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TWO TYPES OF CALCIUM CHANNELS ARE EXPRESSED IN ADULT BOVINE CHROMAFFIN CELLS

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SUMMARY

1. Calcium channel activity was recorded in chromaffin cells in the cell-attached condition, using 110 mM-Ba^{2+} as the permeant ion.

2. One type of calcium channel had a conductance of 16 pS, was completely inactivated at a holding potential of −20 mV and was insensitive to dihydropyridine agonists and antagonists. These characteristics correspond to a calcium channel of the N-type.

3. A second type of calcium channel was active at holding potentials of −30 mV and above, had a channel conductance of 31 pS, and was sensitive to the dihydropyridine agonist, Bay K 8644. The channel opened along two dominant modes with characteristic time constants of 0·5 and 5 ms. The main effect of Bay K 8644 was to increase the probability of both short and long openings with no change in their relative proportions (6 to 1 respectively). These characteristics correspond to a calcium channel of the L-type.

4. ω-Conotoxin affected the activity of both N- and L-type channels. It drastically reduced the number of N-type channel openings and produced complex changes in L-type channel activity. Long openings were less frequent and the conductance during short openings was slightly smaller than that measured in the presence of Bay K 8644.

5. The discussion focuses on modulation of L-type channel activity. Openings of L-type channels are rarely recorded in the cell-attached configuration under control conditions. Addition of Bay K 8644 is needed to reveal the presence of L-type channels. By contrast, L-type currents recorded in the whole-cell configuration are always observed and are insensitive to Bay K 8644. These results indicate that L-type channels are normally inoperable in chromaffin cells.

INTRODUCTION

Whenever the function of voltage-activated Ca^{2+} currents is explored, as for example, excitation–secretion coupling, it remains important to distinguish the respective contributions of ionic flows through specific types of calcium channels. At

the elementary level, two types of calcium channels have been described in some neurons that are elicited at depolarized potentials above −20 mV (Nowycky, Fox & Tsien, 1985; Kostyuk, Shuba & Savchenko, 1988*a*). Of the two, only the L-channel has well-defined properties. This channel, named for its large conductance in isotonic barium, is moderately inactivated at a holding potential of −20 mV and is extremely sensitive to dihydropyridine (DHP) agonist and antagonists. 'N' channel characteristics are nearly opposite to those of the L-channel and vary considerably from one preparation to another (Bean, 1989). Moreover, the 'N' channel is not present in all preparations as is the L-channel (Tsien, Lipscombe, Madison, Bley & Fox, 1988). The 'N' channel has been extensively described in dorsal root ganglion (DRG) cells of chick and mouse (Fox, Nowycky & Tsien, 1987; Kostyuk *et al.* 1988*a*; Green & Cottrell, 1988) and is the main source of Ca^{2+} entry into superior cervical ganglion (SCG) neurons of frog and rat (Lipscombe, Madison, Poenie, Reuter, Tsien & Tsien, 1988; Hirning, Fox, McCleskey, Olivera, Thayer, Miller & Tsien, 1988; Plummer, Logothetis & Hess, 1989). A problem that makes the identification of 'N' channels difficult is the large scatter in the estimated conductances (see Bean, 1989). The N-channel conductance can be as low as 11–13 pS in chick dorsal root ganglion (DRG) neurons (Nowycky *et al.* 1985; Kasai, Aosaki & Fukuda, 1987) and rat SCG (Hirning *et al.* 1988) and as high as twice this value in PC12 tumour cells and rat SCG neurons (Plummer *et al.* 1989). In the central nervous system, a report of Gray & Johnston (1987) points to a 14 pS N-type channel in the hippocampal granule cells. Finally, a further complication is that the 'N' channel does not yet have an established pharmacology; it is insensitive to DHP action and appears to be more sensitive to ω-conotoxin than is the L-channel (McCleskey, Fox, Feldman, Cruz, Olivera & Tsien, 1987).

In the preceding paper (Bossu, De Waard & Feltz, 1991), we concluded that two calcium currents are evoked at high voltage in chromaffin cells. This conclusion prompted us to analyse the underlying Ca^{2+} channels present in these cells when they were maintained in short-term culture (less than 5 days). Under these circumstances, because these cells are spherical just as they are *in situ*, there should be no regionalization in the channel distribution and thus all channel types expressed in this adult endocrine cell should be accessible. Clearly two channel types could be distinguished. We show the small conductance (16 pS) channel, present in all patches, is of an N-type, although it does not carry the majority of the Ca^{2+} entering the chromaffin cells through voltage-dependent channels. This observation, although coherent with the ratio of the ω-conotoxin to the DHP binding sites in this preparation, is somewhat paradoxical when compared to the fact that noradrenaline release can be abolished by the DHP action. The discussion focuses on these discrepancies.

METHODS

The methods are mainly those described in the preceding paper. Most high-voltage-elicited calcium channels have been shown to be more permeant to Ba^{2+} than to Ca^{2+}. Therefore, for elementary current recordings, we routinely used as the pipette solution (in mM): $BaCl_2$, 110; HEPES, 10; Tris-OH to give pH 7·4. This solution was assumed to be isotonic to the following external solution which contained elevated K concentration, in order to zero the membrane

