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INACTIVATION CHARACTERISTICS REVEAL TWO CALCIUM CURRENTS IN ADULT BOVINE CHROMAFFIN CELLS

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SUMMARY

1. Two calcium currents were identified by differences in their inactivation characteristics in adult chromaffin cells maintained in short-term primary culture (3–5 days). Calcium currents were recorded by means of the whole-cell configuration using an intracellular medium highly buffered for pH and pCa.

2. Calcium current evoked from a holding potential of −90 mV inactivated along two components: an initial transient with a time constant of 250 ms followed by a plateau.

3. Steady-state inactivation followed two processes which developed at two distinct membrane potentials. One process was half-inactivated at low voltages around −55 mV and affected mainly the initial transient component. The other process, which affected mainly the sustained component of the calcium current, was half-inactivated at voltages around −10 mV. The proportions of these two processes varied greatly from cell to cell.

4. The dihydropyridine antagonists (nicardipine and nifedipine applied at 10^{-5} M) and the phenylalkylamine D600 (5×10^{-6} M) shifted the half-inactivation value towards −55 mV, indicating the suppression of the sustained component. The snail toxin, ω-conotoxin, had the opposite effect; it shifted the half-inactivation value towards −10 mV.

5. The calcium channel agonist Bay K 8644 (10^{-5} M) either had no effect or induced only a slight increase of the response, as did its (−)-enantiomer (10^{-6} M). To interpret the present results, we suggest that the L-component was maximally activated in our recording conditions.

6. In chromaffin cells, the calcium current recorded in whole-cell conditions is composed of two components with properties close to those of N- and L-type currents described in sympathetic neurons.

INTRODUCTION

The identified sequences of the dihydropyridine (DHP) receptor from skeletal and cardiac muscle (Tanabe, Takeshima, Mikami, Flockerzi, Takahashi, Kangawa, Kojima, Matsuo, Hirosi & Numa, 1987; Mikami, Imoto, Tanabe, Niidone, Mori,

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Takeshima, Narumiya & Numa, 1989) provide an approach to the recognition of membrane proteins responsible for calcium transfer; applied to other tissues, there is little doubt that similar proteins will be isolated from the nervous system in the near future. It is likely that a whole family of channels will be found, partly because the nervous system is highly heterogeneous and partly because multiple regulation sites are involved in calcium entry into neurons (see Miller, 1987). Characterization of the calcium channels in preparations suitable for both biochemical and electrophysiological investigation is required. Endocrine chromaffin cells display excitable properties and can be grown in culture. Expression of neuronal phenotypes in these cells grown *in vitro* can be induced by nerve growth factor (NGF) for rat chromaffin cells (Unsicker, Krisch, Otten & Thoenen, 1978; Doupe, Landis & Patterson, 1985), and by α-melanocyte-stimulating hormone (Demeneix & Grant, 1988) and acidic fibroblast growth factor (Claude, Parada, Gordon, D'Amore & Wagner, 1988) for bovine chromaffin cells. This suggests that these cells are suitable for studying the expression of neuronal calcium channels.

In 1982, an extensive study by Fenwick, Marty & Neher established that voltage-dependent calcium entry into chromaffin cells is promoted principally by a current which activated at high voltage and which showed much cell-to-cell variability in its inactivation kinetics (i.e. stimulus-dependent inactivation). Biochemical studies showed that Ca^{2+} fluxes and noradrenaline release were sensitive to DHP agonist and antagonists (Garcia, Sala, Reig, Viniegra, Frias, Fonteriz & Gandia, 1984; Boarder, Marriott & Adams, 1987; Gandia, Lopez, Fonteriz, Artalejo & Garcia, 1987; Lopez, Moro, Castillo, Artalejo & Garcia, 1989). This suggests that in chromaffin cells part of the secretion is related to the opening of a DHP-sensitive (L-type) Ca^{2+} channel. In the embryologically related sympathetic neurons, Ca^{2+} current also has a high threshold for activation (Marchetti, Carbone & Lux, 1986). However, the current is composed of two components (Hirning, Fox, McCleskey, Olivera, Thayer, Miller & Tsien, 1988), a fast-decaying component, sensitive to the snail toxin, ω-conotoxin (the 'N' Ca^{2+} current) and a slow decaying component sensitive to DHP (the 'L' Ca^{2+} current). In sympathetic neurons, ω-conotoxin blocks the N-current (McCleskey, Fox, Feldman, Cruz, Olivera & Tsien, 1987; Jones & Marks, 1989) and the release of noradrenaline, suggesting a dominant role of this N-current in excitation–secretion coupling (Hirning *et al.* 1988). In contrast, the release of noradrenaline by chromaffin cells and the Ca^{2+} influx in response to various stimuli are ω-conotoxin insensitive (Owen, Marriott & Boarder, 1989; Rosario, Soria, Feuerstein & Pollard, 1989). Therefore one expects the expression of 'N' neuronal-type channels to parallel the neuronal differentiation of chromaffin cells. The use of the N-channel as a marker of neuronal differentiation (Usowicz, Becker, Porzig & Reuter, 1989) has been suggested because of kinetic (Streit & Lux, 1988) and pharmacological (Takahashi, Tsukui & Hatanaka, 1985) changes observed during neurite outgrowth or NGF-induced differentiation. However, indications exist for the presence of an heterogeneous population of Ca^{2+} channels in chromaffin cells (e.g. noradrenaline release and Ca^{2+} entry are not fully abolished by DHP antagonists: Owen *et al.* 1989; Rosario *et al.* 1989). This prompted us to analyse the Ca^{2+} currents in chromaffin cells, since the Ca^{2+} currents evoked in our culture conditions often displayed complex kinetics. Data reported in this first paper allow the distinction at the macroscopic level of two

Ca^{2+} currents, a distinction based on inactivation characteristics and pharmacological properties. The accompanying paper describes the elementary properties of the underlying calcium channels. One of the channel types can be assigned unequivocally to the current component inactivating at about −50 mV. Although present in a large majority of patches, this channel is a minor contributor to the total current in the differentiated endocrine chromaffin cell.

