THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 262, No. 19, Issue of July 5, pp. 9189-9195, 1987
Printed in U.S.A.

Voltage-dependent Activation and Inactivation of Calcium Channels in PC12 Cells

CORRELATION WITH NEUROTRANSMITTER RELEASE*

(Received for publication, December 29, 1986)

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The existence and mechanisms of inactivation of voltage-gated Ca^{2+} channels are important, but still debatable, physiological problems. By using the Ca^{2+} indicators quin2 and fura-2, we demonstrate that in PC12 cells voltage-gated Ca^{2+} channels undergo inactivation dependent on both voltage and $[Ca^{2+}]_i$. Inactivation, however, is never complete and a small number of channels remains open during prolonged depolarization, explaining the steady state elevation of $[Ca^{2+}]_i$ observed in cells depolarized with high KCl. A close parallel exists between Ca^{2+} channel inactivation and the transient nature of neurotransmitter release: (i) secretion is rapidly stimulated during the first 30 s of depolarization, when a transient overshoot in $[Ca^{2+}]_i$ can be demonstrated, while it is negligible during the following period, despite the persistence of an elevated $[Ca^{2+}]_i$; (ii) predepolarization in Ca^{2+}-free medium and subsequent addition of Ca^{2+} (a condition which allows the development of the voltage inactivation) abolishes the fast phase of secretion, while not modifying the steady state $[Ca^{2+}]_i$ eventually attained; and (iii) increases in the intracellular Ca^{2+} buffering decreases the amplitude of the fast secretion phase induced by KCl without altering the steady state $[Ca^{2+}]_i$. We suggest that localized $[Ca^{2+}]_i$ gradients form close to the plasma membrane shortly after depolarization and that the $[Ca^{2+}]_i$ reached in these regions is the relevant parameter in the regulation of secretion.

Voltage-gated Ca^{2+} channels play a pivotal role in the regulation of Ca^{2+}-dependent functions in excitable cells. Through the opening of these channels the depolarization of the plasma membrane, induced by many physiological stimuli, is translated into changes of the cytosolic Ca^{2+} concentrations, $[Ca^{2+}]_i$. This, in turn, switches on a wealth of Ca^{2+}-dependent intracellular reactions, including exocytosis and actomyosin interaction. Thus, a detailed knowledge of the voltage-gated Ca^{2+} channel functioning is expected to be of paramount importance in the fields of cellular biochemistry and physiology.

Until very recently, information on the voltage-gated Ca^{2+} channels was very limited. For example, only during the last 2 years has the heterogeneity of such channels been recognized with the discovery, in addition to the classical high threshold (L type) channels (specifically inhibited by low concentrations of dihydropyridine drugs), of one or more types, distinguishable by their peculiar kinetic properties (1–3). Another aspect which has attracted much interest is the inactivation of the L type channel and the mechanism(s) of its regulation. The results obtained so far concur that inactivation of this channel is slower than that of other voltage-gated channels, for example the classical Na^+ channel. In some cellular systems evidence has been obtained that Ca^{2+} channel inactivation is the direct consequence of the elevated $[Ca^{2+}]_i$ caused by channel activation (4–6), but this mechanism has not gained general acceptance. Alternatively, a voltage-dependent mechanism of inactivation has been envisaged (7, 8). Moreover, fluorescence studies on excitable secretory cells, carried out with Ca^{2+}-sensitive dyes, such as quin2, have led to the unexpected finding that maintained depolarization (for example, with high K^+) induces long-lasting (several tens of min) rises of $[Ca^{2+}]_i$ that are rapidly dissipated by the addition of specific Ca^{2+} channel blockers (9–15). Based on this type of result it has been recently suggested that in bovine chromaffin cells the voltage-gated Ca^{2+} channels are noninactivable (16) (but see the recent report by Artalejo *et al.* (17)).

Until now, activation and inactivation of voltage-gated Ca^{2+} channels have been investigated primarily by electrophysiological techniques as well as by ^{45}Ca flux measurements. In the present work we have employed Ca^{2+}-sensitive dyes, not only quin2 (used already in Refs. 9 and 15), but also the recently introduced fura-2 (19); we have studied in detail the kinetics of the responses induced by depolarization with high extracellular K^+. Our experiments were carried out on cultured cells of the line PC12 (that derive from a rat pheochromocytoma (20) and are therefore similar to chromaffin cells) which possess voltage-gated Ca^{2+} channels mainly of the L type (20). The results obtained clearly demonstrate that these channels are inactivated within a few seconds after depolarization and that this inactivation is both voltage- and $[Ca^{2+}]_i$-dependent. Moreover, the correlation of $[Ca^{2+}]_i$ changes and secretion kinetics revealed interesting clues as to the role of $[Ca^{2+}]_i$ transients for the regulation of exocytosis in PC12 cells.

MATERIALS AND METHODS

PC12 cells were obtained from Dr. P. Calissano (Rome) and subcultured in our laboratory in Falcon plastic flasks (Falcon Labware,

* This work was supported in part by grants from the Ministry of Public Education "Membrane Biology and Pathology Project" (to J. M. and T. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by a grant from the Association for the Development of Neurological Science.

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Division of Becton, Dickinson & Co., Oxnard, CA) in RPMI 1640 (Flow Laboratories, Milano, Italy) supplemented with 2 mM glutamine, 10% horse serum, 5% fetal calf serum, and 100 μl/100 ml gentamycin (40 mg/ml) as described previously (9). Fura-2/AM and fura-2 were obtained from Molecular Probes Inc, Junction City, OR. Quin2/AM and quin2 were purchased from Behring Diagnostics. [^{3}H] Dopamine (specific activity 50 Ci/mmol) was from Amersham International. All other materials were analytical or highest available grade.