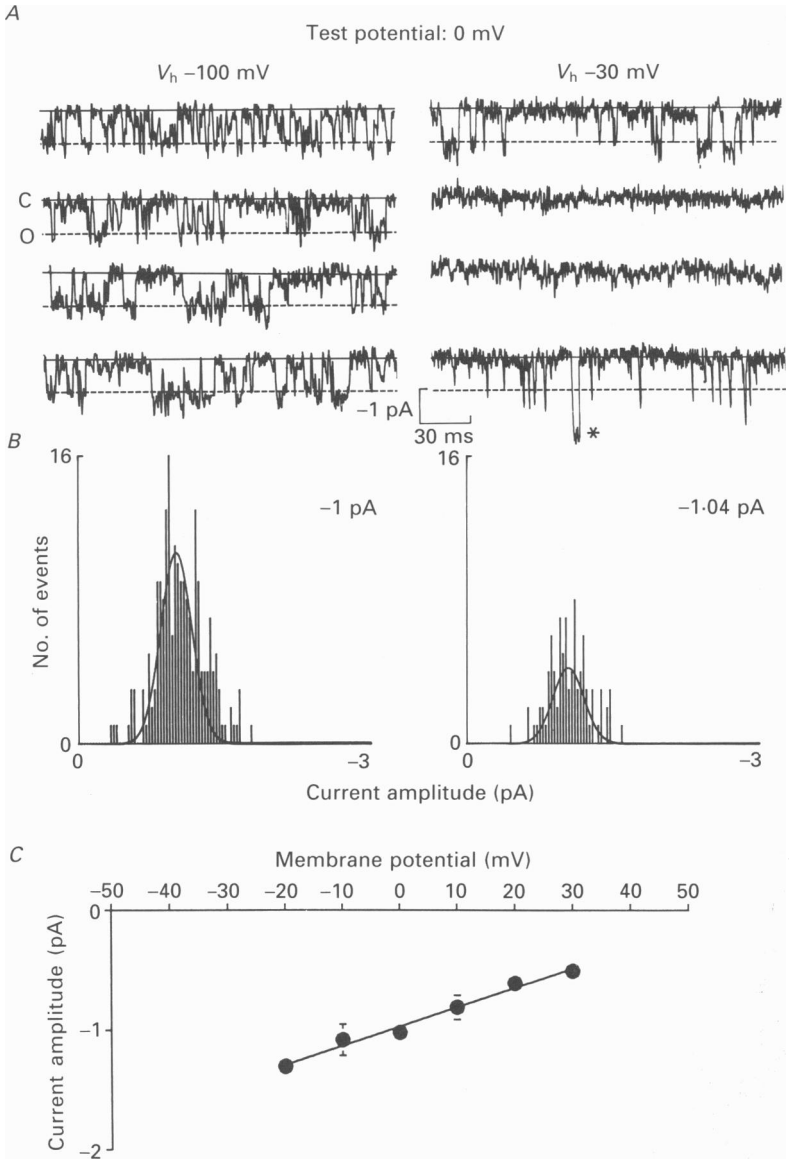

Fig. 1. Characteristics of the inactivating calcium channel evoked in control conditions. *A*, left, typical current traces obtained in cell-attached conditions when depolarizing a patch from a holding potential (V_h) of −100 mV to 0 mV with 500 ms pulses applied at 2 s intervals. All traces displayed activity. The traces shown here were used to establish the corresponding amplitude histogram (see below). Continuous lines show the baseline (C, closed state) and dashed lines show a −1 pA deflection corresponding to the mean amplitude of the elementary events. (O, open state). Sampling frequency, 5 kHz; filter frequency, 1·5 kHz. *A*, right, the same patch was depolarized to 0 mV from a holding potential V_h of −30 mV. Out of a total of ninety-four traces, sixty traces showed no openings (as the two middle traces) and one trace had a large opening of the type underlined by an asterisk. *B*, amplitude histograms corresponding to the events shown in *A*. In each case the data could be fitted (by eye) to a single Gaussian curve. Note that

potential except under the patch. More precisely, bath solution contained (in mM): potassium gluconate, 140; EGTA, 10; $MgCl_2$, 1; HEPES, 20; glucose, 10; KOH to give pH 7·4. The voltage-dependent activities were evoked using a protocol in which the membrane potential was held between −90 and −20 mV, and depolarized to chosen values for 350–500 ms. Pulses were applied at 3 s intervals. Drugs (DHP compounds and ω-conotoxin) were prepared as in the previous paper (Bossu *et al.* 1991) when introduced in the pipette, their final concentrations were adjusted to the indicated values. The Bayer calcium agonist Bay K 8644 was applied either as the racemic compound at 10^{-5} M, or as the (−)-enantiomer (Bay R 5417) at 10^{-6} M to induce pure agonist effect. For recording, the electrode potential was first adjusted to zero when the electrode was introduced in the bath containing 140 mM-potassium gluconate. In the presentation of the results, considering the approximate control on the resting membrane potential, we have not corrected for the −16·5 mV junction potential, which is present between the pipette solution and the potassium gluconate solution.

Only traces with a noise below 150 fA were analysed. All current traces were initially recorded at 10 kHz and stored on a videoscope (PCM Sony). For analysis, sampling was carried out in most cases at 5 kHz and filtered at 1 or 1·5 kHz with a low-pass 8-pole Bessel filter. To examine the activity elicited during the depolarizing periods, current traces were analysed off-line (at a final gain of 400 points/pA) with a Plessey 6220 (LSI 11/23) DEC compatible computer and single traces were plotted on a HP 7470A. The amplitudes of the events were individually determined, using interactive programs allowing the selection of windows in each block, and histograms of the amplitudes established (80 bins). Data could be described by Gaussian curves, for which the positions of the peak and value of the standard deviation were individually adjusted. Successive openings and closings of the channels were first tabulated, then, frequency histograms with 80–100 bins were constructed to estimate the open times. A least-squares method was used to compute the amplitude and time constants of either mono- or biexponential distributions.