METHODS

Chromaffin cells, prepared at the laboratory of D. Aunis (Centre de Neurochimie, Strasbourg), were isolated after enzymatic dissociation of bovine adrenal glands collected at the local abattoir (for exact protocol, see Bader, Ciesielski-Treska, Thierse, Hesketh & Aunis, 1981). The cells were plated on collagen-coated Petri dishes and maintained in DMEM (Dulbecco's modified essential medium) supplemented with 15% heat-inactivated horse serum for 3–5 days before the electrophysiological experiments. Shortly before recording, this solution was replaced by a saline solution (see below). Current recordings, performed on small-diameter cells ($< 20\ \mu$m) so as to minimize space-clamp errors, were obtained using the whole-cell recording configuration. Current traces were amplified with an EPC-7 patch-clamp amplifier (Darmstadt, Germany) and stored on videotape or directly on an IBM personal computer before analysis.

Calcium currents were recorded in isolation by minimizing movements of most other ions and by fixing the calcium gradient with an internal EGTA buffer (30 mM). A highly pH-buffered internal solution was used (100 mM-HEPES) to minimize calcium transfer caused by small changes in pH (Byerly & Moody, 1984). Since the EGTA dissociation constant is pH dependent, better control of pCa was achieved ($K_{Ca} = 1{\cdot}5 \times 10^{-7}$ M at pH 7·2; Martell & Smith, 1974). In a previous paper (Bossu, Rodeau & Feltz, 1989), we showed that solutions highly buffered for pCa allowed better discrimination between two components of the current activated with a high threshold. Finally, the internal solution in the pipette was (in mM): HEPES/CsOH, 100; EGTA, 30; $CaCl_2$, 3 (pCa 7·73); $MgCl_2$, 2; pH 7·2; and the bath solution contained (in mM): Tris-trichloroacetate, 110; $CaCl_2$, 5; $MgCl_2$, 2; TEA (tetraethylammonium chloride), 10; HEPES, 10; glucose, 10; pH 7·4. Note that we have introduced a minimal amount of chloride in keeping with isotonic conditions so as to bring the chloride equilibrium potential to 0 mV. Any calcium-dependent chloride current should be minimal due to reduction both of the elementary Cl^- conductance at such low Cl^- concentrations and of the driving force on Cl^- ions. For comparison some experiments were carried out using solutions with a chloride concentration closer to the physiological range. In the latter cases, the pipette solution contained (in mM): CsCl, 120; TEA, 20; $MgCl_2$, 2; HEPES, 10; EGTA/CsOH, 11; $CaCl_2$, 1; pCa 8, pH 7·2; and the external solution (in mM): choline chloride 130; glucose, 10; HEPES/Tris, 10; $CaCl_2$, 5; $MgCl_2$, 2; TEA, 10; pH 7·4. Drugs were applied in the bath at the final concentrations reported in the text. Stock solutions of DHP (Bayer, Wuppertal, Germany) were prepared in dimethylsulphoxide at a concentration of 10^{-2} M. D600 (Gallopamil hydrochloride; Knoll, Ludwigshaven, Germany) was prepared at 10^{-2} M in 0·1 M-HCl. ω-Conotoxin GVIA (Peptide Inst., Osaka, Japan) was prepared in a saline solution which contained a minimal amount of divalent cations, namely 0 Ca^{2+} and 1 mM-Mg^{2+} (Marqueze, Martin-Moutot, Levêque & Couraud, 1988), and was applied at a concentration of 3×10^{-7} M for 20 min (stock solution kept frozen at 3×10^{-4} M in water until dilution). No attempt was made to correct for junction potentials.

To describe the individual current traces, exponential fits were routinely carried out on the decaying phase of the records, starting 20 ms after the current peak to avoid contamination by the obviously fast activation process. This was achieved using a non-linear regression analysis (Bevington, 1969) according to a routine written by J.-L. Rodeau (Strasbourg). In some instances, the same traces were also fitted using the pCLAMP-4·0 programs of Axon Instruments (Burlingame, USA). Since pulses of 350 ms duration were routinely used to depolarize the cells, a time constant of more than 1 s was of poor significance and could be approximated by a constant. In such cases, similar estimates were obtained using one or the other method. Non-linear regression analysis was also used to describe current–voltage (I–V) and inactivation curves. Fits of I–V curves were obtained assuming an activation curve of a Boltzmann type with an half-activation at a potential $V_{\frac{1}{2}}$, and

an e-fold change around $V_{\frac{1}{2}}$ over k mV. Then if E is the reversal potential of the current and g the maximal conductance,

$$I = g(V-E)/\{1+\exp[-(V-V_{\frac{1}{2}})/k]\}$$

The inactivation curve of a current component was also well described by a Boltzmann-type equation,

$$A = 1/\{1+\exp[(V-V_{\frac{1}{2}})/k]\}$$

where the current amplitude A has decreased to half-amplitude at the potential $V_{\frac{1}{2}}$ with an e-fold change over k mV. In some cases, on examination of the total current, the inactivation curve was better described by the sum of two such equations; the relative amplitude of the current A was then

$$A = a/\{1+\exp[(V-V_{\frac{1}{2}})/k]\}+(1-a)/\{1+\exp[(V-V_{\frac{1}{2}})/k']\},$$

where a and $(1-a)$ represent the amplitudes of both components. Such non-linear regressions were used on individual cells, and mean values of the estimated parameters were used to generate a curve; S.D. on each parameter is given.

RESULTS

Typically, in chromaffin cells, Ca^{2+} currents developed only in response to depolarizing steps bringing the membrane potential above -20 mV from a holding potential of -90 mV and reached peak amplitude (236 ± 97 pA, $n=13$) at $+5\pm10$ mV. Such a current–voltage characteristic shows up in the steepness of the activation curve. Indeed, around -5 mV, the potential corresponding to half-activation $V_{\frac{1}{2}}$, an e-fold change of the conductance was observed over 7 mV (the k value in a fit of the Boltzmann type, see the Methods section). On careful examination, we could not detect in these cells any current evoked at low potential. These observations are in line with the initial description of Fenwick *et al.* (1982) on chromaffin cells, and do not differ with the observations on PC12 tumour cells or on embryologically related sympathetic cells (Marchetti *et al.* 1986). Most strikingly, as illustrated in Fig. 1, and as already pointed out by Fenwick *et al.* (1982), current traces differed from cell to cell on a kinetic basis. In one group of cells, the Ca^{2+} current developed to a maximal value and then maintained a sustained value (Fig. 1, ▲, left). In a second group of cells, the current after reaching a peak value decayed quickly before eventually stabilizing at an almost plateau value (Fig. 1, ●, right). In the two cases, I–V curves (Fig. 1*B*) displayed identical characteristics. But since the inactivation time constants of the calcium current appeared to vary greatly from one cell to another, we analysed the inactivation process first.

