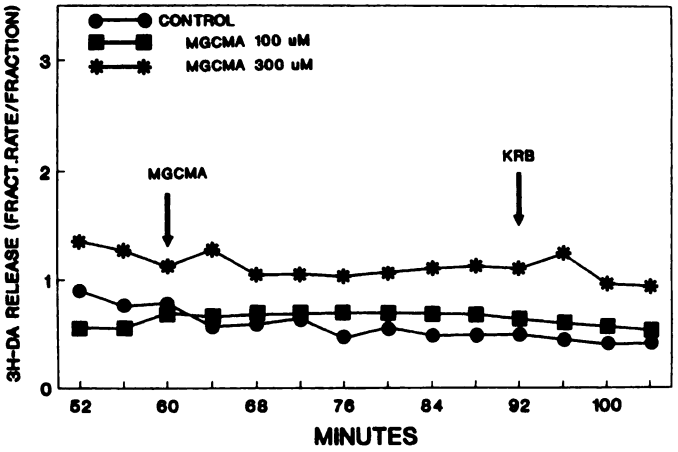

Fig. 8. Effect of MGCMA on [³H]DA release from superfused TIDA neurons. After the 60-min preincubation period, MGCMA (100 and 300 μM) was added to the superfusion medium, as indicated by the *first arrow*. At the 92nd minute from the beginning of the superfusion, the compound was removed from the KRB medium superfusing TIDA neurons, as indicated by the *second arrow*. The area under the [³H]DA release curve from TIDA neurons superfused with 100 and 300 μM MGCMA did not show any statistically significant difference versus that of the control group ($p > 0.05$). Each point of the curve represents the mean of four separate determinations. Standard errors (about 15% of the mean value) were omitted for clarity.

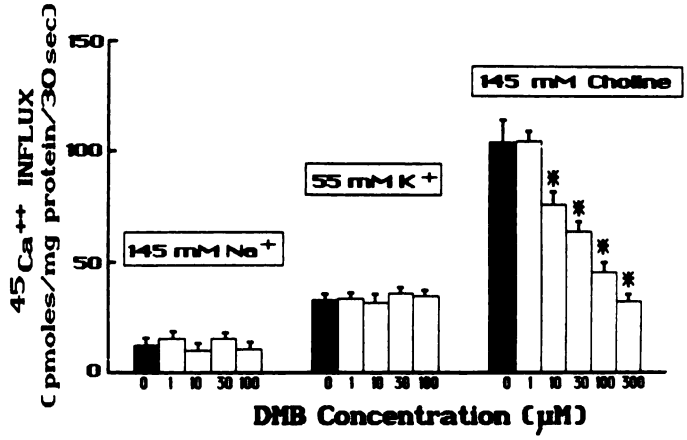

Fig. 9. Effect of DMB on 145 mM choline- and 55 mM K⁺-stimulated $^{45}Ca^{2+}$ uptake in whole brain synaptosomes. Whole brain synaptosomes were preincubated for 60 min in a 145 mM Na^+-containing medium. DMB (0–300 μM) was added to the synaptosomes during the last 20 min of the preincubation period. After the preincubation phase, synaptosomes of each group were exposed to 2 μM $^{45}Ca^{2+}$ in an extracellular medium containing either 145 mM Na^+, 55 mM K^+, or 145 mM choline. After 30 sec, $^{45}Ca^{2+}$ trapped in synaptosomes was determined. Data are the mean ± standard error of three separate experiments. In each experiment, at least three samples for each DMB concentration were evaluated. *, Values statistically different ($p < 0.01$) versus their respective control group ($^{45}Ca^{2+}$ uptake in the absence of DMB).

and deprenyl on [³H]DA release from TIDA neurons. DMB (100 μM), in addition to its stimulatory effect of [³H]DA release, was also able to enhance, although to a lesser exent, the efflux of ³H-metabolites and total ³H from TIDA neurons (Fig. 6).