RESULTS

In conditions where only divalent cation movements were possible, elementary events were recorded in all patches. The different types of activity were identified by their inactivation processes. In control conditions and with the membrane potential held at −20 mV, events appeared to be divided in two groups depending upon whether or not inactivation occurred (as illustrated separately in Figs 1 and 2). Other observations also suggested two channel types.

In Fig. 1*A* and *B*, the channel, which was abolished by holding the membrane potential at −30 mV gave rise to a current with a mean amplitude of 1 pA for a voltage step from −100 to 0 mV. The mean conductance of this channel type estimated on a sample of seven active patches, was 16 pS (Fig. 1*C*). Such activity was very frequent and in fact was recorded in most patches. By contrast, the non-inactivating channel was only present in a few cases, though we routinely maintained the membrane potential of all recorded cells for long periods at −20 mV, a condition we know to enhance its probability of opening; for instance, comparison between the two histograms in Fig. 2*B* shows that the −1·37 pA events on the left were favoured

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depolarizing the holding potential did not modify the amplitude of the individual events. By contrast, inactivation occurred which has been made obvious here by the use of the same scale for the *Y*-axis of the two histograms; each histogram was established over a similar 47 s period. *C*, conductance of the inactivating channels. Channel activity was recorded from seven patches. Histograms were drawn as in *B* for each potential and the mean amplitudes ± S.D. plotted against potential. The regression line through the mean values yielded a conductance of 16 pS.

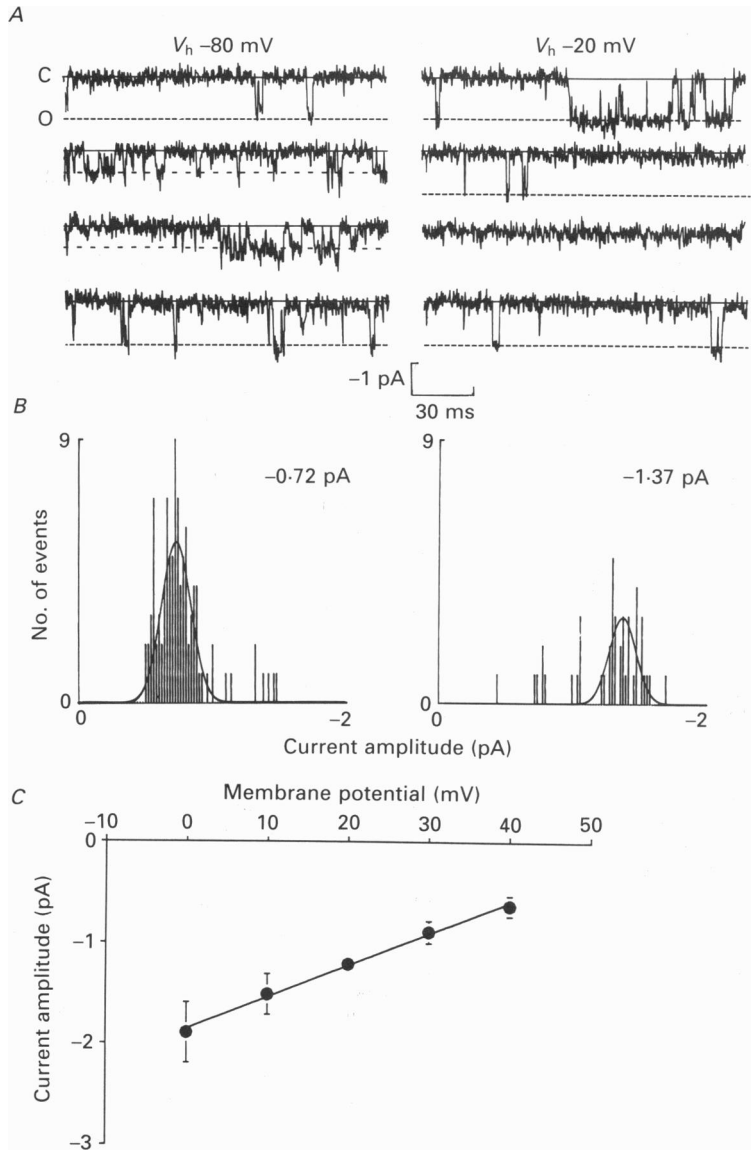

Fig. 2. Characteristics of the non-inactivating Ca^{2+} channels evoked in control conditions. *A*, current traces obtained at a potential of +10 mV in cell-attached conditions by depolarization of a patch from a holding potential (V_h) of either −80 mV (left) or −20 mV (right). Traces on the left contain events of both small and large amplitudes whereas traces illustrated on the right contain only large long-duration events. For each trace, the continuous line points to the baseline. Deflections are shown of −0·7 pA in the middle two traces on the left (widely spaced dashed line) and −1·4 pA (narrowly spaced dashed lines) in the top and bottom traces on the left and in three of four traces on the right. Stimulation and filter conditions are as in Fig. 1. *B*, amplitude histograms of elementary events recorded in the conditions illustrated in panel *A*. The left histogram was generated from 42 s of data and the right one from 63 s of data. Note that by holding the potential at −20 mV (right), the activity corresponding to small events was abolished whereas forty-four large events remained. *C*, conductance of the non-inactivating channels. Mean values ± S.D. (vertical bars; $n = 5$ patches) are plotted against membrane potential. The linear regression obtained from the mean values yielded a conductance of 31 pS. Note the scatter in the event amplitudes for this channel type.

over the few events of the same amplitude on the right. These elementary events evoked at +10 mV had about twice the amplitude of the inactivating events (Fig. 2*A*). The average conductance of the corresponding channel was estimated to be 31 pS (Fig. 2*C*), a value which should be regarded as indicative since only five patches displayed this activity and at low frequency (for instance the forty-four openings used to establish the Fig. 2*B* histogram at the right correspond to an analysis carried over a 63 s period).

