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Background Calcium Permeable Channels in Glomerulosa Cells from Adrenal Gland

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Summary. The cell-attached recording mode of the patch-clamp technique was used to study Ca^{2+} permeable background currents of glomerulosa cells from rat and bovine adrenal gland. With a pipette filled with 110 mM $BaCl_2$ or 90 mM $CaCl_2$, three different types of unitary currents were detected. The B_1 channel demonstrates a nonlinear *I-V* curve. The conductances are 4 and 7 pS at −40 and −70 mV, respectively. The curve of the opening probability *vs.* membrane potential is bell shaped with its maximum at −70 mV. The B_2 channel has a conductance of 6 pS, while the B_3 channel shows a nonlinear *I-V* relationship with conductances close to 17 and 10 pS at HPs of −60 and −20 mV. The three types of currents are insensitive to dihydropyridines.

We suggest that these background currents could be responsible for the basal calcium influx and aldosterone secretion previously observed in nonstimulated glomerulosa cells.

Key Words background calcium channel · glomerulosa cell · adrenal gland · cell-attached recording · single channel

Introduction

In glomerulosa cells, like in most endocrine cells, the increase in intracellular calcium concentration is a primary step in the excitation-secretion coupling process (Kojima, Kojima & Rasmussen, 1985*a,b;* Hausdorff & Catt, 1988; Gallo-Payet & Payet, 1989). This increase is due to the opening of voltage-sensitive surface membrane channels and/or to the release of Ca^{2+} from endoplasmic reticulum by an IP_3-dependent mechanism (Capponi et al., 1984; Payet, Benabderrazik & Gallo-Payet, 1987; Quinn, Cornwall & Williams, 1987*a;* Cohen et al., 1988; Barrett et al., 1989; Gallo-Payet & Payet, 1989; Johnson, Capponi & Valloton, 1989). Moreover, a background Ca^{2+} influx that is sensitive to variations in extracellular calcium concentration (Capponi, Lew & Valloton, 1987) and a basal secretion of aldosterone and corticosterone have been detected (Braley et al., 1986; Capponi et al., 1987; Gallo-Payet & Payet, 1989; Guillon et al., 1990). We have previously described three types of Ca^{2+} channels in rat glomerulosa cells (Durroux, Gallo-Payet & Payet, 1988), which were very similar to those found in neurones (Nowycky, Fox & Tsien, 1985). Two types of calcium channels have been described in bovine glomerulosa cells (Matsunaga et al., 1987). But none of these channels can be responsible for the background Ca^{2+} influx, considering their inactivation voltage range and their kinetics.

Calcium channels, whose activities have been recorded at very negative potentials, have been observed in the outside-out configuration of the patch-clamp technique on *Aplysia* neurones (Chesnoy-Marchais, 1985). Recently, background calcium-permeable channels have also been described in ventricular myocytes with the patch-clamp technique (Coulombe et al., 1989) and in planar lipid bilayers (Rosenberg, Hess & Tsien, 1988). In glomerulosa cells, using the cell-attached configuration, we observed the T, the L, and the N currents (Durroux et al., 1988). Also, we observed three other types of events on a large scale of potential (−100, −10 mV) when the potential remained constant. In this article, we characterize these new types of calcium currents which can be responsible for the background Ca^{2+} in bovine and rat glomerulosa cells. The three single-channel currents can be distinguished by their kinetics, conductances and opening probability-voltage relationships. The smallest events present the characteristics of the B current described by Rosenberg et al. (1988).

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Materials and Methods

Cell Preparation

Long-Evans rats, weighing 200–250 g, were decapitated and their adrenal glands were excised. Zona glomerulosa cells were isolated as previously described (Durroux et al., 1988). In brief, the successive steps of zona glomerulosa isolation and cell dissociation were performed in Eagle's Minimum Essential Medium (GIBCO, Burlington, Ont., Canada). After a 20 min incubation at 37°C in collagenase (2 mg/ml; 4 glomerulosa/ml; Worthington, Freehold, NJ) plus DNAse (25 μg/ml; Sigma, St. Louis, MO), mechanical dissociation was carried out. Cells were filtered and centrifuged for 15 min at 100 × g. The cell pellet was resuspended in Eagle's Minimum Essential Medium containing 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO). The cells were plated at a density of about 7×10^3 in 35 mm tissue-culture dishes and were grown in a humidified atmosphere of 95% air, 5% CO_2 at 37°C. After 24 hr, the culture medium was changed for a medium containing only 3.5% of fetal calf serum, and the cells were used after 1 or 2 days in culture.