Inactivation of the total current

From cell to cell ($n=13$), the inactivation process examined with the protocol described for Fig. 2*A* varied greatly. There was a wide scatter in the values of the pre-pulse potentials $V_{\frac{1}{2}}$ for which the response was reduced to half its maximal amplitude. For instance, in the examples illustrated in Fig. 2*B*, $V_{\frac{1}{2}}$ varied from -18 to -52 mV, and in most cases, was an intermediate value. Closer inspection revealed that each curve might be the sum, in variable proportion, of low- and high-voltage inactivating components. A complex regression according to this hypothesis (namely the superimposition of two Boltzmann-type inactivations) was carried out on two cells (Fig. 3). A non-linear regression was fitted to the eleven experimental points and yielded five parameters, four to describe the two sigmoïds and one to describe the

relative amplitude of these two components. In Fig. 3*A*, the two individual inactivation curves have been drawn and the resulting fit as well. The quality of the fit suggested a composite origin of the underlying Ca^{2+} channels. Furthermore, if the inactivation of the current at the end of a 350 ms depolarizing step was fitted (Fig.

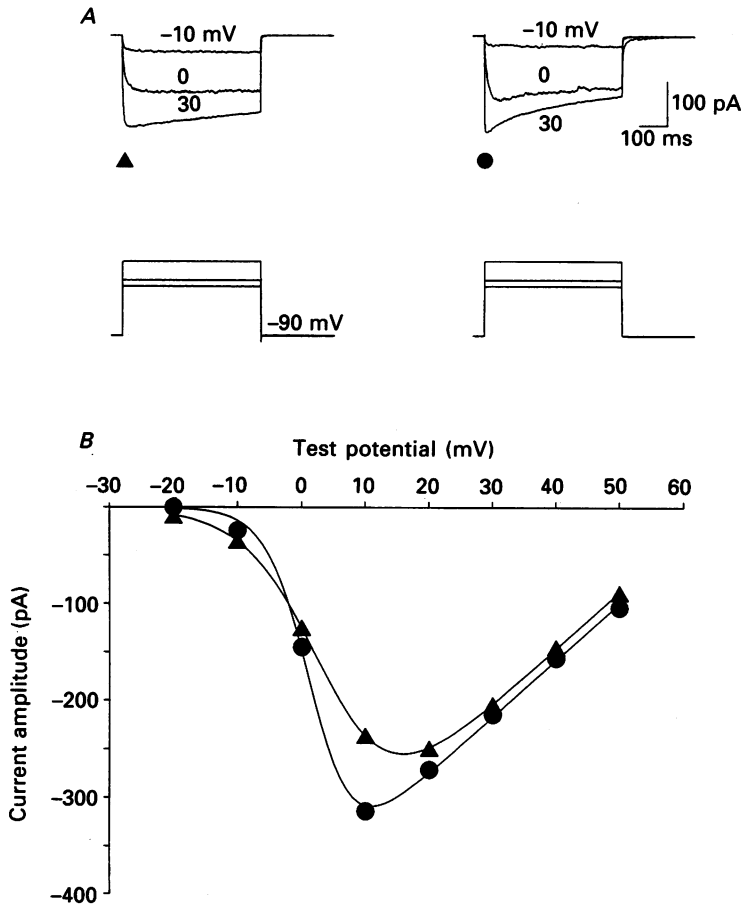

Fig. 1. Calcium current in adult chromaffin cells. *A*, currents evoked by successive depolarizing steps from a holding potential of −90 mV to the value indicated on each trace. Two cells (▲, left and ●, right) in which the evoked Ca^{2+} current showed distinct kinetic properties. *B*, the current–voltage relationship for each series of current traces has been plotted by measuring the peak amplitude of the current at each potential. The continuous lines correspond to fits of the activation curves using parameters calculated from non-linear regressions of the experimental points (see Methods). The maximal conductance, the estimated reversal potential of the current for half-activation $V_{\frac{1}{2}}$ and k were 5·9 nS, 65 mV, −4·2 mV, 5·8 mV (▲), and 5·9 nS, 67 mV, −1·7 mV, 3·4 mV (●) in each case respectively.

3*B*, ○), the fit yielded a curve which closely resembled the curve at the right of Fig. 2. This suggested a dual origin of the inactivation curve. The component related to the sustained phase of the calcium current would be inactivated only at high

potential. The peak current would inactivate along a composite curve with both the characteristics of the sustained current and a transient initial phase. The next set of experiments was aimed at an isolation of this initial component.

Inactivation of the transient phase

Since the high-voltage-activated current in other cells had been shown to be associated with two channel types and, confident in the fact that we had defined the