Furthermore, nialamide and pargyline, two "mixed" MAO-A and MAO-B inhibitors, as well as deprenyl, a selective inhibitor of the MAO-B enzymatic form (29, 30), were ineffective in modifying [³H]DA release from TIDA neurons (Table 1).

Effect of α-PB and MGCMA on basal [³H]DA release from TIDA neurons. When TIDA neurons were superfused under basal conditions with KRB in the presence of α-PB, another amiloride derivative with inhibitory properties on the membrane Na^+-Ca^{2+} antiporter, a dose-dependent (30–100 μM) stimulation of [³H]DA release occurred (Fig. 7).

In contrast, MGCMA, an amiloride derivative that bears substituents on the 5-amino nitrogen atom (12, 31, 32) and possesses a potent inhibitory action on the Na^+-H^+ antiporter[2] even at concentrations of 100–300 μM, which are 100–300 times higher than the IC_{50} value for the inhibition of the Na^+-H^+ antiporter,[2] was unable to modify [³H]DA release from TIDA neurons (Fig. 8). It is interesting to emphasize the fact that this amiloride analogue also failed to affect the Na^+-Ca^{2+} antiporter in cerebral synaptosomes (see Fig. 11).

Effect of DMB on $^{45}Ca^{2+}$ uptake in whole brain synaptosomes induced by 55 mM K^+ or by extracellular Na^+ ion substitution with 145 mM choline. When synaptosomes were preincubated with increasing (10–300 μM) concentrations of DMB, $^{45}Ca^{2+}$ uptake evoked by the removal of extracellular Na^+ ions and their substitution with an equimolar concentra-

[2] Dr. E. J. Cragoe, Jr., personal communication.

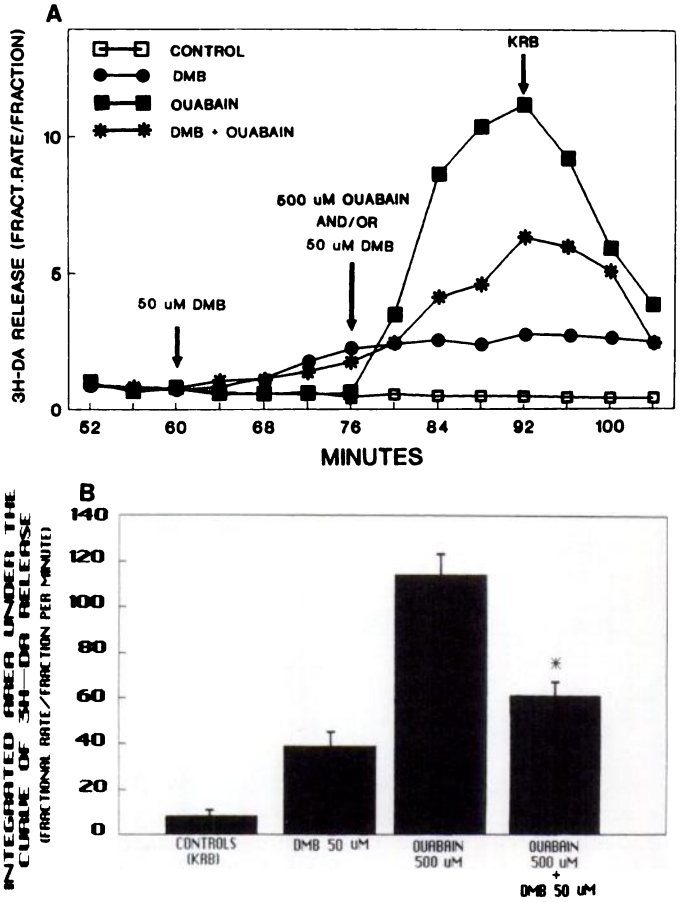

Fig. 10. Effect of DMB on ouabain-induced [^{3}H]DA release from superfused TIDA neurons. After 60 min of preincubation, DMB (50 μM) was added in the superfusion medium of DMB- or DMB- plus ouabain-treated groups. Sixteen minutes later (at the 76th minute from the beginning of the superfusion) ouabain (500 μM) or DMB (50 μM) plus ouabain (500 μM) were added to the superfusion medium of their respective group. The *arrow* at the 92nd min indicates the removal of all the compounds from the KRB buffer superfusing the different groups. Each point of the curve represents the mean of four separate determinations. Standard errors (about 15% of the mean value) were omitted for clarity. B, Same results of A, expressed as integrated areas under the curve of [^{3}H]DA release during the interval from the 76th to the 92nd minute of superfusion. *, Value statistically different versus the ouabain-treated group ($p < 0.01$).