Solutions

Before the experiment, the culture medium was changed for a 130 mM K^+ medium containing (in mM): KCl 130, glucose 10, HEPES 5 (pH 7.4) in order to bring the membrane potential to zero. The pipette solution contained (in mM): $BaCl_2$ 110 or $CaCl_2$ 90, HEPES 5 (pH 7.4). The experiments were carried out at room temperature with the patch-clamp technique in the cell-attached configuration.

Acquisition and Analysis of the Data

The currents were recorded on a tape recorder and were filtered with an Axopatch IB (Axon Instruments, Burlingame, CA) and then digitalized. Analyses were performed on a DEC 11/23 computer, using self made programs. The background current records were split into sweeps. Slow drifts of the baseline were compensated by fitting a baseline to each sweep. Amplitude histograms were built from the baseline corrected records; gaussian curves were fitted to the data. The opening probability during each sweep was calculated from the area between the current record and the baseline. The detection of the openings and closings was made automatically at 50% of the amplitude of the unitary current. Open and closed time histograms were fitted with a Marquardt procedure. L-current records were corrected for linear leakage and for capacitative current by subtracting the averaged sweeps which did not present any opening.

Results

The B_1 Current

The smallest amplitude events were named the B_1 current. They were recorded at various holding potentials (HPs) ranging from −90 to −20 mV (Fig. 1*A*). The kinetics of this channel are highly voltage dependent. When the voltage becomes more negative, the flickering increased, and the openings appear in bursts. At a HP of −20 mV, long duration opening and closing events can be seen (Fig. 1*A*). In some experiments, smaller events can be detected (Fig. 1*A*, traces −90 and −70 mV); however, in this study only the largest events were considered. The current-voltage (*I-V*) relationship of the B_1 channel is not linear (Fig. 1*B*) and the conductance can be estimated to be 7 and 4 pS at HPs of −70 and −40 mV, respectively. Although the zero-current voltage is obviously greater than 0 mV, we cannot determine its value precisely, because of the small amplitude

