The coupling between depolarization-induced calcium entry and neurotransmitter release was studied in rat brain neurons in culture. The endogenous dopamine content of the cells was determined by high performance liquid chromatography utilizing electrochemical detection. The amount of dopamine in unstimulated cells was found to be about 16 ng/mg of protein. Depolarization of the neurons by elevated K+ caused a Ca2+-dependent release of dopamine from the cells. Following 1 min of depolarization, the cellular dopamine content and the amount of [3H]dopamine in cells preloaded with the radioactive transmitter were reduced by 35%.

The release of [3H]dopamine by the neurons was measured at 1.5–6-s intervals by a novel rapid dipping technique. Depolarization in the presence of Ca2+ (1.8 mM) enhanced the rate of neurotransmitter release by 90-fold (0.072 ± 0.003 s⁻¹) over the basal release in the presence of Ca2+. The evoked release consisted of a major rapidly terminating phase (tₚ₀ = 9.8 s) which comprised about 40% of the neurotransmitter content of the cells and a subsequent slower efflux (tₑₓ = 575 s) which was observed during following prolonged depolarization. Predepolarization of the cells in the absence of extracellular Ca2+ did not affect the kinetics of the evoked release. The fast evoked release could be re-elicted in the cells after 20 min "rest" in reference low K+ buffer.

The effects of varying the extracellular Ca2+ concentrations on the kinetic parameters of the evoked release were measured. The amount of neurotransmitter released during the fast kinetic phase was very sensitive to the external Ca2+ (from 0% in the absence of Ca2+ to 40% of the neurotransmitter content at Ca2+ 0.3 mM). The rate constant of the fast release did not depend on the extracellular Ca2+, whereas the rate constant of the slow release increased from 0.0004 ± 0.00001 s⁻¹ at 0.4 mM Ca2+ to 0.0012 ± 0.0002 s⁻¹ at 0.8 mM Ca2+.

The fast evoked release was inhibited by verapamil in a concentration-dependent manner. By contrast, verapamil enhanced the basal and the slow release independent of the presence of Ca2+. Both fast and slow phases of the evoked release were blocked by Ca2+. Addition of Ca2+ within the first 6 s after the onset of depolarization inhibited the fast release but failed to do so when added later on. The results indicate that the amount of neurotransmitter which is released during the fast phase is determined by the amount of Ca2+ entering the cells during the initial stages of stimulation and that subsequently Ca2+ is responsible for the cessation of neurotransmitter release. The possibility that inactivation of the voltage-dependent Ca2+ channel limits the fast phase of neurotransmitter release is discussed.

Entry of Ca2+ into nerve terminals triggers the depolarization-induced release of neurotransmitters (1, 2). Studies on isolated nerve ending preparations from mammalian brain indicate that depolarization concentrations of K+ increase Ca2+ permeability and, consequently, transmitter release (3–5). The small size of the terminals and the complexity of the mammalian central nervous system restrict the possibilities to study the depolarization-release coupling in intact brain neurons. Primary cultures of brain neurons may be ideally suited for studies on the coupling between the depolarization-induced Ca2+ entry and neurotransmitter release. We have previously shown that embryonic rat brain neurons can grow and mature in tissue culture (6, 7). The mature cells are able to accumulate [3H]dopamine in a sodium-dependent benztpine-inhibited process and to release prelabeled [3H]dopamine in a calcium-dependent process which can be evoked by high K+ or electrical field stimulation (9). We present evidence here that these cells also synthesize and metabolize dopamine and in quantities comparable to those of rat brain. In addition the endogenous dopamine is also released upon depolarization.

Our previous studies on the release of dopamine from brain neurons were performed by incubating the cells with depolarizing buffers for 20 min. The rate of transmitter release observed upon depolarization in the presence of extracellular Ca2+ was 3–4 times higher than the basal release without stimulation (6). In order to approximate the physiological time more closely, we have developed a rapid technique to study dopamine release in 1–2-s intervals. We present evidence here that the rate of the evoked release observed using this technique may approach physiologically expected rates.