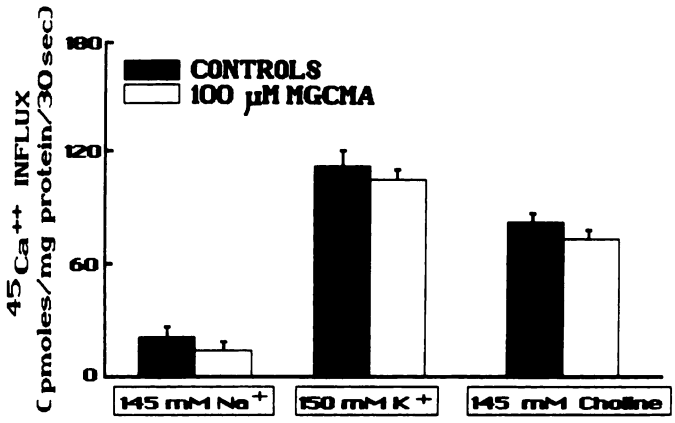

Fig. 11. Effect of MGCMA on 145 mM choline- and 150 mM K^+-stimulated $^{45}Ca^{2+}$ uptake in striatal synaptosomes. Striatal synaptosomes were preincubated for 60 min in a 145 mM Na^+-containing medium. MGCMA (100 μM) was added to the synaptosomes during the last 20 min of the preincubation period. After the preincubation phase, synaptosomes of each group were exposed to 2 μM $^{45}Ca^{2+}$ in an extracellular medium containing either 145 mM Na^+, 150 mM K^+, or 145 mM choline. After 30 sec, $^{45}Ca^{2+}$ trapped in synaptosomes was determined by liquid scintillation counting. Data are the mean ± standard error of three separate experiments. In each experiment, at least three samples for each experimental group were evaluated.

tion of choline was dose-dependently inhibited (Fig. 9). This experimental condition, as has been discussed in the accompanying paper (26), caused an activation of the Na^+-Ca^{2+} antiporter as a Ca^{2+} influx pathway. In contrast, $^{45}Ca^{2+}$ uptake evoked by 55 mM extracellular K^+ ions, which seems to reflect Ca^{2+} ion entrance mainly through voltage-operated Ca^{2+} channels, was completely unaffected by DMB (1–100 μM) (Fig. 9). Similar results on the inhibitory effect of DMB on $^{45}Ca^{2+}$ uptake stimulated by extracellular Na^+ substitution with 145 mM choline were also obtained in experiments performed in synaptosomes obtained from selective dopaminergically innervated brain areas (i.e., striatum, median eminence).[1]

Effect of DMB on ouabain-induced [^{3}H]DA release from TIDA neurons. The cardioactive glycoside ouabain (500 μM), an inhibitor of the Na^+-K^+-ATPase pump (33), stimulated [^{3}H]DA release from TIDA neurons (Fig. 10). However, if the dopaminergic neurons were exposed to ouabain after a preincubation with a concentration of DMB (50 μM) close to the IC_{50} value for the inhibition of Na^+-dependent $^{45}Ca^{2+}$ influx in brain synaptosomes (see Fig. 9), this stimulation was significantly prevented (Fig. 10).