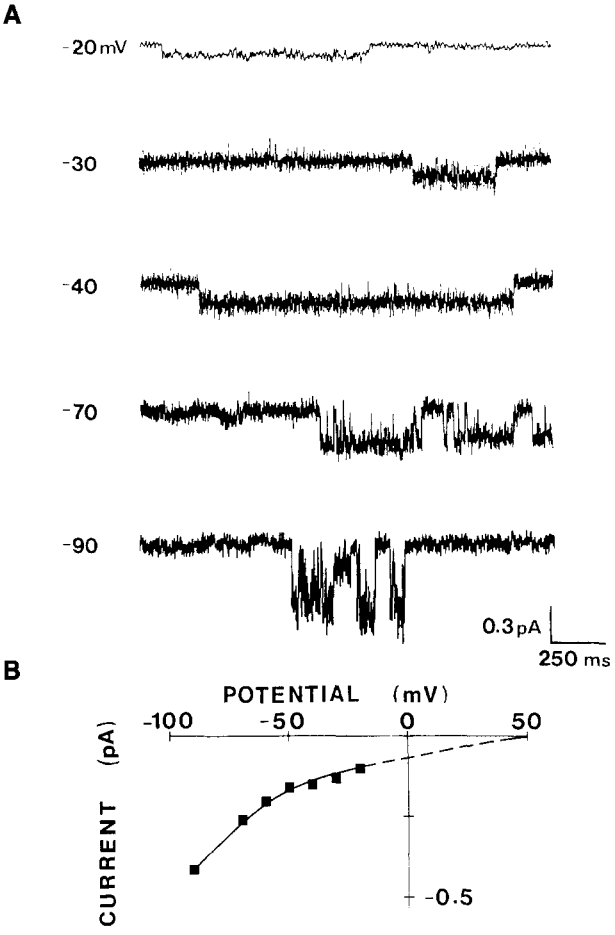

Fig. 1. Recording of the B_1 current in a glomerulosa cell. The pipette was filled with 110 mM $BaCl_2$. (*A*) The B_1 current was recorded at various potentials. The membrane was clamped at the voltage indicated for 5 to 10 min. The sample frequency was 2000 Hz and the current traces were filtered at 1000 Hz with a Bessel filter, except the upper trace at −20 mV which was filtered at 200 Hz. Flickering gets greater when the potential becomes more negative. (*B*) Current-voltage relationship of the B_1 current. The curve is not linear over the voltage range studied and was fitted by eye. The estimated conductances are 7 and 4 pS at HPs of −70 and −40 mV, respectively.

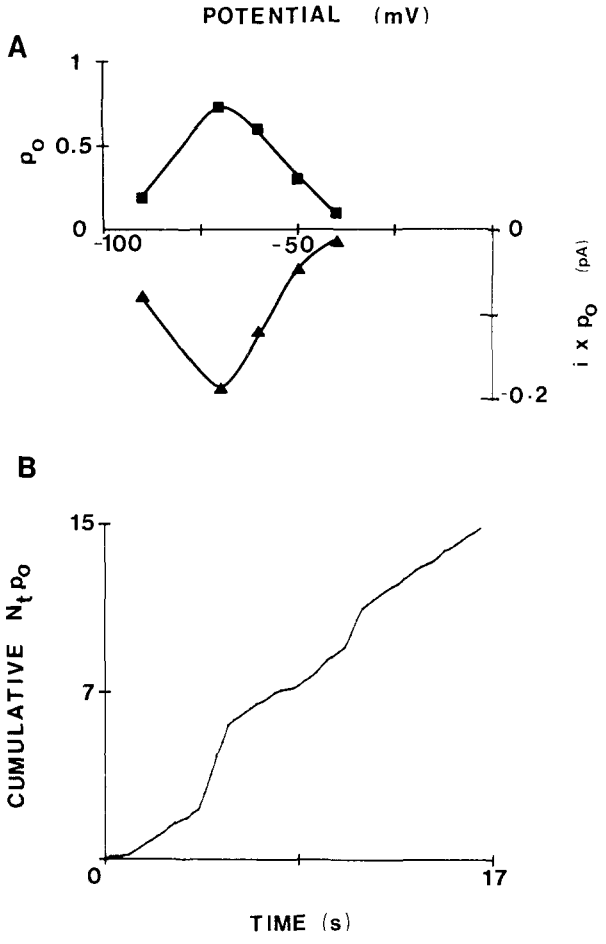

Fig. 2. Opening probability of the B_1 channel as a function of voltage. (*A*) The curve of the opening probability *vs.* voltage is bell-shaped with a maximum at −70 mV. The downward curve represents the current ($i \cdot p_o$) at various membrane potentials; i is the unitary current's amplitude and p_o the opening probability at each voltage. Note that the maximum current is obtained near −70 mV. (*B*) Cumulative channel opening as a function of time. N_t is the number of channels; p_o is the opening probability. HP = −60 mV.

of the current and of the low opening probability of the channel at high voltages. In fact, the B_1 channel is clearly voltage dependent since the opening probability (p_o) is maximum at −70 mV and declines for higher and lower potentials (Fig. 2*A*, filled squares). The current ($i \cdot p_o$)-voltage relationship curve indicates that the maximum calcium influx is also reached at −70 mV (Fig. 2*A*, filled triangles). B_1-channel activity appears in bursts, although no long periods of silence could be detected, as seen from the constant slope factor of the curve of the cumulative opening probability *vs.* time (Fig. 2*B*). Open time (Fig. 3*A*) and closed time (Fig. 3*B*) histograms of the B_1 current could be fitted with three exponentials, suggesting that the channel had more than one open state and more than one closed state. At a HP of −70 mV, the time constants are 8 and 68 ms for the 5 to 300 ms range open time histogram and 2 and 8 ms for the 1 to 30 ms range open time histogram (Fig. 3*A*, inset). The time constant are 9 and 90 ms for the 5 to 300 ms range closed time histogram and 1.5 and 9 ms for the 1 to 30 ms range closed time histogram (Fig. 3*B*, inset).