Measurement of $[Ca^{2+}]_i$ with Quin2 and Fura-2—Quin2 loading was performed essentially as described previously (9). Routinely the cells were incubated with 35 μM quin2/AM which resulted in a final intracellular quin2 content of 400 ± 50 pmol/10^6 cells (n = 12). Loading with fura-2 was performed as follows. PC12 cells at a concentration of 5 × 10^6/ml were incubated in RPMI 1640 at 37 °C under continuous stirring in the presence of 5% fetal calf serum. Fura-2/AM was added from a concentrated stock solution in dimethyl sulfoxide at a final concentration of 1–2 μM. The cell suspension was incubated under continuous stirring for 30 minutes and then pelleted and resuspended in RPMI without serum. Before each experiment an aliquot of the cells (loaded with either quin2 or fura-2) was pelleted and resuspended in a modified Krebs-Ringer bicarbonate medium with and without added $CaCl_2$. The "Ca^{2+}-free medium" composition was 125 mmol/liter NaCl, 5 mmol/liter KCl, 1.2 mmol/liter $MgSO_4$, 1.2 mmol/liter KH_2PO_4, 5.5 mmol/liter glucose, 40 μmol/liter diethylenetriaminepentaacetic acid, 20 mmol/liter Hepes/NaOH buffer (pH 7.4 at 37 °C). The "Ca^{2+} medium" contained, in addition, 1 mM $CaCl_2$. The calibration of quin2 fluorescent signal in terms of $[Ca^{2+}]_i$ was carried out as described previously (9). Fura-2 was calibrated as quin2 using as excitation and emission wavelengths 340 and 505 nm, respectively.

Measurement of Initial Ca^{2+} Influx and Efflux Rates—The curves of fluorescence increase upon addition of $CaCl_2$ to cells depolarized in Ca^{2+}-free medium could be empirically fit by the equation

$$\ln((T - x)/T) = -kt \quad (1)$$

where T = distance in centimeters between fluorescence at time 0 and at steady state, x = distance in centimeters between fluorescence at time 0 and at time t, and k = time constant of the exponential rise (t = time in s). The initial rate, v_0, can then be easily calculated from the following equation.

$$v_0 = kT \quad (2)$$

A single exponential described also the fluoresence decay curve obtained upon addition of verapamil in steady state. The correlation coefficient in all cases was always above 0.99 (see also Fig. 4). The initial rates of fluorescence increase (decrease) can then be converted into Ca^{2+} influx (efflux) by knowing the fluorescence in centimeters of a standard solution of quin2 and assuming a 1/1 stoicheometry between total Ca^{2+} ion influx (efflux) and calcium-quin2 complex formation (dissociation). This assumption is valid if these two conditions are obeyed: (*a*) the amount of quin2 and calcium-quin2 complex is in vast excess of the free Ca^{2+} concentration and (*b*) quin2 represent the major Ca^{2+} buffer of the cell. The first condition is obviously true in all our conditions, since the $[Ca^{2+}]_i$ is in the nanomolar range, while the cellular quin2 concentration is in the millimolar range. The second condition is more difficult to verify since the endogenous Ca^{2+} buffering capacity is unknown. However, the amount of endogenous Ca^{2+} buffers is unlikely to be higher than 10^{-4} mol/liter of cell water, and thus the calcium-quin2 complex formation (dissociation), *i.e.* the rate of fluorescence increase (decrease), should represent to a first approximation the total amount of Ca^{2+} ion influx (efflux). Secretion of [^{3}H]dopamine was measured as described in detail previously (18). All experiments were carried out at 37 °C.

RESULTS

Previous studies (9, 21) demonstrated that in undifferentiated PC12 cells the depolarization-induced increases of ^{45}Ca influx and $[Ca^{2+}]_i$ as well as secretion are nearly completely inhibited by organic Ca^{2+} blockers, in particular by nanomolar concentrations of dihydropyridines. These results were confirmed in the clone of PC12 cells grown in our laboratory (not shown and see Fig. 1). Since dihydropyridines are known to be specific for the L type channels (21), this result suggests that also our clone of cells is essentially endowed only with this type of Ca^{2+} channels.

Depolarization-induced Changes of $[Ca^{2+}]_i$—Fig. 1 shows the kinetics of $[Ca^{2+}]_i$ changes in PC12 cells depolarized by elevating the extracellular $[K^+]$, as revealed by the quin2 technique. The addition of 50 mM KCl to the medium induced a fast rise of $[Ca^{2+}]_i$ (to approximately 450 nM) that was maintained during the subsequent 5–15 min (hereon referred to as the steady state level). In 20 similar experiments the $[Ca^{2+}]_i$ rose to 340 ± 30 nM and to 470 ± 50 nM, from a resting value of 95 ± 10 nM, with 30 and 50 mM K^+, respectively. These persistent $[Ca^{2+}]_i$ increases were fully reversed by nitrendipine (200 nM) or verapamil (20 μM), known voltage-gated Ca^{2+} channels blockers, added at any time after the depolarizing agents (Fig. 1 and see also Ref. 9). When the Ca^{2+} channel blockers were added before depolarization, 50% inhibition was observed at 10 nM nitrendipine or 5 μM verapamil. Similar drug doses were necessary to cause the return of $[Ca^{2+}]_i$ toward basal when added in steady state, though it was noticed that the drugs were slightly more efficient when added in steady state compared to when added before KCl. As shown by electrophysiology, Ca^{2+} currents undergo inactivation, at different rates, depending on the type of Ca^{2+} channels involved. N and T type of Ca^{2+} currents inactivate in a few milliseconds while L type requires a few hundred milliseconds (1–6). The data presented in Fig. 1, however, and previous data obtained in these and other excitable cells with the quin2 technique (9–15) seem to indicate the Ca^{2+} influx through voltage-gated Ca^{2+} channels of L type remains elevated for much longer times, *i.e.* tens of minutes. In order to determine whether the poorer kinetic resolution of Ca^{2+} indicators *versus* electrophysiological measurements could account for this discrepancy, a series of experiments, summarized in Figs. 2 and 3, were carried out. In Fig. 2 50 mM KCl