Effect of MGCMA on $^{45}Ca^{2+}$ uptake in striatal synaptosomes induced by 145 mM choline or elevated K^+ concentrations and on ouabain-induced [^{3}H]DA release from TIDA neurons. Preincubation of striatal synaptosomes in the presence of 100 μM MGCMA, the amiloride analogue that inhibits the Na^+-H^+ antiporter (12, 31, 32),[2] failed to affect $^{45}Ca^{2+}$ uptake evoked either by extracellular Na^+ ion removal and substitution with 145 mM choline or by elevated K^+ concentrations (Fig. 11). Furthermore, when TIDA neurons were superfused with MGCMA (100 μM), the release of previously taken-up [^{3}H]DA induced by ouabain (500 μM) was unaffected (Fig. 12).

Discussion

The results of the present study demonstrated that DMB and α-PB, two amiloride analogues that bear substituents on the terminal guanidino nitrogen atom of the parental molecule, specifically and potently inhibited the Na^+-Ca^{2+} exchanger in cerebral nerve endings. The pharmacological blockade of this ionic exchange mechanism can exert a dual modulation of [^{3}H]DA release from a central neuronal system like the TIDA tract. In fact, if the Na^+-Ca^{2+} exchanger was pharmacologically inhibited when the neurons were under basal conditions, a stimulation of basal [^{3}H]DA release occurred. These results can be explained on the basis of the following consideration. In resting nerve cells, the Na^+-Ca^{2+} exchanger favors the extrusion of Ca^{2+} ions from the cytoplasm in order to maintain constant intracellular Ca^{2+} levels (1, 2, 34). In the presence of the two amiloride analogues with great effectiveness in inhibiting the Na^+-Ca^{2+} antiporter, Ca^{2+} efflux mediated by this ionic antiporter would be blocked, leading to an increase in cytosolic Ca^{2+} levels. The increase in intracellular Ca^{2+} concentrations would trigger [^{3}H]DA release from TIDA nerve terminals. This view seems to be supported by the experiments showing that

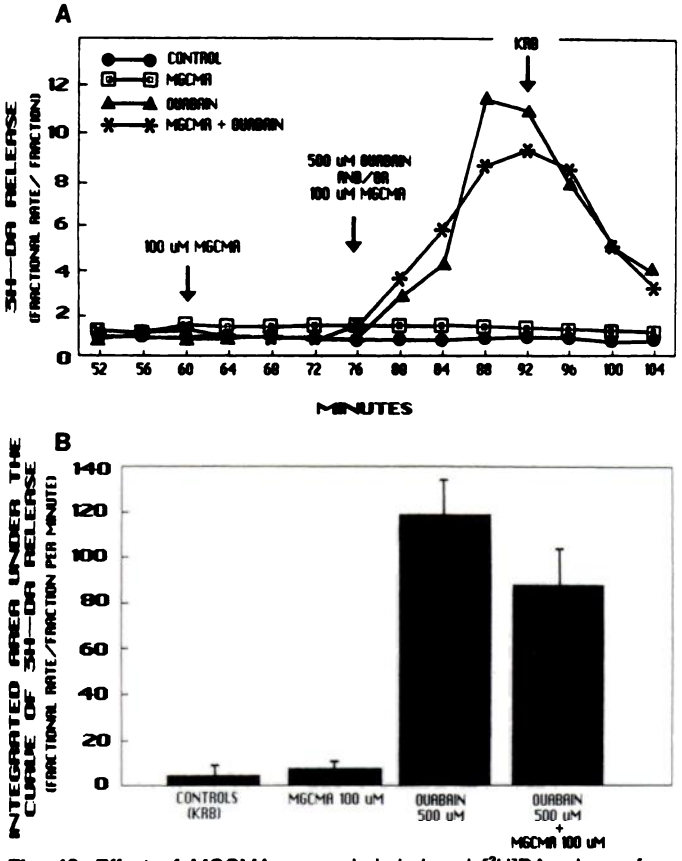

Fig. 12. Effect of MGCMA on ouabain-induced [^{3}H]DA release from superfused TIDA neurons. After 60 min of preincubation, MGCMA (100 μM) was added in the superfusion medium of MGCMA- or of MGCMA (100 μM)- plus ouabain (500 μM)-treated groups. Sixteen minutes later (at the 76th minute from the beginning of the superfusion), ouabain (500 μM) or MGCMA (100 μM) plus ouabain (500 μM) were added to the superfusion medium of their respective group. The *arrow* at the 92nd minute indicates the removal of all the compounds from the KRB buffer superfusing the different groups. Each point of the curve represents the mean of four separate determinations. Standard errors (about 15% of the mean value) were omitted for clarity. B, Same results as in A, expressed as integrated areas under the curve of [^{3}H]DA release during the interval from the 76th to the 92nd minute of superfusion. The area under the [^{3}H]DA release curve from TIDA neurons superfused with ouabain plus MGCMA did not show any statistically significative difference versus that of ouabain alone ($p > 0.05$).