It has been noted in several studies of evoked neurotransmitter release in brain synaptosomes that, despite maintained depolarization, the K+-induced release of various transmitters exhibited a rapid temporal decay (4, 5). Prolonged (20 min) depolarization of the brain neurons with high K+ also induced the release of only 30–40% of the neurotransmitter content (6). This unexplained phenomenon could be the consequence of depletion of the releasable transmitter pool. It could also result from cessation of transmitter release subsequent to

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† To whom correspondence should be addressed.
inactivation of voltage-sensitive Ca²⁺ channels as proposed for synaptosomes (4, 5) or from inactivation of an intracellular component involved in the secretory mechanism. In the accompanying paper (17), we have studied the time course of Ca²⁺ influx and efflux in stimulated brain neurons. The results indicate that the entry of Ca²⁺ through voltage-sensitive calcium channels, which may be inhibited by verapamil and Co²⁺, could account for the observed calcium fluxes. In the present study, the temporal correlation between depolarization-evoked calcium entry and the quantity and kinetics of neurotransmitter release in intact brain neurons was investigated. The results indicate that the Ca²⁺ entry during the initial stages of stimulation activates neurosecretion and limits the amount of the rapidly released neurotransmitter.

MATERIALS AND METHODS

Cell Culture—Whole brains of 16-day-old rat embryos were dissociated and cultured on plastic Petri dishes precoated with poly-L-lysine (Sigma) (10). The cells were grown in serum-free medium consisting of Eagle’s minimal essential medium (Gibco), 60% glucose, 2% antibiotics. The cells were kept in culture up to 30 days in 100% humidity and 5% CO₂. As we have previously shown, under these conditions the cells mature during the first 10–12 days in culture (7). Therefore, the cells used in the present study were grown for 15–18 days prior to the experiments.

Analysis of Endogenous Dopamine in Neurons—The endogenous dopamine content of the neurons was measured by a high performance liquid chromatography system (LKB Sweden) utilizing a 30 cm × 3.9 mm (inner diameter) C-18 Bondapak reverse phase column coupled to an electrochemical detector (TL-4 Amperometric Detector Bioanalytical Systems, West Lafayette, IN) (LCBC®) as described by Nielsen and Johnston (11).

The cells, still attached to the Petri dishes, were rapidly washed twice with 1 ml of calcium-free reference buffer, pH 7.4 (consisting of 123 mM NaCl, 3 mM KCl, 0.5 mM NaH₂PO₄, 0.25 mM Na₂HPO₄, 0.4 mM MgCl₂, and 1 mg/ml glucose) and incubated for 1 min at 37 °C in reference buffer or in depolarizing high K⁺ buffer (same as reference buffer except that the K⁺ concentration was increased to 43 mM and the Na⁺ concentration was reduced to 83 mM to maintain isoosmolarity of the buffer). Both buffers contained Ca²⁺ (1.8 mM). Following the wash the buffers were removed, and 0.3 ml of the mobile phase (consisting of 0.1 M citrate-phosphate buffer, pH 3.6, 10% (v/v) methanol, and 0.04% (w/v) sodium octyl sulfate (Bioanalytical Systems) was added. The cells were removed from the plates with a rubber policeman and transferred to a Teflon-glass homogenizer. The protein content of the pellets was analyzed by a modified Lowry method (12). The dopamine, 5-hydroxytryptamine, their precursors, and metabolites. Standards of known amounts of dopamine, 5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA), and 3,4-dihydroxyphenylacetic acid (DOPAC) (all from Sigma) were dissolved in the mobile phase and analyzed in parallel. The amounts of biogenic amines and their metabolites in each sample were determined by measuring the areas under the peaks obtained from the chromatograms. The retention times of the cell components relative to the authentic standards were found to be similar in both mobile phases.

Kinetics Studies of [³H]Dopamine Release—The cultures were incubated with 250 mM [³H]dopamine (9.9 Ci/mmol, New England Nuclear) in culture medium at 37 °C for 60 min. The incubation medium was then removed and the cells washed four times with calcium-free reference buffer and incubated with reference buffer for 3 × 20 min. The side walls of the Petri dishes were then removed and Co²⁺ (1.5 mM) was present in all the chambers containing the depolarizing buffer. In different sets of experiments the cells were incubated for 4 min with the desired concentration of verapamil or tetrodotoxin in 1 ml of reference buffer containing Ca²⁺ (1.8 mM). The amount of [³H]dopamine released from the cells was determined during repeated incubations of the cells for 1 min at 37 °C in reference or in depolarizing buffers containing verapamil or tetrodotoxin as the depolarizing buffers.