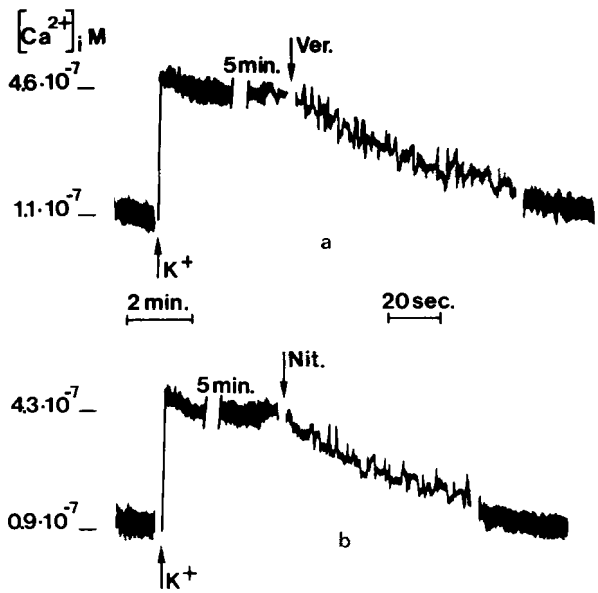

FIG. 1. **Effect of depolarization and Ca^{2+} channel blockers on $[Ca^{2+}]_i$.** Where indicated, 50 mM KCl, 200 nM nitrendipine (*Nit.*), and 20 μM verapamil (*Ver.*) were added. In this and following figures the $[Ca^{2+}]_i$ is given on the *left side* of each panel. Note the difference in noise when the speed of the chart recorder was increased. The traces shown in this and the following figures are representative of experiments performed with very similar results in at least three different batches of cells.

[1] The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)] tetraacetic acid.

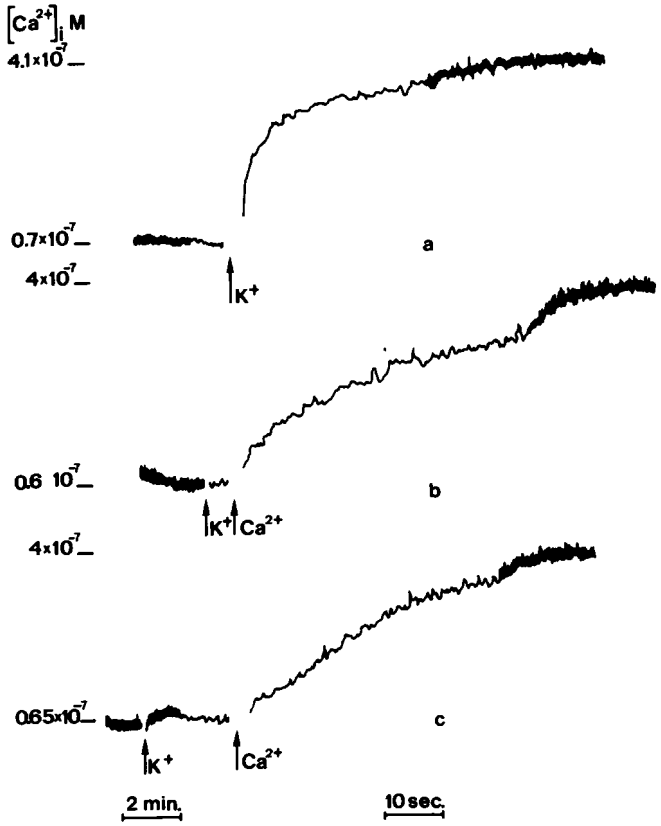

FIG. 2. **Effect of predepolarization on the kinetics of $[Ca^{2+}]_i$ rise.** In *panel a* 1 mM $CaCl_2$ was added to cells 2 min before 50 mM KCl, while in *panels b* and *c* $CaCl_2$ was added after KCl, when indicated.

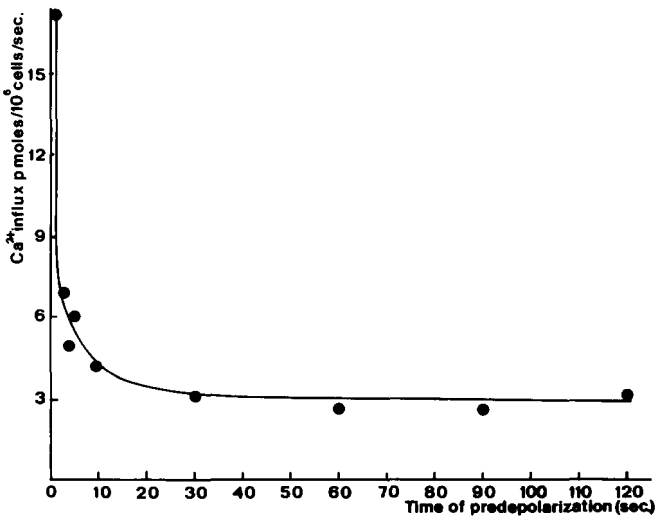

FIG. 3. **Effect of the duration of the predepolarization on Ca^{2+} influx rates.** The cells were depolarized in Ca^{2+}-free medium with 50 mM KCl for different lengths of time before addition of 1 mM $CaCl_2$. The initial rate of Ca^{2+} influx in cells depolarized in Ca^{2+} medium was too fast to be resolved. The absolute values of the Ca^{2+} influx rates were found to be slightly variable among different batches of cells, although the percent inhibition as a function of the predepolarization time was quite reproducible. In six similar experiments the initial Ca^{2+} influx rate, after 2 min of predepolarization, varied between 3 and 6 pmol/10^6 cells/s.