The B_2 Current

The medium amplitude events were named the B_2 current. They differed from the B_1 current by their amplitudes and kinetics. The activity of the B_2 current is shown in Fig. 4*A*. Just as the B_1 current, the flickering increases when the potential gets more negative. The *I-V* relationship curve of the B_2 channel is linear. An extrapolation allows estimation of the zero-current voltage as being close to +15 mV (Fig. 4*B*). The conductance is about 6.6 pS. Contrary to the B_1 channel, we did not determine the p_o for the B_2 channel since bursts of B_2 channel activity could be separated by very long periods of silence as illustrated by the curve of the cumulative opening probability *vs.* time (Fig. 4*C*, plateaus). These silent periods could last up to several minutes.

The B_3 Current

The B_3 current corresponded to the largest events. Flickering could clearly be seen at all membrane potentials (Fig. 5*A*). In this patch, B_2- and B_3-events and superimposition of both are detected as illustrated in Fig. 5*A*, lower. Arrows indicate the B_2 events. The *I-V* curve of the B_3 channel is not linear (Fig. 5*B*). The zero-current voltage is obviously greater than 0 mV. Conductances are 17 and 10 pS at −60 and −30 mV, respectively. As for the B_2 channel, B_3-channel activity can stop for several minutes.

We were able to record T, N, and L unitary currents in glomerulosa cells. We present here L currents recorded in presence of Bay K 8644 (5 μM) in the bath medium and when depolarizing steps from a holding potential of −80 mV to voltages more positive than −40 mV were applied (Fig. 5*C*, upper panel). L-channel activity could not be observed at negative potentials or when the voltage had remained constant for a long time. Moreover the amplitude of the L current is different from those of background currents. The conductance of the L channel is 18 pS (*data not shown*) and the mean open times are 7.3 ms at a HP of −20 mV in presence of Bay K 8644 (5 μM) (*data not shown*).