In the studies of the effect of Co²⁺ on the kinetic parameters of the neurotransmitter release the rapid dipping technique was employed as described above. Co²⁺ (1.5 mM) was present in all the chambers containing the depolarizing buffer. In different sets of experiments Co²⁺ was omitted from the first one, first two, first three, or first nine chambers containing the depolarizing solution and Ca²⁺ (0.72 mM).

Effect of Predipolarization on Neurotransmitter Release—The cells, preloaded with [³H]dopamine and washed for 60 min, were transferred while still attached to the plastic plate as described above to a chamber containing 1 ml of Ca²⁺-free depolarizing solution and incubated at 37 °C for different time intervals (10–120 s). At the end of each depolarization period the cells were dipolarized to the presence of Ca²⁺ (1.8 mM) in the depolarizing solution. The kinetics of the release of [³H]dopamine from the prepolarized cells was then measured by the rapid dipping technique described above.

Expression of the Results and Calculations of the Kinetic Parameters—The sum of all the radioactivity in each chamber (fraction) plus the radioactivity left in the cells at the end of the experiment was taken as the total radioactivity present in the cells at the beginning of the experiment. The radioactivity remaining in the cells at each time point was obtained by subtracting that released in each fraction. The radioactivity released from the cells at each time point was obtained by summing up the radioactivity in the individual chambers. The amount of radioactivity remaining in the cells at various times and the amount of neurotransmitter released into the depolarizing buffers during the depolarization period were expressed as a per cent of the initial radioactivity and analyzed as a sum of exponential terms using the following equations for the kinetic components of the efflux.

$$[³H]\text{dopamine remaining in the cells} (\%) = \sum \left(1 - e^{-kt} \right)$$

$$[³H]\text{dopamine released from the cells} (\%) = \sum \left(1 - e^{-kt} \right)$$

where $K$ and $h$ are constants related to the size and rate of individual releasable [³H]dopamine pools within the neurons. The half-time of release ($t_{1/2}$) of each pool is equal to In(2)/$K$. A good fit of the observed data was obtained using two exponential terms. The apparent rate constants and compartment sizes were obtained from the equa-
by graphical methods of compartmental analysis for unidirectional fluxes as described (13). To assess goodness of fit of the derived theoretical curves were reconstructed by a microcomputer and superimposed on the experimental points as shown under “Results.” The criteria used for the best fit were: 1) a minimum sum of deviation squares was reached (typically, <0.05); 2) the calculated values did not deviate systematically from the experimental data.

RESULTS

Measurement of Endogenous Neurotransmitters and Metabolites in the Brain Neurons—Extracts of the cultured brain neurons under nonstimulating conditions contained several components which co-chromatographed in high performance liquid chromatography using two different mobile phase compositions, with authentic dopamine, DOPAC, 5-HT, and 5-HIAA (Fig. 1). Since HVA was not detectable in the cells under the conditions employed, it was used as an internal standard (Fig. 1). The major components were dopamine (16.3 ng/mg of protein) and serotonin (5.7 ng/mg of protein) (Table I). An additional major peak chromatographed very close to the solvent front. This peak could represent norepinephrine and its major metabolite 3-methoxy-4-hydroxyphenylethanolenglycol, both of which chromatograph very close to the mobile phase front in the chromatography systems used.

Stimulation of the brain neurons by depolarization with high K+ in the presence of Ca2+ (1.8 mM) for 1 min significantly reduced the cellular content of dopamine and serotonin (by about 35%) (Fig. 1, Table I). It should be noted that the depolarization-induced reduction in the endogenous neurotransmitter content correlated very well with the release of [3H]dopamine from preloaded cells (as is shown in the present study). Prolonged depolarization (5 min) did not further reduce the content of the amines and metabolites in the cells (data not shown).

The Kinetics of Neurotransmitter Release—In order to approximate the time course of neurotransmitter release more closely, we examined the effect of length of the time intervals between the transfers of the cells in the serial chambers on the kinetics of the efflux of [3H]dopamine from the depolarized neurons (Fig. 2). Since the first chambers in each set contained nondepolarizing buffer, the amount of basal release of neurotransmitters from the cells was determined under experimental conditions identical to those of the evoked release. In unstimulated cells the rate of efflux of radioactivity in the absence of Ca2+ was found to be 0.0004 ± 0.0001 s−1. The rate of efflux from the unstimulated cells increased in the presence of Ca2+ (0.0008 ± 0.0001 s−1).