were added to the extracellular medium either in the presence of Ca^{2+} (*trace a*), or 5 (*trace b*) and 90 (*trace c*) s before the readdition of Ca^{2+} to a Ca^{2+}-free medium. Under these three conditions profound differences in the initial rates of $[Ca^{2+}]_i$ rise were observed: when K^+ depolarization was induced in the presence of extracellular Ca^{2+} the rate of $[Ca^{2+}]_i$ rise was too fast to be resolved by our experimental set up. In contrast, reintroduction of Ca^{2+} into the medium at a later stage yielded slower (and thus easily measurable) rates. Interestingly, while the initial rates of $[Ca^{2+}]_i$ rise were slowed down in a time-dependent fashion by the predepolarization (see also Fig. 3), the steady state levels eventually attained in all these experiments were approximately the same. In Fig. 3 the initial rate of Ca^{2+} influx is plotted as a function of the duration of the predepolarization period. As mentioned above, the rate of Ca^{2+} influx in cells depolarized in Ca^{2+} containing medium is too fast to be measured, but considerable inhibition was observed already after 3 s, compared to 1 s, and maximal effect within 30 s, without further effect with longer predepolarization. Since during the predepolarization period $[Ca^{2+}]_i$ does not increase above the resting level, these experiments demonstrate the existence of an inactivation mechanism which is totally dependent on depolarization, *i.e.* a voltage-dependent inhibition.

In addition (or alternatively) to voltage-dependent inactivation, voltage-gated Ca^{2+} channels have been reported to possess a $[Ca^{2+}]_i$-dependent inactivation mechanism (4–6). The experimental approach widely used in the past to reveal this mechanism, *i.e.* substitution in the medium of Ca^{2+} with other permeant cations, such as Ba^{2+}, leads in our conditions to an interference with quin2 measurements and is therefore impractical. Indirect information on this matter, however, was obtained by the experiments summarized in Figs. 4 and 5. Cells loaded with quin2 were first depolarized with 50 mM $[K^+]_o$ in Ca^{2+}-free medium for 2 min. Then Ca^{2+} and, upon attainment of the $[Ca^{2+}]_i$ steady state, a Ca^{2+} channel blocker verapamil (or nitrendipine, see Fig. 1) were added, and the decrease of the $[Ca^{2+}]_i$ signal was recorded (Fig. 4). In some experiments (not shown) the membrane impermeant Ca^{2+} chelator EGTA was used instead of verapamil with virtually identical results. Inasmuch as in steady state the rates of Ca^{2+} influx and extrusion through the plasmalemma are identical, the initial rate of $[Ca^{2+}]_i$ decrease upon channel blockade (or Ca^{2+} withdrawal from the medium) is expected to reflect the rate of Ca^{2+} influx through the channel at an elevated $[Ca^{2+}]_i$. On the other hand, as already pointed out above, the initial rate of $[Ca^{2+}]_i$ rise after Ca^{2+} reintroduction into the medium reflects the Ca^{2+} influx through the voltage-gated Ca^{2+} channels at resting $[Ca^{2+}]_i$. Thus, direct comparison of the two rates should provide information on the changes of Ca^{2+} conductance occurring as a consequence of a $[Ca^{2+}]_i$ elevation. In the experiments presented in Figs. 4 and 5 different rates of Ca^{2+} influx and steady state $[Ca^{2+}]_i$ were obtained by adding variable concentrations of $CaCl_2$ 90 s after KCl predepolarization. Both the influx and the efflux curve fit Equation 2 nicely (Fig. 4, *inset*). Fig. 5*a* shows that the initial rate of Ca^{2+} influx increased as $[Ca^{2+}]_o$ rose from 1 to 2.6 mM while the steady state $[Ca^{2+}]_i$ tended to reach a plateau above 1 mM $[Ca^{2+}]_o$ (Fig. 5*b*). The efflux rate induced by verapamil, which should correspond to the influx rate of steady state, also increased as $[Ca^{2+}]_i$ rose. However, when, at any given $[Ca^{2+}]_o$, the ratio between the influx at resting $[Ca^{2+}]_i$ and the efflux in steady state (*i.e.* the influx at elevated $[Ca^{2+}]_i$) was plotted as a function of the steady state $[Ca^{2+}]_i$ (*panel d*), it was observed that the ratio approached 1 at or below $[Ca^{2+}]_i$ of 250–300 nM, but it increased suddenly above these values. This increase in ratio was observed both when the influx rate and the steady state $[Ca^{2+}]_i$ were varied by increasing $[Ca^{2+}]_o$ at constant $[K^+]_o$ (Fig. 5) or when $[K^+]_o$ was varied at constant $[Ca^{2+}]_o$ (not shown). These results

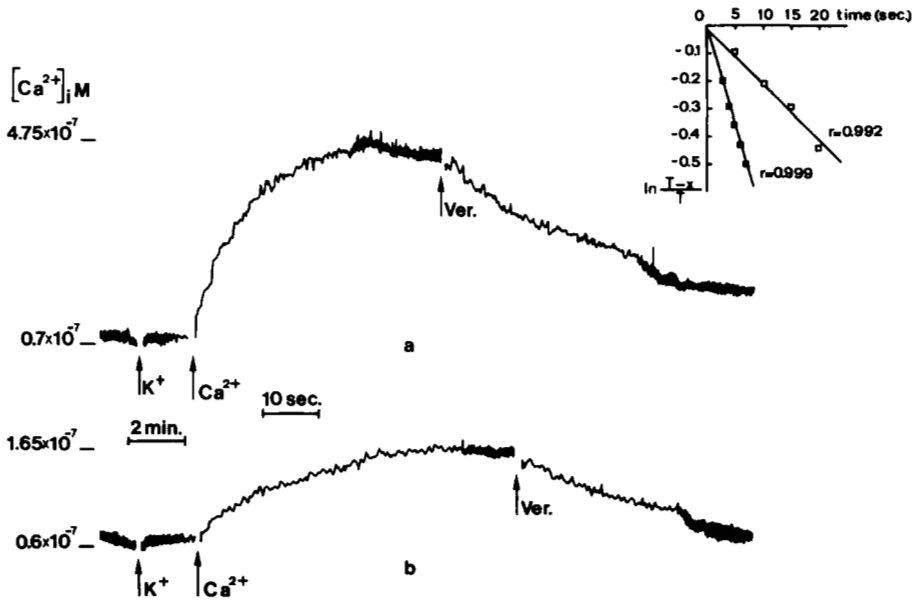

FIG. 4. **Kinetics of Ca^{2+} influx and efflux upon opening and closing of voltage-gated Ca^{2+} channels.** Where indicated, 2.6 mM $CaCl_2$ (*a*), 0.15 mM $CaCl_2$ (*b*), and 20 μM verapamil were added. Note the change in noise when the speed of the chart recorder was changed. In the *inset* the fitting of the experimental curves of influx (■) and efflux (□) (from *panel a*) with Equation 2, described under "Materials and Methods," is presented. r = correlation coefficient. The other symbols were defined under "Materials and Methods."