DMB effectively blocked Na^+-dependent $^{45}Ca^{2+}$ efflux in central nerve endings in a concentration range and with a time-course compatible with those required for the stimulation of [^{3}H]DA release from TIDA neurons.

In addition, DMB-induced [^{3}H]DA release was prevented by the removal of extracellular Ca^{2+} ions, suggesting that the entrance of Ca^{2+} from the extracellular space is a crucial prerequisite for the stimulation of basal [^{3}H]DA release. In addition, the hypothesis that DMB, which inhibits the Na^+-Ca^{2+} antiporter, may induce nonexocytotic release of [^{3}H]DA by displacing it from storage vesicles (35) seems not to be supported by the results obtained with the DA transport inhibitor nomifensine. In fact, when nonexocytotic DA release occurs, the membrane DA carrier operates in a reverse fashion, mediating the efflux of the neurotransmitter (21, 36). Therefore, if DMB produces a nonexocytotic DA release, it would be expected that nomifensine would prevent such a phenomenon. However, this seems not to be the case under our experimental conditions, because nomifensine did not prevent DMB-induced [^{3}H]DA release. The observed stimulatory action of DMB on DA release is in accordance with the results obtained by Floreani *et al.* (37, 38) and Siegl *et al.* (39), who investigated another cellular system, i.e., the cardiac muscle. They found that amiloride and 3′,4′-dichlorobenzamil, which potently inhibited the cardiac Na^+-Ca^{2+} exchange system, both displayed positive inotropic effects, because they increase the systolic force of contraction and the diastolic tension.

In addition, it has been recently shown that amiloride can inhibit monoamine catabolism in peripheral catecholaminergic neurons (40). Therefore, the possibility existed that the stimulation of [^{3}H]DA release observed with amiloride derivatives specific for the Na^+-Ca^{2+} exchanger could be ascribed to the reduced intraneuronal catabolism of dopamine. However, this hypothesis is not supported by the results of the present study showing that DMB, in addition to its stimulatory action on [^{3}H]DA release, also enhanced the efflux of ^{3}H-metabolites of DA from TIDA neurons. Furthermore, the fact that previously taken-up [^{3}H]DA release from TIDA neurons was unaffected by the mixed MAO-A and MAO-B inhibitors nialamide and pargyline, as well as by deprenyl, a selective inhibitor of the MAO-B enzymatic form (29, 30), seems to suggest that the inhibition of MAO activity in superfused TIDA neurons does not account for the stimulatory effect exerted by DMB on [^{3}H]DA release.

The relationship between the effectiveness of DMB and α-PB in inhibiting the Na^+-Ca^{2+} antiporter and their ability to modulate DA release from TIDA neurons is further supported by the result obtained with MGCMA. This 5-*N*-substituted derivative of amiloride (12, 31, 32), which has no effect on the Na^+-Ca^{2+} antiporter but inhibits the Na^+-H^+ antiporter,[2] was devoid of any stimulatory effect on basal [^{3}H]DA release from TIDA neurons. These results also suggest that the Na^+-H^+ antiporter seems not to be involved in the regulation of basal DA release from central neurons.