Depolarization of the cells in the presence of Ca2+ enhanced the release of [3H]dopamine from the cells (Fig. 2, upper panels). However, the evoked transmitter efflux decayed afterwards, in spite of the maintained depolarization. The amount of transmitter released reached a maximal value in the fourth chamber (6 s after the beginning of depolarization) when 1.5-s or 2-s intervals were used, and it reached a maximal value in the third, second, and first chamber when collection intervals were 3, 6, and 30 s, respectively (Fig. 2, upper panels). The total amount of neurotransmitter released was not affected by the choice of time intervals, and at longer time intervals, the maximal release per fraction increased (Fig. 2, upper panels). Thus, the shorter time intervals enabled a better resolution of the kinetics of the evoked release. The best fit analysis of the data obtained with a 2-s interval experiment defined a rapidly releasable dopamine pool with a half-time of 9.6 s and a slowly released pool with a t½ of 577 s. The apparent pool sizes were 40% of the total for the fast and 60% of the total for the slow component (Table II). Similar analysis of the data obtained with shorter intervals (1.5 s (Fig. 2, upper panels) or a 6-s interval (Fig. 2, lower panels)) gave very similar results. The pool size of the fast component in these experiments varied between 40 and 45% of the total neurotransmitter content. The data obtained with 30-s intervals was also in good agreement with this kinetic description of the neurotransmitter release, although this interval was too long to enable the kinetic measurements of the fast release (Fig. 2, lower panel, Table II).

Thus, the shorter intervals were appropriate for measurements of the actual rate constants of the fast component of the evoked release from the neurons, whereas the longer intervals were appropriate for measuring the rate constants of the slow phase. The rate coefficient of the fast release in brain neurons (0.072 s−1) is 2–3 orders of magnitude greater than previously reported for synaptosomes using continuous superfusion techniques (14) and is very close to the values recently reported for the initial release of [3H]dopamine from striatal synaptosomes (0.067 s−1) (4) and for the rapidly terminating release of [3H]acetylcholine from rat brain synaptosomes (0.039 s−1) (5). The fraction of the rapidly terminating phase from the total neurotransmitter store in the depolarized neurons was also in very good agreement with the recently reported value for the K+-evoked release of [3H]acetylcholine from rat brain synaptosomes (5). The kinetic model used in our studies assumes parallel release by the

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**FIG. 1.** LCEC separation of extracts from cultured brain neurons. Cells were incubated in reference (open chromatogram) or in depolarizing (filled chromatogram) buffers for 1 min and then homogenized in the mobile phase (containing 0.1 M citrate-phosphate, pH 3.6, 10% methanol, and 0.04% sodium octyl sulfate). Two-thirds of the supernatant obtained after centrifugation of the homogenate of each culture dish were used. The peaks which were identified as dopamine (DA), DOPAC, 5-HT, and 5-HIAA are indicated. HVA (1 ng/dish) served as an internal standard.

**TABLE I**

<table>
<thead>
<tr>
<th>Neurotransmitter</th>
<th>Dopamine (ng/mg protein)</th>
<th>Serotonin (ng/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated cells</td>
<td>16.3 ± 1.1</td>
<td>5.7 ± 0.5</td>
</tr>
<tr>
<td>Depolarized cells</td>
<td>10.5 ± 1.3</td>
<td>3.4 ± 0.5</td>
</tr>
<tr>
<td>% released</td>
<td>35 ± 5</td>
<td>40 ± 6</td>
</tr>
</tbody>
</table>
"fast" and "slow" processes. Since the rate constant of the fast component is about 60 times larger than the rate constant of the slow component, the results of the compartmental analysis may also fit a series model.