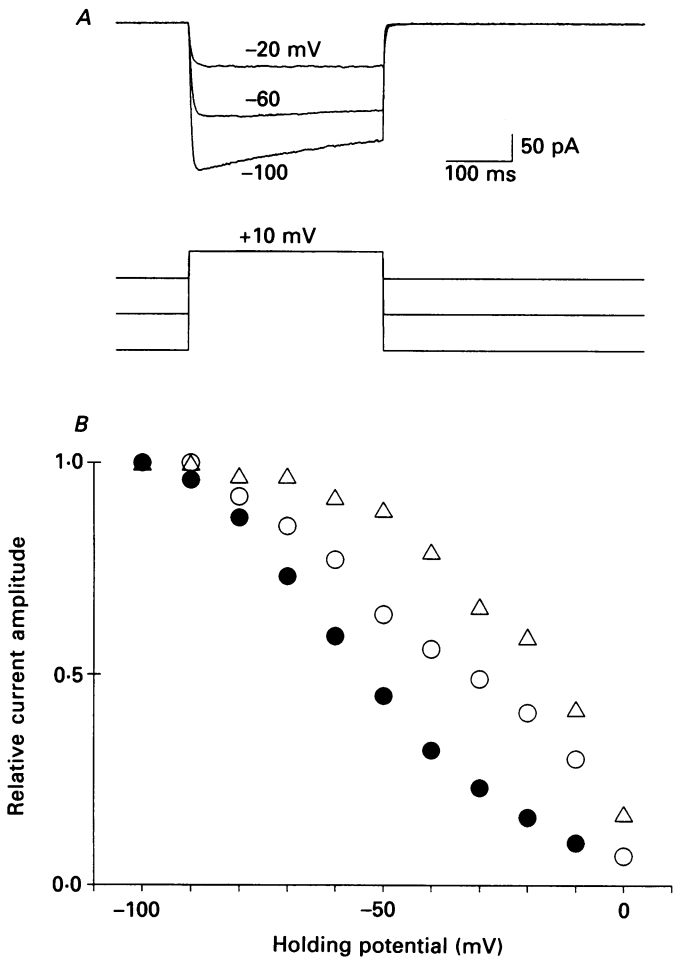

Fig. 2. Inactivation characteristics vary from cell to cell. *A*, successive current traces were obtained using the protocol illustrated in the middle panel trace in which a depolarizing step potential to +10 mV, which evoked a maximal response, was preceded by a pre-pulse (as indicated on the current trace at the top). The holding potential is likely to correspond to steady-state conditions since it was maintained at least 30 s before the test pulse was applied. *B*, the steady-state inactivation curves show the amplitude of the response relative to the maximal response as the pre-pulse potential is varied. Three different cells with half-inactivation at −52 (●), −33 (○), and −18 mV (△).

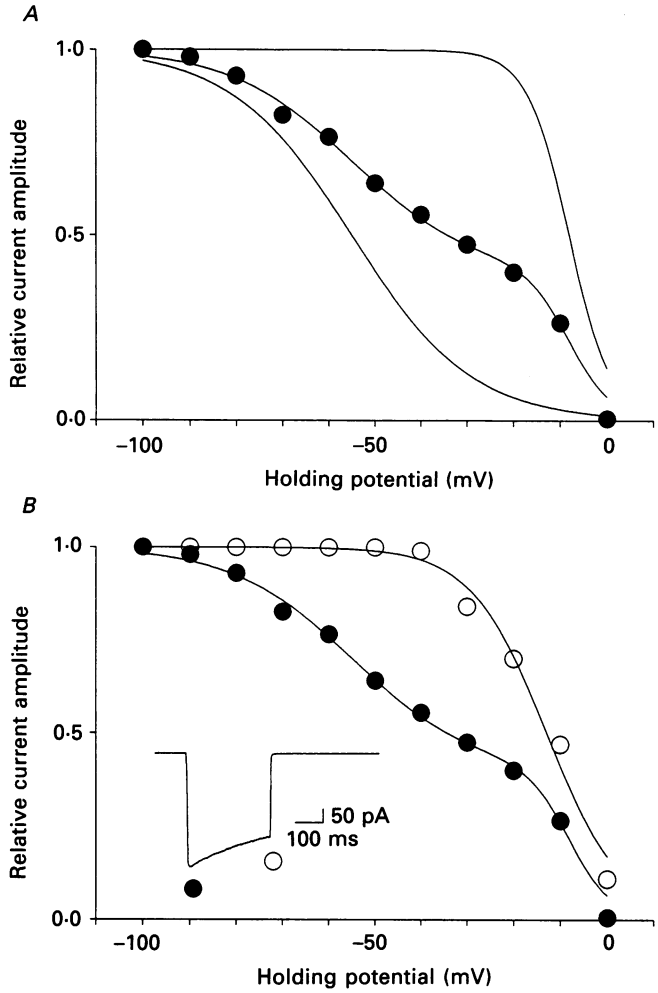

Fig. 3. A composite steady-state inactivation curve is analysed in terms of two superimposed processes. *A*, points show averaged values obtained from two cells which obviously showed steady-state inactivation with two components of almost equal amplitude. The linear regression analysis was carried out assuming that the two inactivation processes were superimposed, each of a Boltzmann type, It yielded the two individual curves shown by continuous lines; the corresponding parameters (see Methods section) were: $V_{\frac{1}{2}} = -8$ mV, $k = 4{\cdot}5$ mV and $V_{\frac{1}{2}} = -55$ mV and $k = 13$ mV for the curves at the right and the left respectively. The final fit to exponential data was obtained with a relative amplitude of 0·6 (and 0·4) for the components inactivating at low (and high) voltage respectively. *B*, same cells and same analysis but carried out on the current amplitude at the end of the depolarizing pulse (○). The fit, obtained assuming a single process, corresponds to an inactivation process with $V_{\frac{1}{2}} = -13$ mV, $k = 8$ mV. Data at peak current (●) and corresponding fit already presented in *A* are shown again for comparison.

conditions for stable recordings (see Methods), we tentatively interpreted the decay of the calcium current in terms of two components. This prompted us to undertake a kinetic description of the decaying part of the responses after they had reached a peak value. An exponential fit was performed on the individual traces recorded at