On the assumption that there were two distinct populations of channels, our aim was to use classical calcium agonists and antagonists as pharmacological tools to facilitate further distinction. Figure 3 shows current traces recorded from two distinct patches which only displayed a small amplitude, inactivating activity even though either (−)-Bay K 8644 or nicardipine had been added to the pipette solution. It could be shown (Fig. 3*B*) that the amplitudes of the elementary events recorded when the membrane potential was varied were distributed along the same regression line as in control conditions, when either a calcium agonist or antagonist had been added. Also, the event duration was in all conditions just below 1 ms at +10 mV (Fig. 3*C*). A similar value was observed in control conditions when the potential was varied between +10 and −10 mV. One should keep in mind, nevertheless, that such a value was possibly overestimated since in the analysis all events shorter than 0·4 ms have been discarded because no reliable estimate of their amplitude could be made. Furthermore some infrequent long openings (> 10 ms) were recorded in control conditions (see Fig. 1*A*) and in the presence of nicardipine (Fig. 3*A*).

In contrast, the large amplitude and non-inactivating activity was dramatically affected by an application of Bay K 8644 when it was introduced in the pipette at quite high concentration, 10^{-5} M for the racemic compound and 10^{-6} M for the (−)-enantiomer. This activity, rare in control conditions, was then observed in almost all patches recorded (8 cells). As in heart cells or sympathetic neurons, bursts of activity with prolonged openings were frequently evoked, as illustrated in Fig. 4. Further, a latency of some 30 s to a few minutes systematically elapsed after drug application before any effect was observed, even though high concentrations were used.

Conductance and gating properties of the DHP-sensitive events were easily resolved in the presence of Bay K 8644. The main reason for this, as in heart cells, was an increased probability of opening combined with a lower threshold for evoked activity (it could be elicited at around −20 mV). The main characteristics are summarized in Fig. 5. Elementary conductance was estimated to be 24 ± 1 pS by least-squares regression through the mean amplitude of the elementary currents recorded at six different membrane potentials (Fig. 5). In fact, the extreme conductance values encountered when considering single cells (22 and 29 pS) point to a wide scatter in the individual measurements. Characteristically, in control conditions, the open mode of this activity could be described as a combination of short and long openings with time constants of 0·5 and 5 ms respectively. The main effect of Bay K 8644 resulted in an increased probability of openings, with no obvious change in the open times or in the relative proportions of long and short events which remained at about 1 to 6 (Fig. 5*B*).

Finally the effect of ω-conotoxin was examined. Pre-treatment of cells with ω-conotoxin reduced the overall channel activity and conspicuously affected mainly

the inactivating component. In most patches, it could be characterized at test potentials (+10 to +40 mV) at which the activity in control conditions is too frequent to characterize the elementary events (not illustrated).