Effects of Changing the Extracellular Ca2+ Concentration—
In order to further characterize the evoked transmitter release, we examined the effect of varying extracellular Ca2+ concentration (0–1.8 mM) on the kinetic parameters of the evoked processes (Fig. 3). In the absence of extracellular Ca2+, no depolarization-enhanced efflux of [3H]dopamine from the cells was observed (as is shown later on, in Fig. 5). The rate of [3H]dopamine release from the slowly released pool of the depolarized neurons was dependent upon the extracellular Ca2+ concentration (Fig. 3A). This rate constant increased from 0.0004 s⁻¹ at low extracellular Ca2+ (e.g. less than 0.4 mM) into 0.0012 s⁻¹ at Ca2+ above 1.0 mM (Table II). On the other hand, the rate constant of the release from the fast component was unaffected by the change in the extracellular Ca2+ concentration (Fig. 3B, Table II).

A markedly different response to changes in extracellular Ca2+ was observed in the size of the releasable pool of the fast component; the size of the fast pool increased from 0 in the absence of Ca2+ to about 43% of the neurotransmitter content of the cells at 0.3 mM Ca2+ (Fig. 3B, Table II). Above 0.3 mM Ca2+ the pool size was relatively unaffected by changes in extracellular Ca2+. The concentration of extracellular Ca2+ needed to increase the fast releasable pool size to half of its maximal value was 0.11 mM (Fig. 3B, inset).

Effects of Verapamil and Tetrodotoxin on [3H]Dopamine Release—The dependence of the kinetic parameters of neurotransmitter release on extracellular Ca2+ indicated that Ca2+ entry into the cells may be involved. Our studies of the depolarization-induced Ca2+ fluxes in these cells (17) implicated the voltage-dependent Ca2+ channel as the main component in the stimulated Ca2+ influx. We, therefore, studied the effects of the Ca2+ channel blocker, verapamil, and of the Na+ channel blocker, tetrodotoxin, on the evoked neurotransmitter release. Verapamil (1–100 μM) inhibited the fast component of release in a concentration-dependent manner (Fig. 4). The concentration of verapamil which caused 50% inhibition of the fast release was 10 μM (Fig. 4). The concentration dependency of the inhibition by verapamil of the fast stimulated release of [3H]dopamine was closely related to its inhibition of depolarization-induced Ca2+ entry into the neurons (17).

In addition, verapamil affected the rate of the slow efflux of [3H]dopamine from the depolarized cells (Fig. 4). In this case, increasing the verapamil concentration from 10 to 100 μM enhanced the slow efflux by 4-fold. Verapamil also enhanced the efflux of [3H]dopamine from the unstimulated cells (Fig. 4, the fractions collected prior to the depolarization). The latter effect was, however, independent of the external Ca2+.

Neither the kinetic parameters nor the amount of [3H]
Kinetics of Neurotransmitter Release

The cells were preloaded with [3H]dopamine and washed. The cells were then stimulated by dipping in a series of chambers containing depolarizing buffer and Ca2+. The cumulative amounts of [3H]dopamine released into the different chambers as a function of the time since the beginning of depolarization were analyzed by a sum of exponential terms using the following equation: % release = R1(1 - e^(-k1t)) + R2(1 - e^(-k2t)), where R1 and R2 are the different pool sizes in percent of the total cell content of [3H]dopamine, and k1 and k2 are the rate constants of the same pools, respectively. In this table, the data of representative experiments presented in Figs. 2, 3, and 5 were used. All experiments were performed three times. The average deviations of the calculated values of R1, R2, and those of k1, k2 were 3%.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Fast component</th>
<th>Slow component</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R1 (mM)</td>
<td>k1 (s^-1)</td>
</tr>
<tr>
<td>Interval*</td>
<td>s</td>
<td>s</td>
</tr>
<tr>
<td>2 s</td>
<td>43</td>
<td>0.072</td>
</tr>
<tr>
<td>6 s</td>
<td>40</td>
<td>0.072</td>
</tr>
<tr>
<td>30 s</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>Cobalt†</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>0.1 mM</td>
<td>24</td>
<td>0.072</td>
</tr>
<tr>
<td>0.2 mM</td>
<td>30</td>
<td>0.072</td>
</tr>
<tr>
<td>0.3 mM</td>
<td>40</td>
<td>0.072</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>40</td>
<td>0.072</td>
</tr>
<tr>
<td>0.8 mM</td>
<td>45</td>
<td>0.072</td>
</tr>
<tr>
<td>1.8 mM</td>
<td>45</td>
<td>0.072</td>
</tr>
<tr>
<td>Without cobalt</td>
<td>42</td>
<td>0.072</td>
</tr>
</tbody>
</table>

*The time intervals between the sequential dip intervals varied between 2 and 30 s. The depolarizing buffer contained 1.8 mM Ca2+.
†The Ca2+ concentration in the depolarizing buffer varied between 0-1.8 mM. The dipping interval was 2 s.
‡At different time points after the beginning of depolarization and thereafter, Co2+ (1.5 mM) was present in the depolarizing buffer. The depolarizing buffer contained 0.72 mM Ca2+. Dipping intervals were 2 s.