The Na^+-Ca^{2+} exchange system does not only operate as a pathway for the extrusion of intracellular Ca^{2+} ions but can also work in a reverse way (4). In fact, when the transmembrane Na^+ electrochemical gradient is reduced, as it is upon the inhibition of the Na^+-K^+-ATPase pump by ouabain (33), the Na^+-Ca^{2+} exchanger functions as a Ca^{2+} influx pathway. The increase in intracellular Ca^{2+} concentration that follows the inhibition of the Na^+-K^+-ATPase elicits dopamine release (41). In the present experiments, the Na^+-Ca^{2+} exchanger inhibitor DMB, at a concentration close to the IC_{50} value for the inhibition of the Na^+-Ca^{2+} antiporter, was able to cause a 50% inhibition of ouabain-induced [^{3}H]DA release. The ability of DMB to prevent the stimulatory effect on [^{3}H]DA release produced by the inhibition of the Na^+-K^+-ATPase pump can be explained on the basis of the ability of DMB to block Ca^{2+} influx mediated by the Na^+-Ca^{2+} antiporter. Interestingly, an analogous phenomenon has been described for the amiloride-induced reversal of the inotropic and toxic action of ouabain (37).

In contrast, the Na^+-H^+ inhibitor MGCMA, which failed to affect the activity of the Na^+-Ca^{2+} antiporter in central nerve endings and displayed no effect on basal [^{3}H]DA release, was unable to prevent ouabain-induced [^{3}H]DA release from TIDA neurons, suggesting that, also under these conditions, the pharmacological modulation of the activity of the Na^+-H^+ antiporter

does not appear to play a crucial role in neurotransmitter release.

In conclusion, the results of the present experiments suggest that pharmacological inhibition of the Na^+-Ca^{2+} antiporter may affect dopamine release from the central nervous system in opposite ways, depending on whether the Na^+-Ca^{2+} exchanger is operating as a Ca^{2+} efflux pathway, as occurs under resting conditions, or as a Ca^{2+} entrance pathway, as occurs upon the reversal of the transmembrane Na^+ electrochemical gradient.