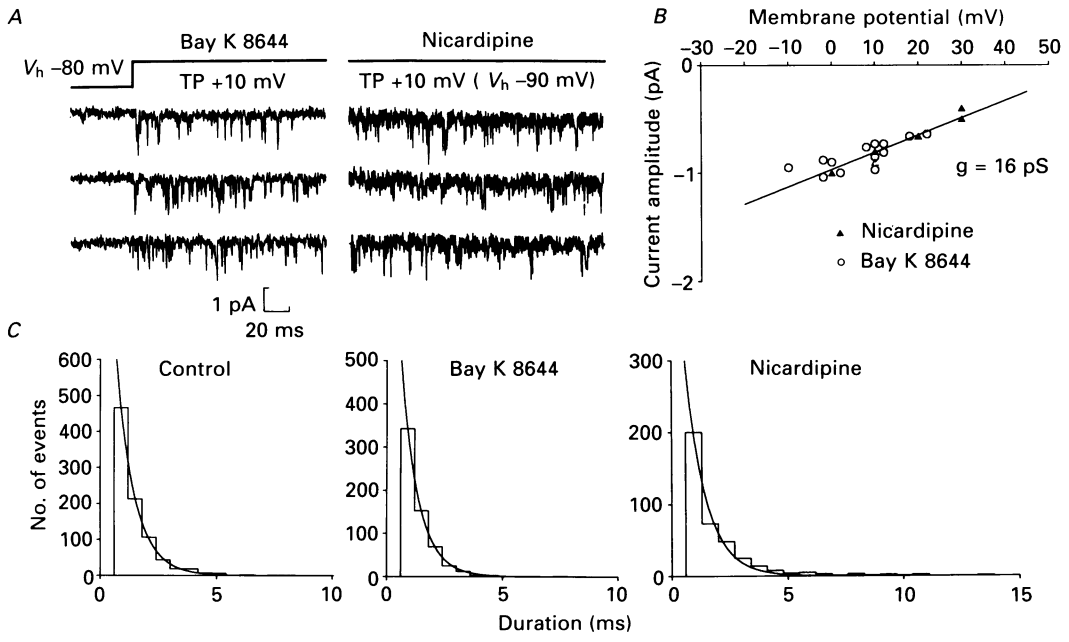

Fig. 3. Amplitude and gating properties of the inactivating channel are not changed by dihydropyridines. *A*, current traces obtained for a test pulse (TP) to +10 mV from a holding potential (V_h) of −80 mV. On the left, the (−)-enantiomer of Bay K 8644 was added to the pipette solution at 10^{-6} M and on the right, nicardipine at 10^{-5} M (two different cells). Top panel shows the stimulation protocol. The horizontal scale bar represents 20 ms. The vertical bar represents a 1 pA deflection for the individual traces and a 0·2 pA deflection for the average trace. *B*, unitary current amplitudes of N-channels recorded in the presence of the calcium agonist (○, 8 cells) and the antagonist (▲, 2 cells) are compared to the regression line representing current amplitudes obtained in control conditions (cf. Fig. 1). *C*, open time histograms are given at +10 mV (from an holding potential of −80 to −90 mV) in control conditions (5 cells) and in the presence of (−)-Bay K 8644 (4 cells) and nicardipine (2 cells). Decay constants obtained using a mono-exponential fit were 0·78, 0·74 and 0·88 ms respectively. Note the long openings in the open time histogram for nicardipine (such openings are also seen in the current traces in control conditions, see Fig. 1*A*).

The activity recorded in isolation when holding the membrane potential at −20 mV was also altered by ω-conotoxin as shown qualitatively in Fig. 6. First, activity was compared to control conditions (Fig. 6*A*). In the presence of ω-conotoxin, few events were recorded during voltage steps below +10 mV. At the most depolarized potentials (+30, +40 mV), current traces were devoid of the long openings normally present in control conditions. As a result, unitary amplitudes were estimated using a very narrow range of potential (+10 and +20 mV). These estimated amplitudes were systematically smaller than in the control condition (see

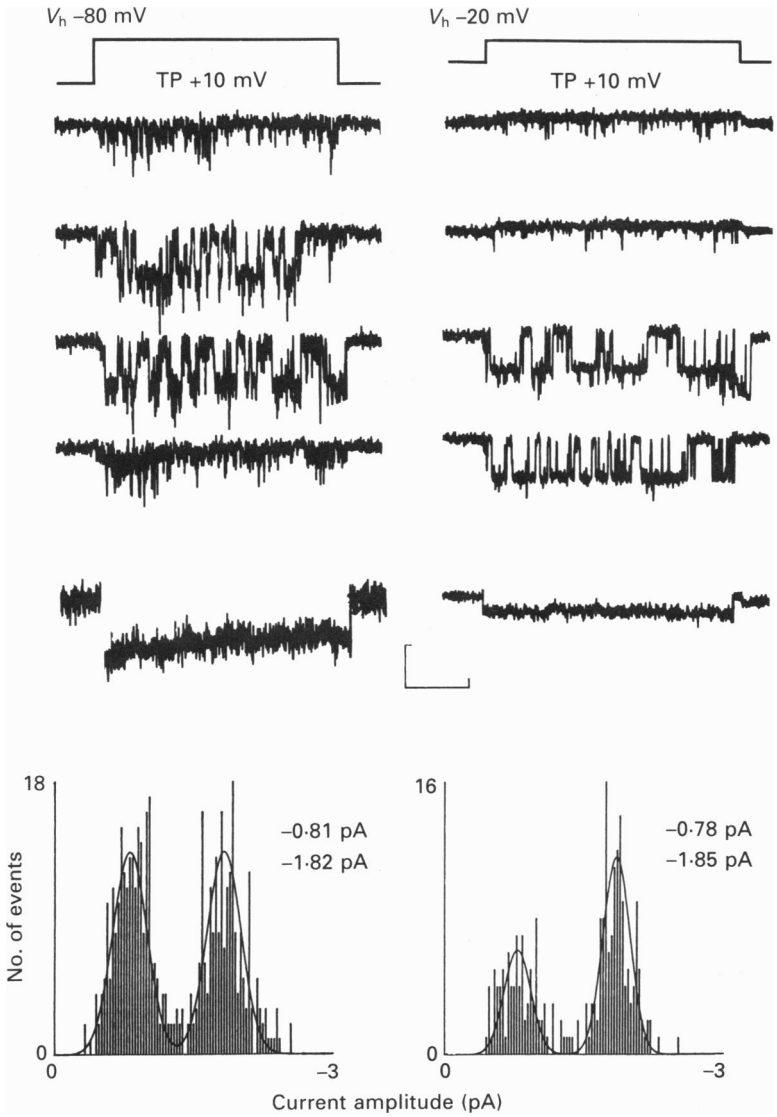

Fig. 4. Calcium channels evoked in presence of 10^{-6} M-(−)-Bay K 8644. Current traces illustrate activity evoked when following the protocol given on the top panel. The long events, characteristic of the presence of the calcium agonist, are shown superimposed on the brief activity typically recorded when holding the membrane potential at −80 mV (at the left) and abolished by holding the potential at −20 mV (at the right). Average traces at the bottom and amplitude histograms correspond to the activity recorded over 52 s periods. Note that depolarizing the cell to −20 mV gives rise to typically sustained activity (average trace) corresponding exclusively to large events (see histogram) and long-duration events (as seen on the individual traces at the right). Sampling frequency, 5 kHz; filter frequency, 1 kHz. Time unit bar, 80 ms; current amplitude, 2 pA for the individual traces and 0·5 pA for the average trace.

the regression line in Fig. 6). ω-Conotoxin has also been shown to reduce the sustained current in chromaffin cells (Hans, Illés & Takeda, 1990). We tentatively assume that this effect mainly results from a reduced probability of long openings, instead of from the appearance of bursts of short grouped events. Long openings were present on further addition of Bay K 8644 (Fig. 6*B*) and subsequently were observed at a wide range of potentials. To characterize the conducting properties of the Ca^{2+} channels in the presence of the calcium agonist, and in view of the above indications, we chose to divide the well-resolved events to short (< 5 ms) and long (> 5 ms)