suggest that PC12 cells, in addition to the voltage-dependent mechanism, are also endowed with an appreciable $[Ca^{2+}]_i$-dependent inactivation mechanism of voltage-gated Ca^{2+} channels.

Ca^{2+} Channel Activation-Inactivation and Transmitter Release—Our previous work on PC12 cells (9, 18) had already revealed a poor temporal correlation between the K^+-induced secretion of catecholamines (that is maximally activated during the first few seconds after depolarization and drops to much lower levels thereafter) and the $[Ca^{2+}]_i$ elevation, as measured by quin2 (that is persistent). Comparison of the data of Figs. 1 and 6 confirms these previous findings. In cells depolarized by the addition of 30 or 50 mM K^+ in Ca^{2+}-containing medium, a burst of secretion occurred within the first 30 s. Thereafter, the rate of release from the cells depolarized with 30 mM K^+ was indistinguishable from that of controls, while with 50 mM K^+ secretion continued at rates slightly above controls, but severalfold lower than the initial rate. When the cells were predepolarized with the same concentration of K^+ in a Ca^{2+}-free medium, and Ca^{2+} was reintroduced 2 min after, the initial burst of secretion was completely lost, and only the slow secretion induced by 50 mM K^+ was observed (Fig. 6).

In order to interpret these results, it should be kept in mind that the quin2 signal reflects the mean $[Ca^{2+}]_i$ within individual cells as well as an average of the entire cell population. Thus, comparison between secretion and $[Ca^{2+}]_i$ is valid only when the $[Ca^{2+}]_i$ changes in all the cells simultaneously and is homogeneously distributed within individual cells (21). The abrupt opening of Ca^{2+} channels induced by depolarization is expected to cause a marked rise of $[Ca^{2+}]_i$ immediately beneath the plasma membrane, with diffusion to, and equilibration with, the rest of the cytosol taking place only after some delay. This is tantamount to saying that, shortly after depolarization (*i.e.* when the burst of secretion occurs), $[Ca^{2+}]_i$ gradients are expected in the cytosol, with maxima close to the plasmalemma, where exocytosis is being stimulated (23, 24). Measurement of local $[Ca^{2+}]_i$ gradients in the millisecond time scale is not yet technically feasible. Indirect information on this problem, however, is provided by the experiments shown in Fig. 7. As seen in *panel a* addition of 50 mM K^+ to cells loaded with the new Ca^{2+} indicator fura-2 induced an immediate spike of fluorescence increase which declined to the steady state within 30–60 s. Because of its higher fluorescence, with respect to quin2 (30 times on a molar basis (19)), excellent signals can be obtained with intracellular fura-2 concentration of the order of 50–100 pmol of dye/10^6 cells, *i.e.* 5- to 10-fold lower than the concentration of quin2 usually employed. The $[Ca^{2+}]_i$ overshoot observed with fura-2 was abolished by predepolarization in Ca^{2+}-free medium (Fig. 7*b*). With quin2 $[Ca^{2+}]_i$ overshoots were observed, but only when unusually low concentrations were used (Fig. 7*c*). Thus, the existence of transient elevations of average $[Ca^{2+}]_i$ induced by depolarization can be revealed simply by decreasing the intracellular Ca^{2+} buffering capacity. These overshoots in $[Ca^{2+}]_i$ correlate kinetically and therefore might be causally related to the burst of secretion. However, our previous experiments, carried out in quin2-loaded cells under conditions in which no overshoot was revealed, have shown the persistence of the secretion burst. In order to further investigate this problem, PC12 cells were loaded with different quin2 concentrations. Fig. 8 shows that the burst of secretion was severely curtailed (≃60%) only at unusually high (1400 pmol/10^6 cells) quin2 loading; whereas, at intracellular concentrations sufficient to abolish the $[Ca^{2+}]_i$ overshoot, the secretion of [^{3}H]dopamine was only marginally affected.

DISCUSSION

Previous studies on the inactivation of the voltage-gated Ca^{2+} channel in various excitable cell systems, carried out by electrophysiological and ^{45}Ca flux techniques, yielded variable results. In some cases evidence was obtained indicating that inactivation occurs as a consequence of the raised $[Ca^{2+}]_i$ and, in others, as a consequence of depolarization ($[Ca^{2+}]_i$- and voltage-dependent mechanisms of inactivation) (4–6). Finally, in a few studies the channels were reported to remain open, *i.e.* to be noninactivable (25–26).