DISCUSSION

The Content of Endogenous Neurotransmitters in the Cultured Neurons.—We have previously shown that about 20% of the mature cultured rat brain neurons exhibit high affinity uptake of [3H]dopamine. The accumulation of the radioactive neurotransmitter was sodium dependent and was inhibited by benztpoline, a specific inhibitor of the catecholamine uptake system (6). To date, the content of the endogenous neurotransmitters in the primary cultures of the rat brain neurons have not been reported. Our measurements of the content of biogenic amines in the cultured neurons indicate that they contain endogenous dopamine and serotonin, as well as their metabolites DOPAC and 5-HIAA. The amount of dopamine in the cells (16.3 ± 1.1 ng/mg of protein) is higher than that reported to be present in adult rat brain hypothalamus (6.6 ± 0.3 ng/mg of protein) and is lower than the concentration of dopamine in rat brain striatum (64.9 ± 5.4 ng/mg of protein) (11). The amount of serotonin in the cells (6.7 ± 0.5 ng/mg of protein) is similar to the values reported for adult rat brain hypothalamus or striatum (4.90 ± 0.4 and 6.9 ± 0.5 ng/mg of protein, respectively) (11). Hence, the endogenous neuro-
transmitters content of the cultured neurons may reflect their abundance in the rat brain.

Stimulation of the cultured cells with high K⁺ in the presence of Ca²⁺ reduced the amount of dopamine and serotonin in the cells by about 35%. However, it did not significantly affect the cellular contents of their metabolites. The reduction in the neurotransmitter content was very similar to the values predicted from studies of the release of radioactive dopamine (e.g., the fast evoked release of [³H]dopamine during 1 min of stimulation in the presence of 1.8 mM Ca²⁺ was about 40% of the content of [³H]dopamine in the cells). The release of the radioactive neurotransmitter could, therefore, serve as a probe for the release of the endogenous neurotransmitter by the stimulated neurons.

**Kinetic Parameters of Stimulated Dopamine Release: Are They Physiologically Significant?**—In the present report we describe a rapid technique which enables the measurement of dopamine release from cultured brain neurons over intervals as short as 1 s. Analysis of the time course of the release of [³H]dopamine by the depolarized brain cells indicated that the Ca²⁺-dependent release behaves as if the neurotransmitter was located in at least two kinetically distinct pools, one which undergoes fast release, and another, whose release is 60-fold slower. The rate constant of the fast component of the evoked release as determined in our experiments was 0.072 s⁻¹ (Fig. 2, Table II). This suggests that the neurotransmitter present at a certain moment in this pool is released at a rate of 7.2%/s. To be physiologically relevant, this rate constant should account for the transmitter release following presynaptic action potentials, where it is known that the rate of transmitter release rises rapidly to a peak for 1 ms and then drops back to very low levels in another few milliseconds (15). Based upon the kinetic data from which the amount of fast released dopamine pool could be estimated at 40% of the total [³H]dopamine pool and the LCEC data on the amount of endogenous transmitter present in the cells (which is equivalent to about 0.06 fmol/cell), the size of the dopamine pool which is released by the fast mechanism was estimated to be 0.024 fmol/cell. Thus, the amount of dopamine released during 5 ms by a single cell is equivalent to 5000 molecules. It should be noted that this value is a minimal estimate, since the population of dopaminergic neurons in the culture is approximately 20%. The amount of dopamine released per “dopaminergic” neuron during 5-ms depolarization may, therefore, be around 20,000 molecules. In addition, diffusion barriers which may be present at the cellular glycocalyx could slow down the movement of the neurotransmitter into the extracellular incubation medium; such barriers were not in-