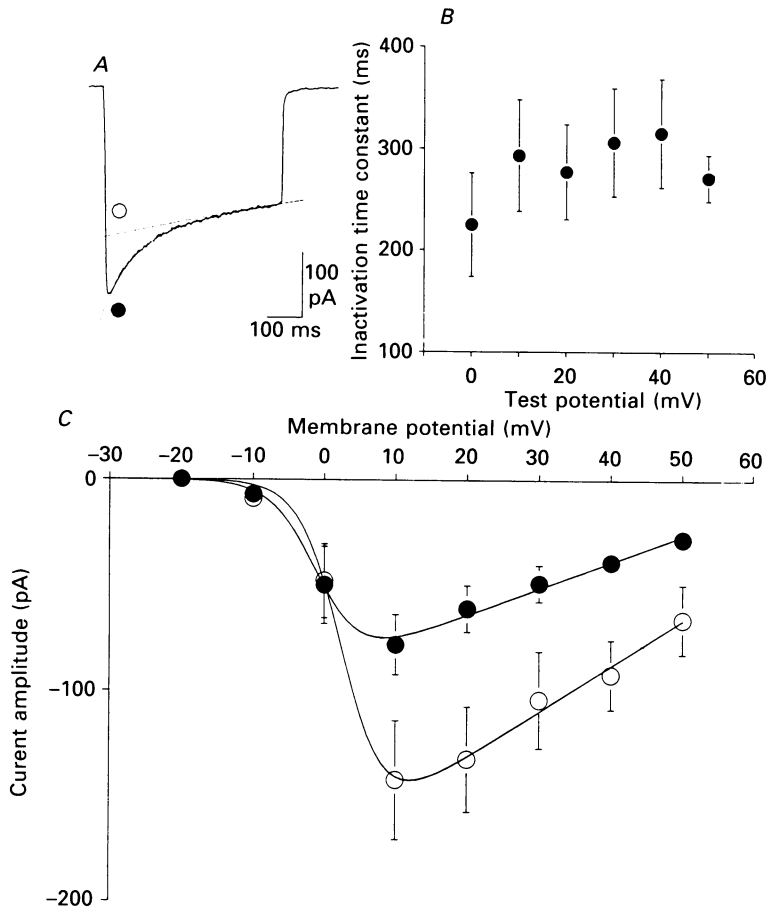

Fig. 4. Kinetic analysis of the Ca^{2+} currents. *A*, the decaying part of a current trace has been fitted by two exponentials a fast (f) and a slow (s) one, each defined by two parameters: the extrapolated amplitude A of the exponential to the time the potential was stepped to a depolarized value and the time constant. The slow exponential and the sum of the two exponentials used in the fit have been drawn (dotted lines) on the current trace; here $A_f = 93$ pA, $\tau_f = 82$ ms, $A_s = 227$ pA, $\tau_s = 1{\cdot}9$ s for a jump to 0 mV from -90 mV. *B*, shows the value of τ_f when the voltage was varied. τ_s exceeded in all cases 1·5 s (6 cells). *C*, the current–voltage characteristics have been plotted for A_f (●) and A_s (○). Data from the same cells as in *B*. Each point is the mean ± S.E.M. Characteristics of the activation curves were for the fast component $V_{\frac{1}{2}} = -0{\cdot}9$ mV and $k = 3{\cdot}2$ mV and for the slow component $V_{\frac{1}{2}} = +2{\cdot}8$ mV and $k = 3{\cdot}0$ mV. Estimated E_{Ca} values were +71 and +80 mV respectively. Maximal conductance for the transient component was $1{\cdot}26 \pm 0{\cdot}1$ nS, and for the sustained component $2{\cdot}16 \pm 0{\cdot}2$ nS.

different membrane potentials in eleven cells. In five cells the decay of the total Ca^{2+} current was well fitted by a single exponential with a very slow time constant (several seconds), and it was described as a plateau in our experimental conditions (350 ms pulse duration, see Methods). For the six remaining cells, the decay was well fitted

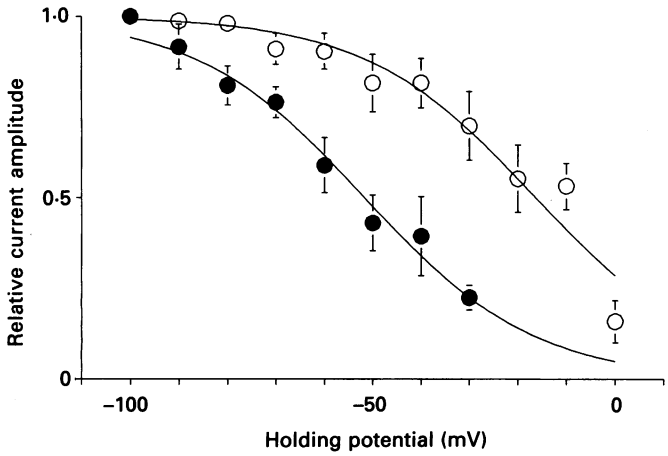

Fig. 5. Steady-state inactivation curves for the slow and fast components. The amplitudes of A_f and A_s have been measured when the holding potential was varied ($n = 6$). Inactivation of A_s (○), assuming a single process, developed with $V_{\frac{1}{2}} = -17$ mV and $k =$ 17 mV, and of A_f (●) with $V_{\frac{1}{2}} = -52$ mV and $k = 17$ mV. Corresponding fits are shown with continuous lines. Points give mean values, and vertical bars ± S.E.M.

by the sum of two exponentials (Fig. 4*A*), the plateau component (A_s) following a fast component (A_f) decaying with a characteristic time constant of about 250 ms. The latter component accounted for 30% of the total Ca^{2+} current and its decay was almost insensitive to membrane potential (Fig. 4*B*). The activation curves of these two components are almost superimposable (Fig. 4*C*; note the similarity of the activation parameters calculated for both A_f and A_s estimated at varied membrane potential. The estimated reversal potential for both slow and fast components was between +70 and +80 mV. Half-activation occurred around 0 mV and k was similar (3 mV in both cases).

When considering the activation curves, this kinetic analysis proved to be of poor discriminative value, so we turned to the analysis of the respective inactivation curves of both these components. This analysis was carried out on the six cells where two kinetic components could be clearly identified, and the results are displayed in Fig. 5. Clearly each component inactivated in a distinct range. The estimated half-inactivation for the fast component was −52 mV, whereas for the slow component it occurred at the more depolarized potential of −17 mV (a value close to the value obtained in Fig. 3*B*). The picture henceforth arising was that of two distinct populations of channels with quite similar activation characteristics, but developing with distinct kinetics and also inactivating at distinct potentials. The following pharmacological experiments were designed to obtain further evidence for two distinct components of the Ca^{2+} current.