Our previous studies with quin2 had revealed that depolarization, brought about in PC12 cells by exposure to high K^+ medium, induced rapid $[Ca^{2+}]_i$ rises, which then remain elevated for tens of min (9, 15, 18). This persistent elevation was interpreted as being due to the prolonged opening of Ca^{2+} channels because (i) it is rapidly dissipated by the addition of specific Ca^{2+} channel blockers; and (ii) it represents a true steady state since it is independent on the amount of intracellular Ca^{2+} buffer (9). Concomitantly with our findings in PC12 cells, similar observations were made by us and others in various excitable secretory cells, such as adrenal chromaffin

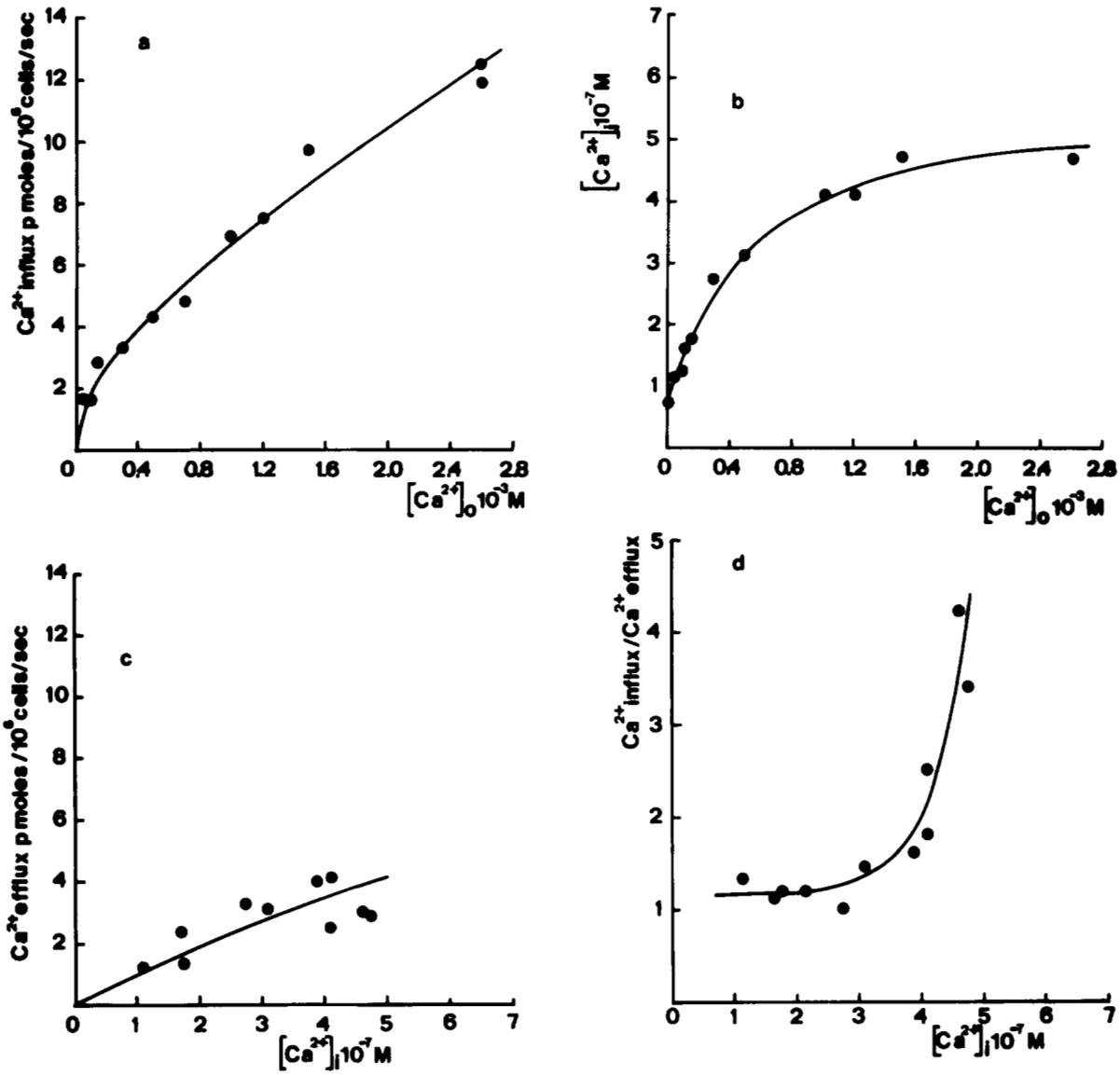

FIG. 5. **Effect of $[Ca^{2+}]_o$ on the influx and efflux rates, steady state $[Ca^{2+}]_i$, and influx-efflux ratio.** The cells were depolarized with 50 mM KCl in Ca^{2+}-free medium for 2 min before adding different concentrations of $CaCl_2$. Ca^{2+} efflux was initiated by the addition of 20 μM verapamil.

and insulinoma cells, GH_3 mammotrophs, as well as brain synaptosomes (9–15). The attainment of a new steady state, at higher levels of $[Ca^{2+}]_i$ with respect to the resting level, appears to be widespread and possibly general in excitable secretory systems depolarized for prolonged periods of time. Thus, a clear discrepancy seems to exist between $[Ca^{2+}]_i$ measurements and electrophysiological data. Up to now the reason for such a discrepancy had not been explained.

Recently, in order to account for the depolarization-induced $[Ca^{2+}]_i$ steady state, Burgoyne and Cheek (16) have proposed that in chromaffin cells (the nontumoral counterparts of PC12 cells) voltage-gated Ca^{2+} channels are not inactivable. The results that we have now obtained, starting from initial observations very similar to those of Burgoyne and Cheek (16), are clearly incompatible with this interpretation. In our studies we analyzed not only $[Ca^{2+}]_i$ levels, but also (and especially) the rates at which $[Ca^{2+}]_i$ changes take place. The most important observation we made was the recognition of the profound difference of initial rates of $[Ca^{2+}]_i$ rise in the cells depolarized while bathed in a Ca^{2+}-containing medium with respect to the cells exposed to Ca^{2+} after predepolarization in a Ca^{2+}-free medium. The results clearly demonstrate that considerable inactivation occurs within a few seconds. In order to interpret these results it should be noted that: (*a*) in PC12 cells (which, as those used in the present work, have not been treated with nerve growth factor) most, if not all, voltage-gated Ca^{2+} channels appear to be of L type, inhibitable with dihydropyridines (21) and (*b*) during predepolarization in Ca^{2+}-free medium, $[Ca^{2+}]_i$ does not rise significantly. Thus, the marked differences of the initial rates of $[Ca^{2+}]_i$ rise described above can be due neither to the variable activation of heterogeneous channels, nor to channel inactivation dependent on the raised $[Ca^{2+}]_i$, but only to voltage inactivation of the channel. Even if other Ca^{2+} channels (T or N) were present in our PC12 clone, the kinetics of their inactivation (1) (20–30 ms) is beyond the time resolution of our experiments. At variance with the observed differences in Ca^{2+} influx rates, the steady state levels were approximately the same in the cells predepolarized in Ca^{2+}-free and depolarized in Ca^{2+}-containing media.