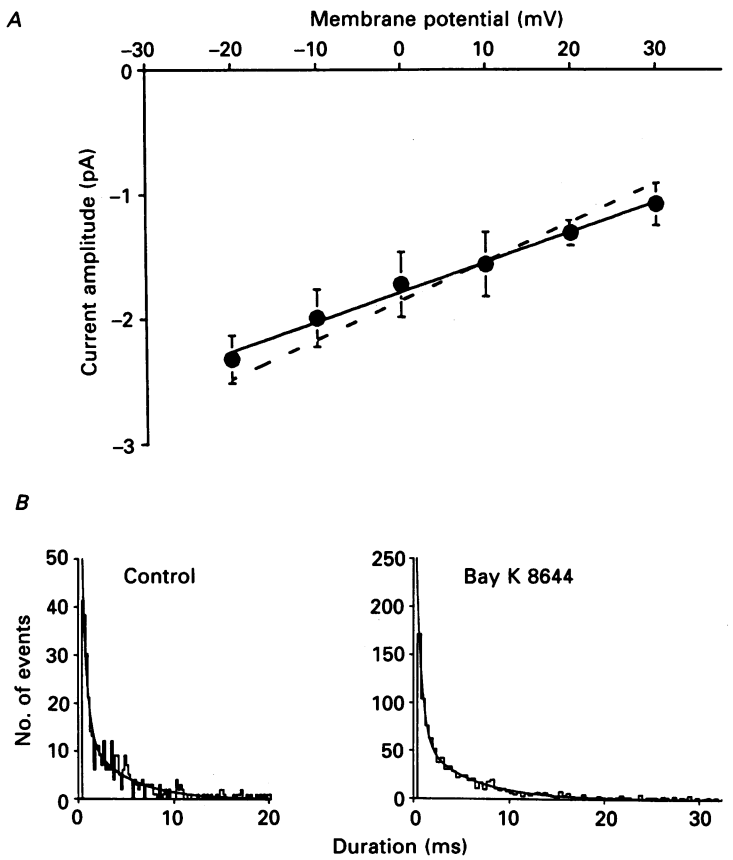

Fig. 5. Characteristics of the non-inactivating activity in the presence and absence of 10^{-6} M-(−)-Bay K 8644. *A* shows the current–voltage relationship obtained from corresponding amplitude histograms (as in Fig. 4). Each point (●) shows the mean amplitude and vertical bars the S.D. (8 cells). The continuous line is the regression line which yields an estimated conductance of 24 pS. Compare this conductance to the conductance of 31 pS obtained in control conditions (dashed line). Note the large scatter in the experimental values: in fact, extreme slopes were 22 and 29 pS respectively. *B* shows the open times in control conditions (left) and in the presence of (−)-Bay K 8644 where a larger number of events are recorded (right). Test pulse to +10 mV and from a holding potential of −20 mV. The open time histograms when fitted using a biexponential fit were described by almost identical parameters. Time constant values (τ) were for the fast (f) and slow (s) exponentials: were: τ_f and τ_s 0·5 and 4·3 ms (4 cells) and 0·56 and 5·13 ms (6 cells) in the absence and presence of the calcium agonist respectively. Amplitude ratios (A_f/A_s) were 6·1 and 5·2 respectively.