Inactivation characteristics in presence of Ca^{2+} antagonists

Dihydropyridine antagonists, phenylalkylamines and ω-conotoxin have been used to block selectively each component of the Ca^{2+} current. To assess the effect of a substance, we used a sampling procedure in which inactivation curves were

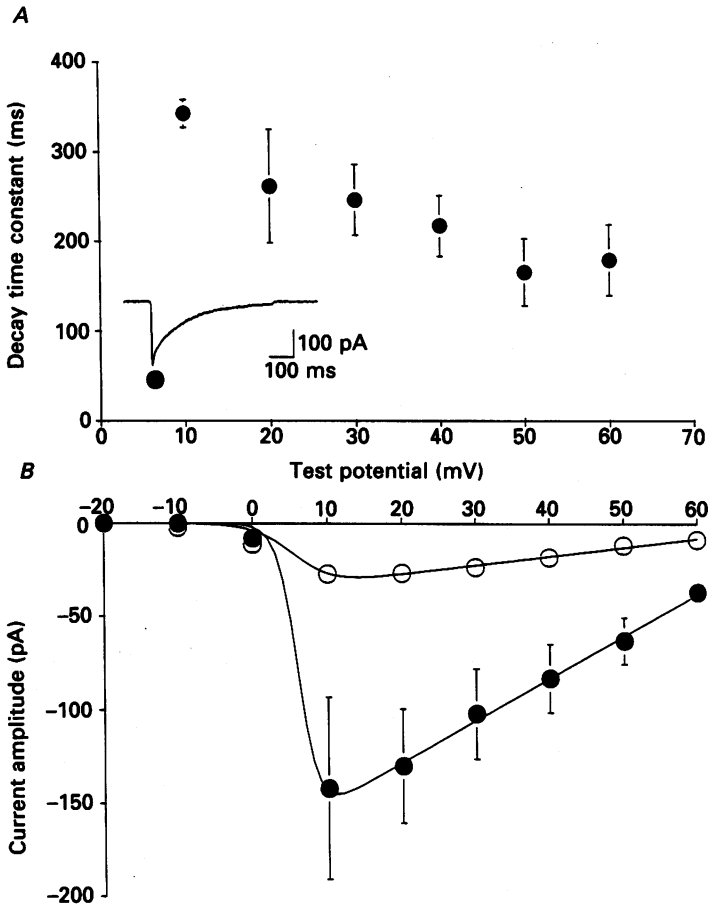

Fig. 6. Characteristics of the Ca^{2+} current in the presence of nicardipine, 10^{-5} M. *A*, inset illustrates a Ca^{2+} current trace in presence of the dihydropyridine antagonist. The current appears as a prominent peak followed by an exponential decay nearly reaching baseline level before the end of the 350 ms pulse. The value of the time constants of the fast decaying components on such traces has been plotted at various membrane potentials. Points show mean values ± S.E.M. obtained from six cells. *B*, activation curve for the fast (●) and the slow (○) components in presence of nicardipine. Data from six cells (mean values ± S.E.M.). Characteristics of the activation curves were $V_{\frac{1}{2}} = 6{\cdot}0$ mV and $k = 1{\cdot}34$ mV, and $V_{\frac{1}{2}} = 5{\cdot}7$ mV and $k = 2{\cdot}6$ mV for the fast (●) and slow components (○) respectively. Note the reduced amplitude of the slow component.

established for several cells in succession after the drug had been applied to the perfusion medium. Using this approach, we had no internal control. In our previous observations, we observed variability from cell to cell in a single Petri dish in the

kinetics of the current and in the inactivation curve. Once the drug had been applied, we observed only slight variations in the characteristics of the observed responses. Therefore we are confident the curves presented here show an actual effect of the drug, and do not simply reflect the sampling procedure.

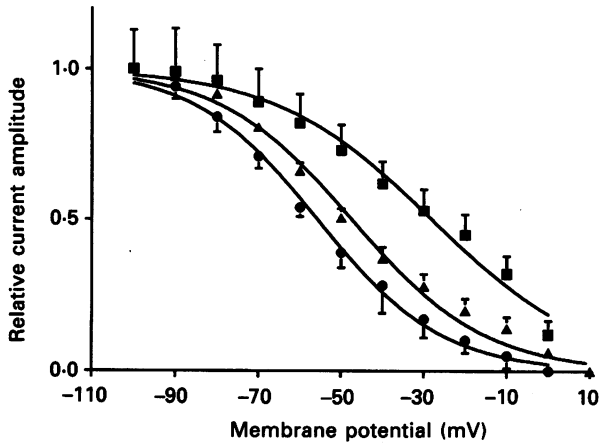

Fig. 7. Steady-state inactivation curves of the Ca^{2+} current remaining in the presence of 10^{-5} M-nicardipine (●), 10^{-5} M D600 (▲) and after a treatment with ω-conotoxin (■). Boltzmann-type fit yielded characteristic values for $V_{\frac{1}{2}}$ and k of −56 and 15 mV, −47 and 15·9 mV, and −28 and 19 mV respectively. Mean amplitudes ± S.E.M. are given in each case (n = 6, 5 and 7 cells).

After nicardipine application at 10^{-5} M, the current typically inactivated to zero within the 350 ms of the depolarizing pulse (inset in Fig. 6*A*). The decay of the Ca^{2+} current in this condition could be described mostly by a single exponential with a time constant of about 250 ms at all potentials (Fig. 6*A*), associated with a small plateau component. *I–V* curves for the fast-decaying component and the plateau phase as deduced from kinetic analysis were established (Fig. 6*B*). When comparing *I–V* curves in control conditions (Fig. 4*C*) and in the presence of nicardipine, the main effect of this DHP antagonist proved to be a reduction of the plateau component (compare the mean values of 150 pA in control, and 24 pA in presence of nicardipine). This suggests that after nicardipine blockade, mostly the transient Ca^{2+} component remained. This was confirmed on a single cell where the plateau component had been measured before (104 pA) and after (31 pA) nicardipine application, and the decaying component was shown to be unaffected. Further the inactivation curve of the remaining current after nicardipine treatment could be fitted with a single Boltzmann equation (Fig. 7, ●) with parameters close to those of the inactivation curve for the fast decaying component estimated from kinetic analysis in control conditions (compare with Fig. 5, ○).

Similar reduction of the plateau phase and shift of the inactivation curve towards more hyperpolarized potentials was observed in the presence of nifedipine (data not shown).

In the presence of the phenylalkylamine D600 applied at 5×10^{-6} M (Fig. 7, ▲), the inactivation curve so obtained could again be fitted with a dominant sigmoid with

$V_{\frac{1}{2}}$ in a hyperpolarized domain (−47 mV to be compared to −56 mV after nicardipine). This suggests that D600 blocks specifically the slow component of the Ca^{2+} current which inactivates at depolarized potentials. However, at the concentration we used, this effect was less pronounced than with DHP antagonists.