**FIG. 3.** Effects of varying the extracellular Ca²⁺ on the kinetics of the release of [³H]dopamine from the depolarized brain neurons. A, the percentages of [³H]dopamine remaining in the cells at different time points after initiating the depolarization in the presence of 0.1 mM Ca²⁺ (●) or of 1.8 mM Ca²⁺ (○) are presented. B, initial time course of [³H]dopamine released from depolarized neurons in the presence of the following concentrations of Ca²⁺: 0.1 mM (●), 0.2 mM (○), 0.3 mM (△), 0.4 mM (▲), 0.5 mM (◆), 0.8 mM (■), and 1.8 mM (□). The solid lines in panels A and B are theoretical curves generated from coefficients and exponential constants given in Table II. The size of the “fast” released neurotransmitter pool (in per cent of initial cellular content) at different concentrations of extracellular Ca²⁺ is shown in the inset. The data in the inset was obtained by compartmental analysis of the representative experiments shown in panel B. Experiments were repeated 3 times.
FIG. 4. Effects of different concentrations of verapamil on \(^{3}H\)dopamine release by the brain neurons. Cells were preloaded with \(^{3}H\)dopamine and washed. The cells were then incubated twice for 1-min intervals in reference buffer containing Ca\(^{2+}\) (0.72 mM) and then twice in the same buffer containing different concentrations of verapamil. The cells were then stimulated by repeated 1-min incubations in depolarizing buffer containing matched concentrations of verapamil and Ca\(^{2+}\) (0.72 mM). The amount of \(^{3}H\)dopamine released upon each incubation (fraction) is presented as a per cent of the initial \(^{3}H\)dopamine present in the cells. The incubations performed in the presence of verapamil are indicated by a blank bar. Incubations in depolarizing buffer are indicated by a filled bar. The concentrations of verapamil were: 0, no verapamil; \(\bullet\), 10 \(\mu\)M; \(\Delta\), 50 \(\mu\)M; \(\bigtriangleup\), 100 \(\mu\)M. Experiments were repeated 3 times.

The Biphasic Release of Neurotransmitter from Stimulated Brain Neurons: Correlation with Calcium Entry—The results reported in this work verify both qualitatively and quantitatively the previous observations about the "phasic" nature of K\(^{+}\)-evoked transmitter release. Namely, despite maintained depolarization, the evoked transmitter efflux exhibits a rapid temporal decay. These phenomena could not be attributed to depletion of the intraneuronal neurotransmitter store since release from the cells could be re-evoked after a "recovery period" in the absence of stimulation. Several explanations could be given for the observed phenomena. (a) Depolarization-dependent inactivation of the Ca\(^{2+}\) channel results in the early decay of the Ca\(^{2+}\)-dependent neurotransmitter release. (b) Two neurotransmitter pools exist, a fast and a slowly releasing one, and the transfer from the latter to the former pool is slow relative to the rate of the fast release. (c) Inactivation of an intracellular component is involved in the stimulated fast release. What, then, is the mechanism...
that terminates the fast release?

Studies of K+-stimulated Ca2+ entry into synaptosomes prepared from rat brain striata indicated two components of Ca2+ influx: a fast channel which is inactivated within 1 s, and a slow channel, not inactivated up to 20 s of depolarization (4). Similar studies on synaptosomes from rat brain indicated at least three components of Ca2+ entry: fast, slow inactivating, and noninactivating pathways, with t50 values of 5, 40, and 300 s, respectively (5). Our studies on the kinetics of depolarization induced Ca2+ entry into the brain neurons indicated the presence of a single noninactivated pathway (17), which was inhibited by verapamil and by Co2+. The inhibition pattern was similar to that of the voltage sensitive Ca2+ channels in other neuronal preparations (2). The presence of an additional, rapidly inactivated Ca2+ channel was not detected in brain neurons. It should be borne in mind, however, that such channels might be masked by larger concentrations of the noninactivated type, and by the rapidly exchanging voltage independent component observed in the brain neurons (17).

The release of dopamine from the brain neurons is similar to the stimulated Ca2+ uptake in its being inhibited by verapamil and Co2+ (Figs. 4 and 5). Moreover, it is not inactivated upon prepolarization in the absence of extracellular Ca2+ if the neurotransmitter stores are not depleted (Fig. 6). On the other hand, the release differs from Ca2+ uptake in that its rate decreases upon sustained depolarization and in its dependence on the extracellular concentration of Ca2+.