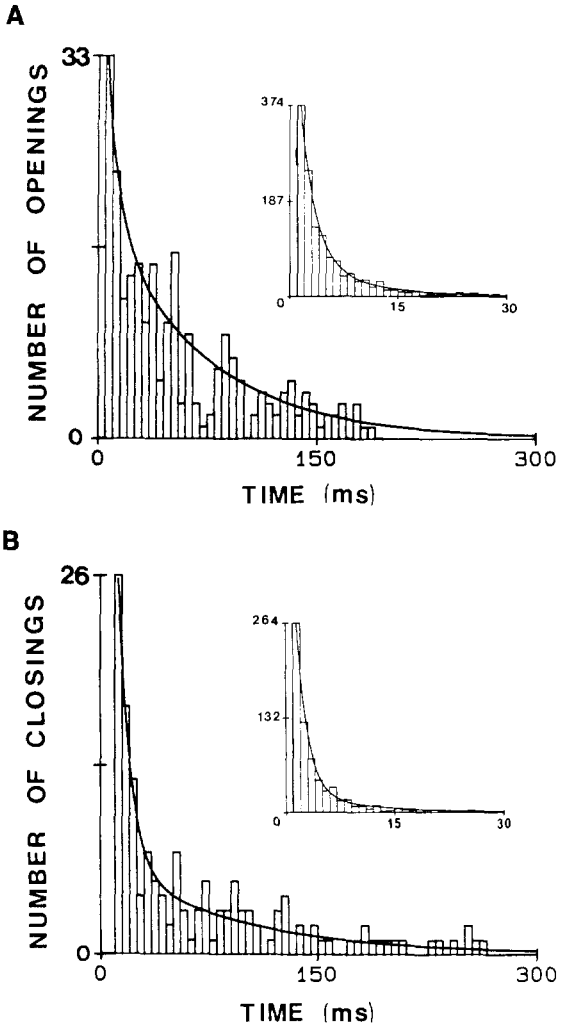

Fig. 3. Kinetics characteristics of the single B_1 current. (*A*) Open time histograms: 5 to 300 ms range histogram; the distribution of open time is fitted by the sum of two exponentials. Time constants: 8 ms and 68 ms. Inset: 1 to 30 ms range histogram; the smooth curve is a biexponential fit. Time constant: 2 and 8 ms. (*B*) Closed time histograms: 5 to 300 ms range histogram; the distribution of closed time is fitted by the sum of two exponentials. Time constants: 9 and 90 ms. Inset: 1 to 30 ms range histogram; the smooth curve is a biexponential fit. Time constant: 1.5 and 9 ms. HP is −70 mV.

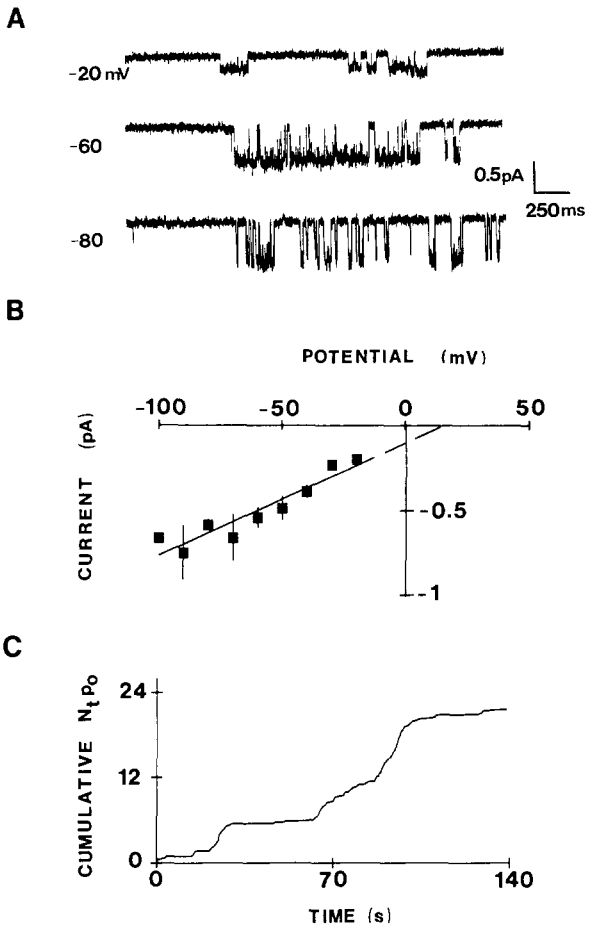

Fig. 4. Recordings of the B_2 current. The pipette was filled with 110 mM $BaCl_2$. (*A*) B_2 current was recorded at the potentials indicated. The sample frequency was 2000 Hz and the traces were filtered at 500 Hz. (*B*) Current-voltage relationship. The unitary conductance is 6.6 pS, and the estimated zero-current voltage is close to +15 mV. (*C*) Cumulative channel opening probability. Note that the channel stopped its activity during long periods as indicated by the plateau on the curve. HP = −80 mV.

Pharmacological Characteristics

The activities of three channels comparable to those described above have also been recorded with a 90 mM $CaCl_2$-filled pipette. Figure 5*D* illustrates such an activity at a HP of 0 mV. Although it was difficult to determine which type of current it was, these events looked like B_3 currents, because of the amplitude of the current and of the flickering. Similar activities were also recorded when glomerulosa cells were bathed in a normal external medium (*data not shown*).