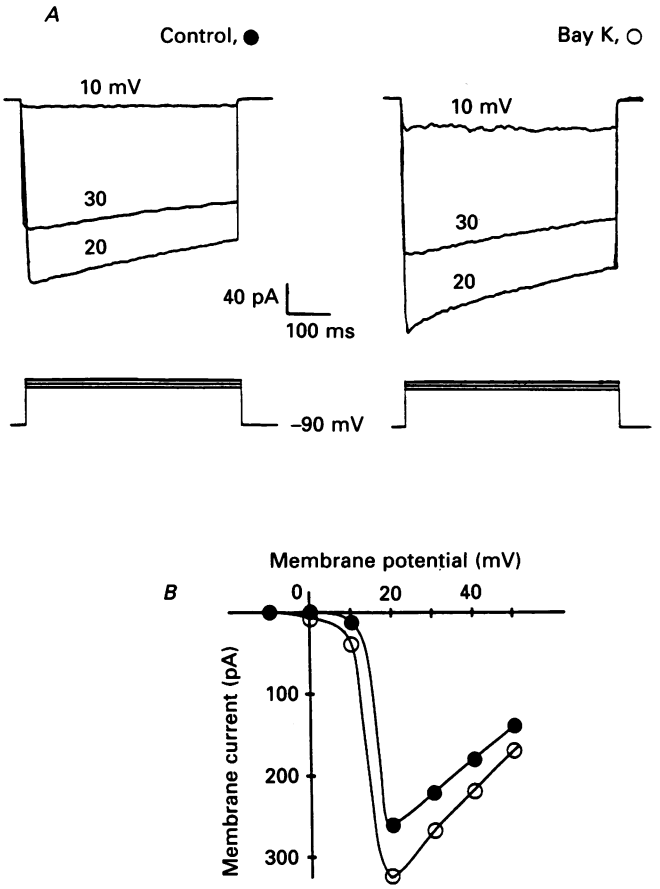

Fig. 8. Effect of the dihydropyridine agonist (−)-Bay K 8644 (10^{-6} M) on the chromaffin cell Ca^{2+} current. The (−)-enantiomer was used instead of racemic to avoid eventual mixed agonist and antagonist effects. *A*, individual current traces in control conditions (●) and after the racemic agonist application (○) obtained at the potentials indicated on traces. *B*, same cell as in *A*. *I–V* curves for the peak current before (●) and after application (○) of Bay K 8644 (continuous lines are eye-fitted). Test pulse duration, 350 ms.

Since ω-conotoxin has been reported to block a class of channels different from the ones affected by dihydropyridines, we also looked at its effect on the Ca^{2+} current evoked in chromaffin cells. Cells were tested after 3×10^{-7} M-ω-conotoxin had been applied for at least 20 min. The inactivation curve established after ω-conotoxin application had a mid-point corresponding to half-inactivation around −28 mV (Fig. 7, ■). When compared to the previous results, the data suggested that most of the transient component had been abolished. However, closer examination of the

inactivation curve revealed that some of this component remained. In fact, the inactivation curve measured at the end of the 350 ms pulse was still shifted towards depolarized potentials ($V_{\frac{1}{2}} = -20$ mV).

Effect of DHP agonist

Two results indicated that the DHP agonist, Bay K 8644, should have a pronounced effect on the Ca^{2+} current. D600 and DHP antagonists had drastic effects on the steady-state inactivation characteristics and the decay of the Ca^{2+} current and ω-conotoxin had only slight effects. To our surprise, the racemic Bay K 8644 (10^{-5} M) had no effect ($n = 3$). As illustrated in Fig. 8, we observed in two cells only a slight increase in the current (compare control traces to traces recorded after Bay K 8644 application, Fig. 8*A*) and no shift in the *I–V* curve (Fig. 8*B*).

DISCUSSION

Voltage-dependent calcium entry into chromaffin cells has been characterized previously by pharmacological means usually by measurement of responses produced by elevated K^+ concentration. Either $^{45}Ca^{2+}$ influx or release of labelled noradrenaline from pre-loaded chromaffin cells was measured. These biochemical data indicate that Ca^{2+} entry is mainly through DHP-sensitive (L-type) Ca^{2+} channels (see reference in Owen *et al.* 1989). Our present electrophysiological data demonstrate the possible existence of two distinct voltage-activated Ca^{2+} currents based firstly on combined kinetic and inactivation arguments and secondly on pharmacological observations. In half of the cells we studied, up to 40 % of the total Ca^{2+} current could be accounted for by a transient component (decaying by e-fold in about 250 ms) which was inactivated at high voltage, was almost completely abolished by ω-conotoxin application, but remained after application of DHP antagonists. These characteristics point to a current of the N-type. The second component of the current is dominant and corresponds to Ca^{2+} entry decaying within a few seconds; moreover, it is abolished by DHP antagonists but remains in the presence of ω-conotoxin. In the first section we shall discuss the validity of the kinetic approach that we have chosen before showing that additional properties (pharmacology and inactivation properties) allow identification of distinct Ca^{2+} currents in chromaffin cells. Finally attention will be given to the paradoxical observation that the whole-cell response is sensitive to DHP antagonists but almost insensitive to Bay K 8644.

Kinetic analysis

Kinetic analysis of Ca^{2+} currents in whole-cell recordings is usually of little value. Firstly, Ca^{2+} currents show a progressive reduction in amplitude and alterations in kinetics during the recording period (Fenwick *et al.* 1982). Secondly, even when recordings are performed immediately after rupturing the cell membrane, pipette solutions of slightly different composition and therefore of the internal medium are known to modify profoundly the kinetics of the response. These problems make comparisons between various reports difficult. We introduced 100 mM-HEPES into the intracellular medium in order to provide better control of the internal pH and consequently of the internal pCa. As a result we observed that Ca^{2+} currents recorded

in nodose neurons and in chromaffin cells stabilized within minutes (Bossu *et al.* 1989; this report). Therefore, good control of the internal pH appears to maintain the activity of the Ca^{2+} channels even without an external supply of ATP. If one further assumes that an ATP-dependent phosphorylation is necessary for the opening of Ca^{2+} channels, then a basal production of ATP must have been maintained by the cell in our internal solution.