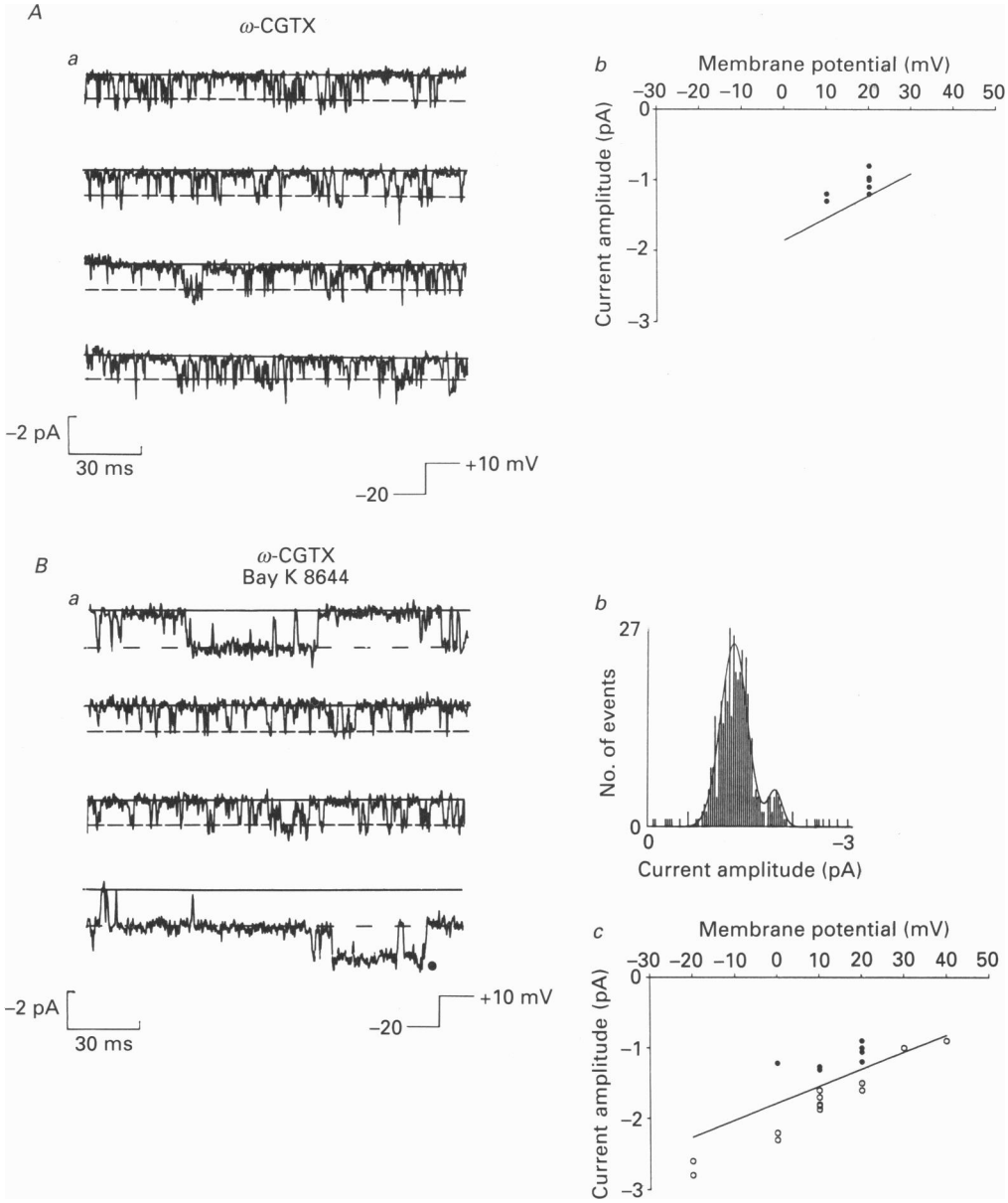

Fig. 6. Characteristics of the Ca^{2+} elementary currents remaining after 20 min incubation of the chromaffin cells with ω-conotoxin (ω-CGTX), 3×10^{-7} M, in the absence (*A*, left) and the presence (*B*, right) of 10^{-6} M-(−)-Bay K 8644 added to the pipette solution. Non-inactivating events were recorded in isolation by holding the membrane potential at −20 mV. *A a* illustrates current traces evoked in presence of ω-conotoxin, by voltage steps to +10 mV. Continuous lines follow the baseline. Amplitude histograms show a single peak (−1·4 pA, dashed lines). *A b*, in this *I–V* relationship, the amplitudes of the elementary events in the presence of ω-conotoxin alone (●) is compared with those obtained for the non-inactivating channel in control conditions (continuous line, 31 pS; see Fig. 2*C*). *B*, in presence of both ω-conotoxin and Bay K 8644, current traces (in *a*) show elementary events distributed along a double-peaked amplitude histogram (illustrated in *B b*) with mean amplitudes of the corresponding events around −1·3 and −1·9 pA. These amplitudes have been reported on current traces using narrowly spaced

openings. Short events were smaller in amplitude than controls (grouped in a single population: cf. regression line). In contrast long events were larger (see Fig. 6*B*). In summary, these observations indicate that in amplitude ω-conotoxin stabilizes a slightly less conducting conformation of the large channel, when compared to the substate opened in control conditions or to the long openings favoured by the calcium agonist. In the absence of ω-conotoxin, the amplitude histograms show a wide scatter between individual events and also large variations from cell to cell are observed (see S.D. on elementary current–voltage curves). This variability could originate from spontaneous fluctuations between the two states.

DISCUSSION

Using inactivation characteristics as a way of classifying Ca^{2+} channels, we have distinguished two types of events in bovine chromaffin cells maintained in short term-primary culture (3–7 days). We shall discuss the characteristics of inactivating and non-inactivating Ca^{2+} channels recorded in the cell-attached configuration and then show how they support the concept of a modulatable DHP-sensitive activity.

Characterization of the channel that inactivates at a hyperpolarized potential

In our chromaffin cell preparation, the Ca^{2+} channel, with a conductance of 16 pS, has a high threshold of activation and is linked to full inactivation at a holding potential of −20 mV. This channel is insensitive to DHPs and its probability of opening is reduced by ω-conotoxin. We will refer to it, because of its inactivating properties, as an N-channel using the nomenclature of Tsien and his colleagues (1988). Its conductance and open time suggest that it is the channel described by Fenwick, Marty & Neher (1982). By contrast, Hoshi & Smith (1987) observed exclusively single amplitude events that did not inactivate at −20 mV and were sensitive to DHP. The reason for these differences may be related to different growth conditions.

The 16 pS conductance is typical of the channels described in most cell types derived from the neural crest (see Introduction). The larger conductance state observed by Plummer *et al.* (1989) in mammalian sympathetic and PC12 cells appears to be an exception. These authors further point to variability in its mode of opening. From the large conductance state which dominates at more depolarized potentials (+20 mV), the channel may change to a less conducting state (of some 14 pS) at less depolarized test pulses (−10 mV), or sometimes also spontaneously during a single pulse to +20 mV. These observations may indicate that the biophysical characteristics of a channel can be modified during the culture period; one wonders what the exact role of nerve growth factor (NGF) is in this process since the development of mammalian superior cervical ganglion (SCG) cells requires NGF.

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and widely spaced dashed lines, respectively. The filled circle at bottom right shows a second −1·9 pA event recorded on top of a large −1·9 pA event. In *B c*, for the *I–V* relation, elementary events have been classified further according to their open time duration. Mean amplitudes for events < 5 ms (●) and > 5 ms (○) are compared to values obtained with Bay K 8644 alone for the non-inactivating channel (continuous line, 24 pS; see Fig. 5*A*).

Also, in long-term culture of PC12 cells (1–3 weeks in presence of NGF), a selection process at the successive division steps may take place. Considered together, these observations point to a possible plasticity in the expression of this Ca^{2+} channel.