References

1. Carafoli, E. Intracellular calcium homeostasis. *Annu. Rev. Biochem.* **56**:395–433 (1987).
2. Gill, D. L., E. E. Grollman, and L. D. Kohn. Calcium transport mechanisms in membrane vesicles from guinea-pig synaptosomes. *J. Biol. Chem.* **256**:184–192 (1981).
3. Carafoli, E. The homeostasis of calcium in heart cells. *Mol. Cell. Cardiol.* **17**:203–212 (1985).
4. Blaustein, M. P. The cellular basis for cardiotonic steroid action. *Trends Pharmacol. Sci.* **8**:289–292 (1985).
5. Nachshen, D. A., and M. P. Blaustein. Some properties of potassium-stimulated influx in presynaptic nerve endings. *J. Gen. Physiol.* **76**:709–728 (1980).
6. Erdreich, A., R. Spanier, and H. Rahamimoff. The inhibition of Na-dependent Ca uptake by verapamil in synaptic plasma membrane vesicles. *Eur. J. Pharmacol.* **90**:193–202 (1983).
7. Caroni, P., F. Villani, and E. Carafoli. The cardioactive antibiotic doxorubicin inhibits the Na^+-Ca^{2+} exchange of dog heart sarcolemmal vesicles. *FEBS Lett.* **130**:184–186 (1981).
8. De la Pena, P., and J. P. Reeves. Inhibition and activation of Na^+-Ca^{++} exchange by quinacrine. *Am. J. Physiol.* **252**:C24–C29 (1987).
9. Schellemberg, G. D., L. Anderson, and P. D. Swanson. Inhibition of Na^+-Ca^{2+} exchange from rat brain by amiloride. *Mol. Pharmacol.* **24**:251–258 (1983).
10. Debetto, P., M. Floreani, F. Carpenedo, and S. Luciani. Inhibition of the Na^+-Ca^{2+} exchange in cardiac sarcolemmal vesicles by amiloride. *Life Sci.* **40**:1523–1530 (1987).
11. Garty, H., and D. J. Benos. Characteristics and regulatory mechanisms of the amiloride-blockade by Na^{2+} channel. *Physiol. Rev.* **68**:309–373 (1988).
12. Vigne, P., C. Frelin, E. J. Cragoe, Jr., and M. Lazdunski. Structure-activity relationships of amiloride and certain of its analogues in relation to the blockade of the Na^+-H^+ exchange system. *Mol. Pharmacol.* **25**:131–136 (1984).
13. Nachshen, D. A., and P. Drapeau. The regulation of cytosolic pH in isolated presynaptic nerve terminals from rat brain. *J. Gen. Physiol.* **91**:289–303 (1988).
14. Kaczorowski, G. J., F. Barros, J. K. Demethers, and M. J. Trumble. Inhibition of Na^+-Ca^{2+} exchange in pituitary plasma membrane vesicles by analogues of amiloride. *Biochemistry* **24**:1394–1403 (1985).
15. Siegl, P. K. S., E. J. Cragoe, Jr., M. J. Trumble, and G. J. Kaczorowski. Inhibition of the Na/Ca exchange in membrane vesicles and papillary muscle preparations from guinea-pig hearts by analogues of amiloride. *Proc. Natl. Acad. Sci. USA* **81**:3238–3242 (1984).
16. Schellenberg, G. D., L. Anderson, E. J. Cragoe, Jr., and P. D. Swanson. Inhibition of synaptosomal membrane Na^+-Ca^{2+} exchange transport by amiloride and amiloride analogues. *Mol. Pharmacol.* **27**:537–543 (1985).
17. Kleyman, T. R., and E. J. Cragoe, Jr. Amiloride and its analogs as tools in the study of ion transport. *J. Membr. Biol.* **105**:1–21 (1988).
18. Taglialatela, M., S. Amoroso, L. M. T. Canzoniero, G. F. Di Renzo, and L. Annunziato. Membrane events and ionic processes involved in dopamine release from tuberoinfundibular neurons. II. Effect of the inhibition of the Na^+-Ca^{++} exchange by amiloride. *J. Pharmacol. Exp. Ther.* **246**:689–694 (1988).
19. Annunziato, L. Regulatory mechanisms of tuberohypophyseal dopaminergic neurons, in *Central and Peripheral Regulation of Prolactin Secretion* (R. M. Mac Leod and U. Scapagnini, eds). Raven Press, New York, 59–68 (1980).
20. Cuello, A. C., A. S. Horn, A. V. P. Mackay, and L. L. Iversen. Catecholamines in the median eminence: new evidence for a major noradrenergic input. *Nature (Lond.)* **243**:465–467 (1973).
21. Annunziato, L., F. Cerrito, and M. Raiteri. Characteristics of dopamine release from isolated nerve endings of the tuberoinfundibular neurones. *Neuropharmacology* **20**:727–731 (1981).
22. Beani, L., C. Bianchi, A. Giacomelli, and F. Tamberi. Noradrenaline inhibition of acetylcholine release from guinea-pig brain. *Eur. J. Pharmacol.* **48**:179–193 (1978).