In whole-cell recordings from sympathetic and sensory neurons, two distinct Ca^{2+} currents ('N' and 'L') have been identified by their kinetics (see Swandulla & Armstrong, 1988 and Belluzzi & Sacchi, 1989). The N-component decays characteristically with a time constant of about 50 ms in DRG sensory neurons (Fox, Nowycky & Tsien, 1987; Gross & MacDonald, 1988) and about 200 ms in sympathetic neurons (Hirning *et al.* 1988; Plummer, Logothetis & Hess, 1989). The somewhat slower time constant observed in sympathetic neurons agrees well with the time constant of 250 ms we observed in chromaffin cells. Nevertheless, one should recognize that this similarity may be coincidental since variations in the decay time constants are known to depend on the cytoplasmic Ca^{2+} concentration and therefore on the whole-cell recording conditions (Bossu *et al.* 1989).

Inactivation characteristics

Two inactivation time constants are an indication of two underlying channel activities (rather than of a single channel with complex opening kinetics). Moreover, each component was half-inactivated at distinct potentials, some 45 mV apart: −52 mV for the transient component and −8 mV for the sustained component. Since these values were obtained in steady-state conditions in which we took care to reduce cumulative effects that could lead to extinction of the response, they appear to be characteristic of two distinct types of underlying elementary events. As expected from the use of complex saline solutions for the internal and external media (see Methods), no direct comparison of the potentials leading to half-activation could be made between the data presently obtained and those obtained with a cytoplasmic medium closer in composition to Locke's solution. Nevertheless, both approaches (with either 110 mM-HEPES/Tris or as in Fig. 9, 130 mM-CsCl) revealed, the presence of two components, each inactivating at a distinct potential. Calcium currents in undifferentiated PC12 cells also display a dual type of inactivation process (Janigro, Maccaferri & Meldolesi, 1989).

Pharmacological properties

The two kinetic components also differed in their pharmacological sensitivity. The slowly decaying component inactivated at high threshold, was selectively abolished by DHP antagonists and remained in the presence of ω-conotoxin. Virtually opposite properties were obtained for the transient component.

The most important observation in the present work was that Ca^{2+} currents in chromaffin cells were insensitive to Bay K 8644 at a relatively high concentration of 10^{-5} M. In comparison, effective concentrations in the cardiovascular system were much lower. In sharp contrast, part of the current is abolished by DHP antagonists, which reveals a DHP sensitivity of the activated channels. Furthermore, half-inactivation for the remnant current occurs at more hyperpolarized potentials, at a

holding potential where the DHP-insensitive N-current is expected to be inactivated. The increased effectiveness of the DHP at more depolarized potentials has been reported for all charged DHP antagonists (see references in Triggle & Rampe, 1989) and would account for this shift in the inactivation curve. Similarly the blocking

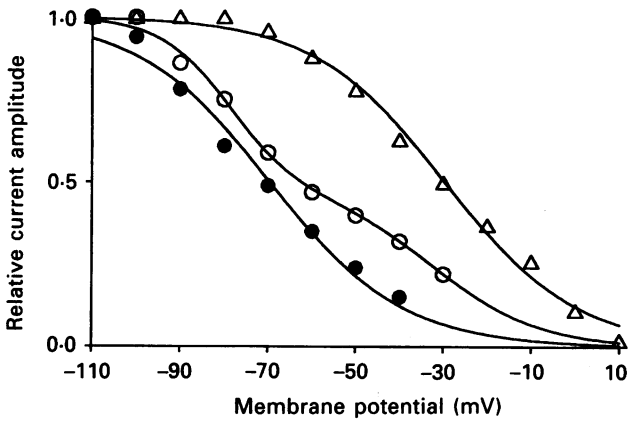

Fig. 9. Steady-state inactivation of Ca^{2+} current displays cell-to-cell variability when recordings are made in a choline chloride external solution and with caesium-loaded cells ($n = 3$ cells). The two extreme inactivation curves are fitted by a single Boltzmann equation, with distinct $V_{\frac{1}{2}}$ values (●, −70 mV; △, −30 mV). The middle curve (○) is fitted by a weighted sum of two Boltzmann equations (see Methods), with $V_{\frac{1}{2}} = -79$ mV, and $k = 8$ mV for the first component (fraction 0·52) and $V_{\frac{1}{2}} = -32$ mV and $k = 12$ mV for the second one (fraction of 0·48).

effect of the phenylalkylamine D600 though not (or at least less) voltage dependent (Kostyuk, 1984; McCobb, Best & Beam, 1989) allows the recording in isolation of the current component inactivating in a hyperpolarized domain as well. A straightforward conclusion would be then to assume that for unknown reasons in these cells on patch rupture all the Ca^{2+} channels of an L-type are activated, and that the DHP-sensitive component is revealed only through an antagonist action of the DHP which leaves only the N-component. This assumption is contradictory to the conclusion of some authors who interpret a slight effect of the Ca^{2+} agonist Bay K 8644 as indicating an absence of DHP-sensitive channels (Aosaki & Kasai, 1989; Plummer *et al.* 1989). We think the recordings of the channel activity we show in the following paper (Bossu, De Waard & Feltz, 1991) support our present hypothesis. They reveal a sensitivity to Bay K 8644, which is seen as an opening of channels otherwise maintained quiescent in intact chromaffin cells (for instance via a GTP-dependent pathway; Dolphin & Scott, 1989).

Up to now it has not been possible to demonstrate pharmacologically that the Ca^{2+} current evoked in chromaffin cells is composed of two distinct components, a DHP-sensitive L-component, and a DHP-insensitive N-component. We know, nevertheless, that in this cell type, ω-conotoxin abolishes maximally half the current (Hans, Illes & Takeda, 1990). We further show that the Ca^{2+} current remaining after the toxin application proved to inactivate at depolarized potentials, as if one was then dealing mostly with an L-type activity. A similar conclusion was reached by

Sher, Pandiella & Clementi (1988) who followed, by Fura-2 fluorescence, Ca^{2+} entry into PC12 cells evoked by elevated K^+ concentration; they showed that the main effect of ω-conotoxin was to abolish the initial DHP-insensitive transient rise in internal Ca^{2+} (see also Pun, Stauderman & Pruss, 1988, on chromaffin cells). In contrast ω-conotoxin is without effect on catecholamine-release experiments from loaded cells $^{45}Ca^{2+}$ flux essays carried out on PC12 (Greenberg, Carpenter & Cooper, 1984) and chromaffin (Owen *et al.* 1989) cells. This apparent inconsistency is probably related to the fact that the detection of any sustained Ca^{2+} entry is enhanced in techniques which do not allow enough time resolution.

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