The relationship between Ca2+ entry and the stimulated neurotransmitter release could be further examined in the experiment where Co2+ was added to the stimulated cells at different times in the course of depolarization. The addition of Co2+ at early stages of the stimulation (i.e., within the first 6 s) reduced the amount of the fast released neurotransmitter. This is compatible with the ion's effects on Ca2+ uptake by the neurons. However, the release of neurotransmitter was not immediately stopped after the addition of Co2+. Instead, it followed the same kinetics as that of release induced by depolarization in the presence of lower concentrations of Ca2+ (e.g., reduced amount of transmitter was released by the fast process). If the coupling between Ca2+ entry and neurotransmitter release was very tight, it should be expected that the neurotransmitter release would decay immediately in the presence of Co2+ due to the artificial "inactivation" of the inward Ca2+ flux.

The fact that neurotransmitter release from depolarized neurons persisted past the addition of Co2+ could, in theory, be due to massive influx of Ca2+ during the very first seconds of stimulation. Flooding of the nerve terminal with Ca2+ might overload the Ca2+ removal systems in the nerve terminals and consequently delay transmitter release shut-off upon addition of Co2+. Considering the results obtained from the kinetic measurements of the Ca2+ fluxes into the depolarized neurons (17), the amount of Ca2+ entering a cell during 1 ms of depolarization in the presence of Ca2+ (1.8 mM) can be estimated at 8000–9000 molecules. Whether this flux is large enough to increase the local intracellular Ca2+ concentrations above those required for neurosecretion cannot be directly answered. However, if Ca2+ channel inactivation is assumed to exist and its effect on transmitter release is delayed due to such flooding, it would be expected that the kinetics of termination of the fast release would be determined by initial influx of Ca2+ into the cells. This was not substantiated by at least two facts. (a) The release declined with time at low as at high extracellular Ca2+ although the influx of Ca2+ into the depolarized cells was reduced (Fig. 3). (b) The termination of the fast phase was unaffected by the timing of the addition of Co2+ into the depolarizing medium (Fig. 5). Thus, the possibility that Ca2+ channel inactivation is responsible for the decline in transmitter release is questionable.

The possibility of pre-existing "fast" and "slow" releasing neurotransmitter pools in the neurons is inconsistent with
the affinity constant of 0.8 mM for the extracellular Ca\(^{2+}\). Thus, release during the early phase of stimulation, especially at low Ca\(^{2+}\) entry, may in principle be a limiting factor for dopamine 

saturated at concentrations around the amount 

The evoked Ca\(^{2+}\) uptake by the neurons indicated a single 

apparent half-maximal saturation for Ca\(^{2+}\) (about 0.7 mM). 

increase in the rate constant 

extracellular Ca\(^{2+}\) exhibited two different affinities to the ion: 

of the fast released neurotransmitter was half- 

saturated at concentrations around 0.1 mM, whereas the increase in the rate constant of the slow release had a higher apparent half-maximal saturation for Ca\(^{2+}\) (about 0.7 mM). The evoked Ca\(^{2+}\) uptake by the neurons indicated a single affinity constant of 0.8 mM for the extracellular Ca\(^{2+}\). Thus, Ca\(^{2+}\) entry, may in principle be a limiting factor for dopamine release during the early phase of stimulation, especially at low extracellular Ca\(^{2+}\) concentrations. At longer periods, Ca\(^{2+}\) influx determines the steady-state level of the neurotransmitter efflux. 

Alternatively, the situation in the brain neurons may be analogous for the phenomena described in the squid giant axon, although different time scales were experimentally used. In the latter, a presynaptic action potential is accompanied by an influx of Ca\(^{2+}\) which lasts several seconds. Furthermore, extracellular Ca\(^{2+}\) concentration increases linearly during repeated stimulations. Nevertheless, transmitter release lasts only about 2 ms and then exhibits a temporal decay (15). Recently, a mathematical model of intracellular calcium diffusion with influx during an action potential, binding to fixed cytoplasmatic sites, and active extrusion through the surface was presented to predict the distribution of presynaptic Ca\(^{2+}\) in the squid axon following stimulation (8). By assuming a square law relation between submembrane calcium and transmitter release the model predicted the temporal decay of neurotransmitter release as well as the relatively long persistence of free intracellular Ca\(^{2+}\).

REFERENCES