23. Smith, J. E., J. D. Lane, P. A. Shea, W. J. McBride, and M. H. Aprison. A method for concurrent measurement of picomole quantities of acetylcholine, choline, dopamine, norepinephrine, serotonin, 5-hydroxytryptophan, 5-hydroxyindolacetic acid, tryptophan, tyrosine, glycine, aspartate, glutamate, alanine, and *gamma*-aminobutyric acid in single tissue samples from different areas of rat central nervous system. *Anal. Biochem.* **64**:149–169 (1975).
24. Dunkley, P. R., J. W. Heath, S. H. Harrison, P. E. Jarvie, P. E. Glenfield, and J. A. P. Rostas. A rapid Percoll gradient procedure for the isolation of synaptosomes directly from an S_1 fraction: homogeneity and morphology of subcellular fractions. *Brain Res.* **441**:59–71 (1988).
25. Sanchez-Armass, S., and M. P. Blaustein. Role of sodium-calcium exchange in the regulation of intracellular calcium in nerve terminals. *Am. J. Physiol.* **252**:C595–C603 (1987).
26. Taglialatela, M., G. Di Renzo, and L. Annunziato. 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. *Mol. Pharmacol.* **38**:385–392 (1990).
27. Bradford, M. M. A rapid sensitive method for the quantification of microgram quantities of protein utilizing the principle of dye-binding. *Anal. Biochem.* **72**:248–254 (1976).
28. Cragoe, E. J., Jr., O. W. Woltersdorf, J. B. Bicking, S. F. Kwong, and J. H. Jones. Pyrazine diuretics. II. *N*-Amidino-3-amino-5-substituted-6-halopyrazinecarboxamides. *J. Med. Chem.* **10**:66–75 (1967).
29. Knoll, J. Analysis of the pharmacological effects of selective monoamine oxidase inhibitors, in *Monoamine Oxidase and Its Inhibition* (Wolstenholme and Knight, eds.). Elsevier, Amsterdam, 135–161 (1976).
30. Mendlewicz, J., and M. B. H. Youdim. L-Deprenyl, a selective monoamine oxidase type B inhibitor in the treatment of depression: a double blind evaluation. *Br. J. Psychiatry* **142**:508–511 (1983).
31. Baud, L., A. Healy, E. J. Cragoe, Jr., E. J. Goetzl, and C. H. Koo. Leukotriene D_4-induced increase in the cytoplasmic pH of human myelocytic leucocytes. *J. Cell. Physiol.* **136**:355–360 (1988).
32. Frelin, C., P. Barbry, P. Vigne, O. Chassandre, E. J. Cragoe, Jr., and M. Lazdunki. Amiloride and its analogs as tools to inhibit Na^+ transport via the Na^+ channel, the Na^+/H^+ antiport and the Na^+-Ca^{++} exchanger. *Biochemie* **70**:1285–1290 (1988).
33. Repke, K. R. H., and W. Shonfeld. Na^+/K^+-ATPase as the digitalis receptor. *Trends Pharmacol. Sci.* **5**:393–397 (1984).
34. Carafoli, E. Calcium-transporting systems of plasma membrane, with special attention to their regulation. *Adv. Cyclic Nucleotide Protein Phosphorylation.* 543–549 (1984).
35. Zubieta, J. K., E. J. Cragoe, Jr., and L. X. Cubeddu. Effects of amiloride and 5-(*N*,*N*-hexamethylene)amiloride on dopaminergic and cholinergic neurotransmission. *J. Pharmacol. Exp. Ther.* **247**:88–95 (1988).
36. Raiteri, M., F. Cerrito, A. M. Cervoni, and G. Levi. Dopamine can be released by two mechanisms differentially affected by the dopamine transport inhibitor nomifensine. *J. Pharmacol. Exp. Ther.* **208**:195–202 (1979).
37. Floreani, M., and S. Luciani. Amiloride: relationship between cardiac effects and inhibition of Na^+-Ca^{++} exchange. *Eur. J. Pharmacol.* **105**:317–322 (1984).
38. Floreani, M., M. Tessari, P. Debetto, S. Luciani, and F. Carpenedo. Effects of *N*-chlorobenzyl analogues of amiloride on myocardial contractility, Na-Ca-exchange carrier and other cardiac enzymatic activities. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **336**:661–669 (1987).
39. Siegl, P. K. S., E. J. Cragoe, Jr., M. J. Trumble, and G. J. Kaczorowski. Inhibition of Na^+-Ca^{2+} exchange in membrane vesicle and papillary muscle preparations from guinea pig heart by analogs of amiloride. *Proc. Natl. Acad. Sci. USA* **81**:3238–3242 (1984).
40. Palaty, V. Inhibition of monoamine oxidase by amiloride. *Can. J. Physiol. Pharmacol.* **63**:1586–1589 (1985).
41. Taglialatela, M., S. Amoroso, G. Kaparos, F. Maurano, G. F. Di Renzo, and L. Annunziato. Membrane events and ionic processes involved in dopamine release from tuberoinfundibular neurons. I. Effect of the inhibition of the Na^+, K^+-adenosine triphosphatase pump by ouabain. *J. Pharmacol. Exp. Ther.* **246**:682–688 (1988).

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