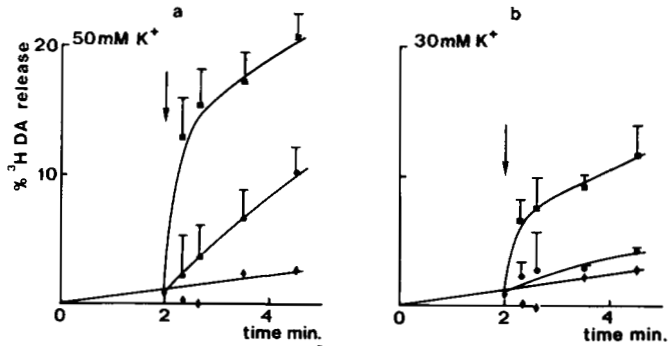

FIG. 6. **Effect of predepolarization on [^{3}H]dopamine release.** Cells incubated in Ca^{2+}-free or Ca^{2+}-supplemented medium and challenged at time 0 with 30 (*panel a*) or 50 (*panel b*) mM KCl. *^{3}H DA*, tritiated dopamine. ●, cells incubated in Ca^{2+}-free medium. ■, cells incubated in Ca^{2+} medium. ♦, unstimulated cells. The *arrows* indicate the addition of either 1 mM $CaCl_2$ to cells depolarized in Ca^{2+}-free medium or KCl to cells incubated in the continuous presence of Ca^{2+}. Secretion was terminated by centrifugation through silicon oil as described previously (9). The release is expressed as percent of total ^{3}H DA cellular content (mean of two experiments carried out in triplicates (+ S.D.)).

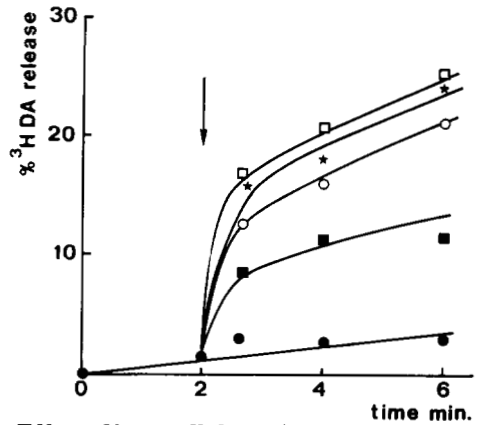

FIG. 8. **Effect of intracellular quin2 concentration on secretion induced by KCl.** Cells were loaded with different concentrations of quin2/AM and challenged with 50 mM KCl in Ca^{2+} medium. The *arrow* indicates the moment of KCl addition. The final intracellular quin2 contents were: □, 0; ★, 350; ○, 900; and ■, 1400 pmol/106 cells, respectively. ●, basal secretion. Typical experiment carried out in triplicates.

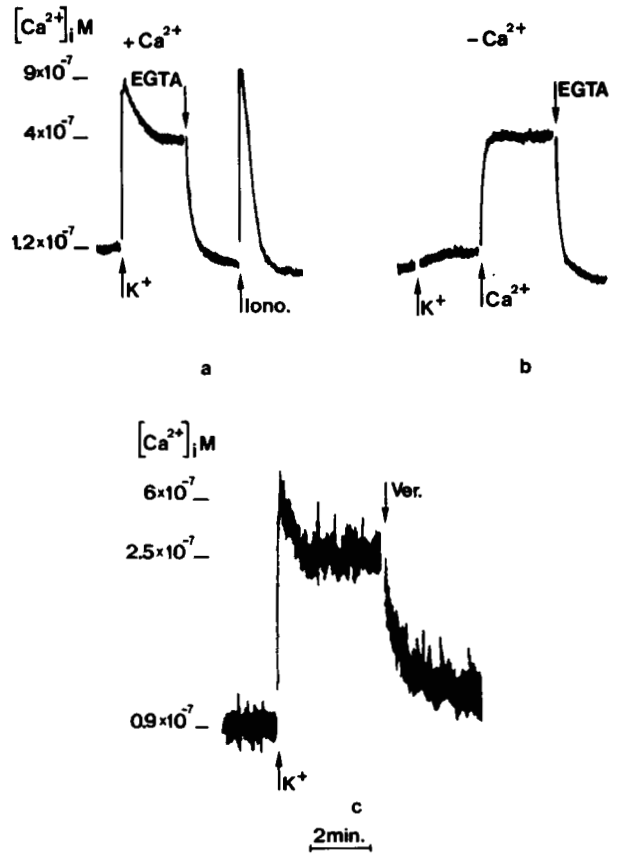

FIG. 7. **Kinetics of $[Ca^{2+}]_i$ rises as revealed by fura-2 or low quin2 concentration.** In *traces a* and *c* the cells were challenged with 50 mM KCl in Ca^{2+} medium while in *trace b* KCl was added in Ca^{2+}-free medium and 1 mM $CaCl_2$ was added 2 min later. EGTA, where indicated, was 1 mM. Intracellular fura-2 content = 70 pmol/10^6 cells. Intracellular quin2 content = 140 pmol/10^6 cells. Given that the PC12 cell volume is 500 μm^3/cell (38), these values correspond to an intracellular concentration of 140 and 280 μM, respectively. Note the difference in noise between *trace c* and those previously shown, for example Fig. 1*a*, where the quin2 content was about three times higher, *i.e.* 400 pmol/10^6 cells, equivalent to an intracellular concentration of 800 μM. *Ver.*, verapamil. *Iono.*, ionomycin.