Characteristically, this channel has short open times of less than 1 ms which is in agreement with all reported data. Lipscombe, Kongsamut & Tsien (1989), have reported the presence of additional long openings; we observed these rarely. The shorter open times we encountered may have been due to the spontaneous release of catecholamines which altered the gating properties of this channel (Lipscombe *et al.* 1989).

Although the exact conducting properties of this N-type channel are not known, its inactivation at hyperpolarized potentials leads one to believe that it contributes to the inactivating fraction of the whole-cell current. However, the characteristics of the total current are impossible to assess from our knowledge of the N-channel. Reciprocally, from total current records, we have no precise way of estimating the density of the population of N-channels. In single-channel recordings, the N-channel is present in most if not all patches. Our present culture and recording conditions would have brought up the estimated number of N-channels to at least one per 10 μm^2. Indeed it has been suggested (Carbone & Lux, 1987) that the inactivation process is more pronounced in conditions where most of the cell is depolarized by an elevated K^+ concentration applied in the bath medium. Although widely spread, this activity corresponds to short-lasting events; summation of the individual events would therefore possibly lead to a relatively small current. Using whole-cell current recordings we tentatively estimated that the inactivating N-component is a small component of the voltage-evoked Ca^{2+} current in chromaffin cells, in the ratio of 1 to 3 or even less. We are aware, in this latter approach, that results are biased by a tendency to underestimate the N-component. First, this may arise from space-clamp artifacts minimized by studying only cells of small diameter. Second, by using test pulses of 350 ms, the decay of N-component, which proceeds over a few hundred milliseconds may have been included as part of the sustained component. Together, roughly similar figures arise from both cell-attached and whole-cell recordings.

Characteristics of the non-inactivating channel

The large-conductance L-channel, observed in chromaffin cells maintained in primary culture has a mean conductance of 24 pS; a similar conductance has been reported in PC12 cells by Plummer *et al.* (1989). However, in the preparation we used, the conductance of the L-channel varied characteristically over a large range from 22 to 32 pS. This agrees with other reports. For instance, Hoshi & Smith (1987) recorded in conditions where L-type elementary currents never exceeded 1 pA at +10 mV; such a current amplitude corresponds to even smaller values than we generally observed. A lower conductance of 18 pS was also observed in mouse DRG (Kostyuk *et al.* 1988*a*) and in PC12 cells (Kostyuk, Shuba, Savchenko & Teslenko, 1988*b*). These authors carried out their recordings in 60 mM-$BaCl_2$, a concentration that may not have allowed maximal conductance of the Ba^{2+} channel; however, similar amplitudes have been observed in chick DRG using 95 mM-$BaCl_2$ (Carbone & Lux, 1987). The largest conductance values we observed corresponded in most cases to events recorded in the presence of Bay K 8644.

Gating properties of the L-channel in chromaffin cells correspond to those

originally described by Hess, Lansmann & Tsien (1984) in the heart, and to those found in peripheral neurons (see the kinetic analysis of Fox *et al.* 1987 and Kostyuk *et al.* 1988*b*). In the presence of Bay K 8644 this channel has a higher probability of opening, longer openings during step depolarization and a prolonged activity, on return to the holding potential. The identity of the gating properties in all preparations becomes more obvious on application of an agonist of the Ca^{2+} channel, with the development of a fast component of about 1 ms and a slower component ranging from 3 to 20 ms depending on the recording conditions. Such open times have already been described in chromaffin cells by Hoshi & Smith (1987) and in rat SCG neurons by Plummer *et al.* (1989).

Comparison to binding studies

Our present electrophysiological results are consistent with some binding data. For example, freshly plated PC12 cells, in the absence of NGF, show a density of ω-conotoxin sites of 18 fM/10^6 cells and half that number for DHP sites (Usowicz, Becker, Porzig & Reuter, 1989). These figures produced in PC12 cells expressing an endocrine phenotype and are likely to extend to freshly isolated chromaffin cells since they too express an endocrine phenotype. This results indicates to us that the relative density of the ω-conotoxin and DHP binding sites is in accord with the probability of our finding a patch with an N- or an L-channel.

Considering retrospectively the possibility of using N-channels as markers of a neuronal differentiation, it is clear that the electrophysiological approach will be of little value. Although N-channels were present in every patch made on chromaffin cells (our results), their relative contribution to the total Ca^{2+} current was difficult to ascertain. Moreover, after neurite outgrowth, space-clamp conditions would prohibit accurate measurements of Ca^{2+} currents. Janigro, Maccaferri & Meldolesi (1989) reached similar conclusions with regard to PC12 cells.

Our main conclusion is that these observations support the hypothesis put forward in the preceding paper (Bossu *et al.* 1991). In addition to numerous N-channels, we found evidence in chromaffin cells for a population of L-channels that could only be revealed on introduction of Bay K 8644. We suggest that this population of DHP-sensitive channels constitutes a reservoir of channels in our cell-attached control conditions; these channels are then drive to their activatable conformation by whole-cell recording conditions. Maximal recruitment of L-channels would be induced either by dialysis of the cell in the whole-cell configuration, or by Bay K 8644 in the cell-attached configuration. Though in control conditions this L-type activity occurs at low frequency, this does not exclude its contribution to a physiological process. Using Fura-2 monitoring of the intracellular Ca^{2+} concentration, Pruss & Stauderman (1988) showed that on prolonged K^+-induced depolarization a DHP-sensitive Ca^{2+} entry could be maintained. This result is in accord with all release data and suggests that on maintained depolarization the turnover of successive openings of the L-channels becomes frequent enough to elevate internal Ca^{2+} concentration and cause catecholamine release.

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