The total complement of voltage-gated Ca^{2+} channels has been calculated to be around 1650/single PC12 cell (27). According to our data (Fig. 3), between 1 and 30 s of predepolarization with 50 mM KCl, the initial rate of Ca^{2+} influx decreases by about 80%. This value is an underestimation of the true inactivation because our method of measuring the initial rates is adequate for cells predepolarized in Ca^{2+}-free medium but becomes inaccurate with very fast rates of Ca^{2+} influx, as is the case when cells are depolarized in Ca^{2+} medium. Thus, in steady state the number of open channels in PC12 cells can be calculated to be of the order of a few tens of channels/cell.

In addition to the voltage-dependent mechanism, our results appear consistent with the existence in PC12 cells as well, of the $[Ca^{2+}]_i$-dependent mechanism of Ca^{2+} channel inactivation (4, 6, 17). In this respect the data are clearly less stringent compared to those concerning the voltage inactivation. We found that a 4-fold increase in the $[Ca^{2+}]_i$ decreases the influx rate by a factor of 4. Such an effect can not be a direct consequence of the decreased Ca^{2+} electrochemical gradient because (*a*) according to the constant field theory (28), the rate of Ca^{2+} influx through the channels should be almost unaffected by changes of $[Ca^{2+}]_i$ up to 100-fold (4, 6, 28) and (*b*) the decrease of the influx rate becomes appreciable only above a threshold level of $[Ca^{2+}]_i$. At a first glance it would appear that the $[Ca^{2+}]_i$-dependent mechanism of inactivation is far less important than that due to membrane potential. However, it should be emphasized that all our experiments were carried out with prolonged depolarizations and cannot be directly extrapolated to "physiological" conditions. In the latter cases the $[Ca^{2+}]_i$-dependent mechanism could play a more important role, in particular shortly after depolarization. In fact it has been calculated that $[Ca^{2+}]_i$ reaches very high concentrations at the mouth of the channels, beneath the plasma membrane (23, 24), and this in turn should cause a rapid inactivation of the Ca^{2+} current. If depolarization is prolonged, as is the case with quin2 experiments, the $[Ca^{2+}]_i$ should tend to equilibrate within cells and the $[Ca^{2+}]_i$ inhibition to decrease. However, prolonged depolarization causes voltage-dependent inactivation to ensue, and in the long run, this type of inactivation will tend to predominant. A similar conclusion was reached recently for smooth muscle by Jmari *et al.* (29) and Ganitkevich *et al.* (30).

Another consequence of depolarization that has attracted a

great deal of interest in recent years is the transient nature of the evoked secretory response. This observation is extremely puzzling in view of the persistent rise of $[Ca^{2+}]_i$ (9, 18). The results obtained in the present study by the use of two Ca^{2+} indicators, quin2 and fura-2, might help to understand such a contradiction. The response of fur-2 and quin2 differed during the initial seconds of the response but coincided thereafter. With fura-2 the $[Ca^{2+}]_i$ change induced by depolarization was always biphasic, with a brisk overshoot (coinciding with the period of maximal release stimulation) followed by a plateau (steady state). With quin2, on the other hand, the overshoot was observed only at very low intracellular loadings. Ca^{2+} indicators unavoidably increase the intracellular Ca^{2+} buffering capacity. The buffering is higher with quin2 than with fura-2 (usual intracellular concentrations $\cong$1 mM and $\cong$100 μM, respectively). Increases of the cytosolic Ca^{2+} buffering capacity are expected to blunt changes of $[Ca^{2+}]_i$ that involve limited amounts of Ca^{2+} (31). Our data demonstrate that this occurs immediately after depolarization. The amount of Ca^{2+} that flows across the membrane during this initial phase was clearly enough to cause an overshoot of the fura-2 signal, but not of that of quin2. Later, the persistent (many min) opening of a small fraction of the Ca^{2+} channels allowed the attainment of a steady state, and therefore similar results were obtained with the two indicators.

Because of simple geometrical reasons, the brisk opening of the channels after depolarization is expected to generate initially localized Ca^{2+} gradients, with maxima in the cytosolic regions close to the plasma membrane (23, 24). In PC12 cells, the data that we have now obtained suggest that these gradients are responsible for the rapid initial phase of secretion. In fact, this phase was totally inhibited in the cells predepolarized in Ca^{2+}-free medium, *i.e.* before being exposed to Ca^{2+}, but only partially reduced in cells heavily loaded with quin2. Under the first condition, the gradients are expected to be blunted by the extensive inactivation of the channels, under the second, by the dye. Interestingly, predepolarization was much more effective at inhibiting the first phase of secretion than the increase in intracellular quin2 concentration. The simplest interpretation of these findings is that, although quin2 is very effective in buffering average $[Ca^{2+}]_i$ (in fact no overshoot is usually observed with this dye), the local $[Ca^{2+}]_i$ increases beneath the plasma membrane are so large as to overcome the blunting of the dye.

Last but not least, the study of the late slow secretion phase sustained by the steady state $[Ca^{2+}]_i$ provided information on the $[Ca^{2+}]_i$ threshold for secretion in intact PC12 cells. Such a slow phase was observed when cells, predepolarized with 50 mM $[K^+]_o$, were exposed to Ca^{2+} (measured $[Ca^{2+}]_i$ steady state = 450 nM), but not when predepolarization was with 30 mM $[K^+]_o$ ($[Ca^{2+}]_i$ steady state = 350 nM). Thus, the $[Ca^{2+}]_i$ threshold for secretion should lay in between the two values, *i.e.* be around 400 nM. This value agrees nicely with those determined in permeabilized chromaffin cells and platelets (32–36) and, more recently, in intact neutrophils treated with the Ca^{2+} ionophore ionomycin (37).

Acknowledgments—We are indebted to Drs. F. Wanke, A. Ferrone, S. Treves, and D. Pierobon for stimulating discussion, to Dr. P. Calissano for the gift of PC12 cells, Dr. R. Y. Tsien for the suggestion for the use of fura-2, F. Mazzari for typing, and G. Ronconi for skillful technical assistance.

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