We compared the sensitivity of the background channels and of the L channel to dihydropyridines. It is well known that L channels are very sensitive to dihydropyridines. Firstly, the L channel is generally detectable in glomerulosa cells in the presence of Bay K 8644. Secondly, as illustrated in Fig. 5*C*, lower panel, the L current is very sensitive to nifedipine since addition of 5 μM nifedipine completely blocks the L current. On the contrary, no effects of dihydropyridines can be detected on the background channels. At a HP of −40 mV, neither the amplitude nor the kinetics of the B_3 current are changed after addition of 50 μM nifedipine to the bath medium (Fig. 6*A*, lower trace *vs.* upper trace). Moreover, the slope of the cumulative opening probability is not changed over an 18-min period despite the addi-

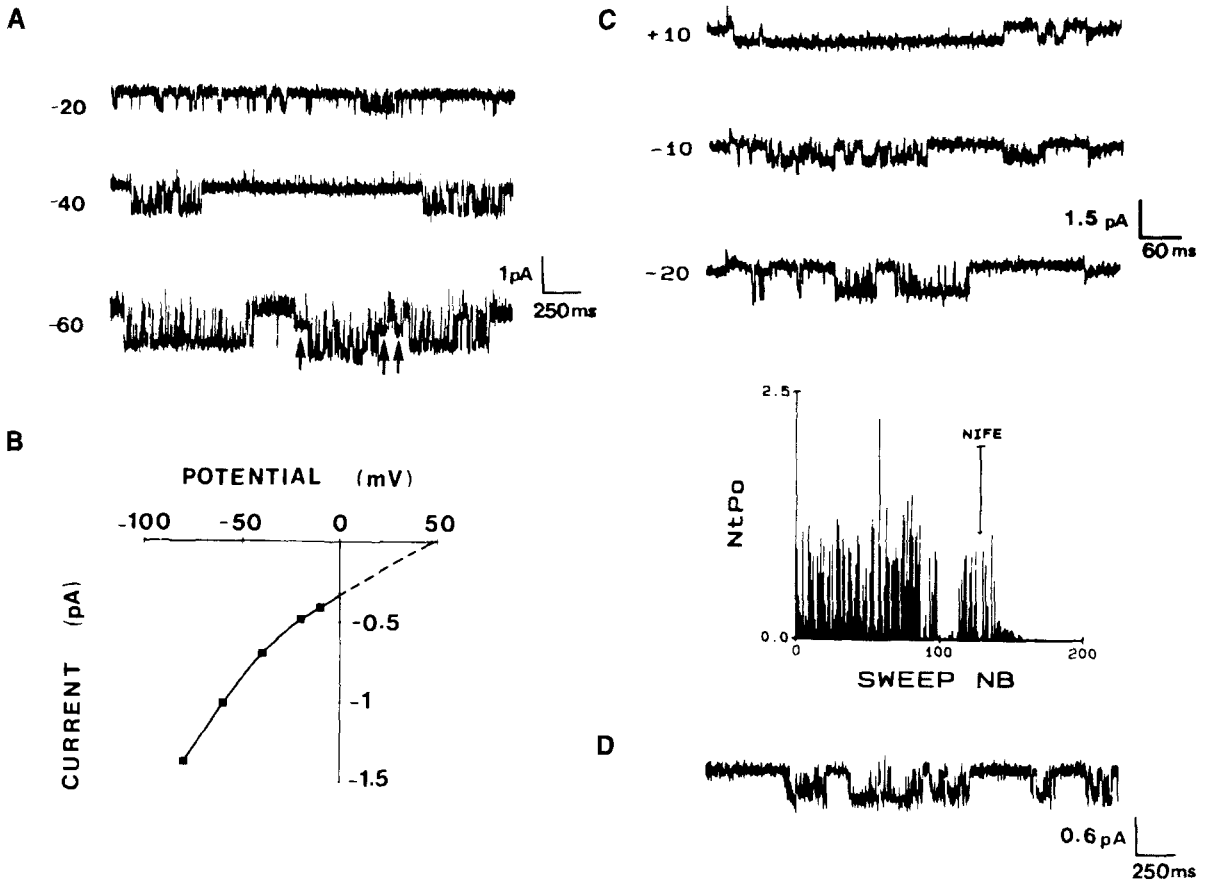

Fig. 5. Recordings of the B_3 current. The pipette was filled with 110 mM $BaCl_2$. (*A*) B_3-current recording at three different voltages. Superimposition of the B_2 and the B_3 currents can be detected on the lower trace. Arrows indicate the B_2 events. The signal was sampled at 1000 Hz and filtered at 500 Hz. (*B*) Current-voltage relationship. The curve is not linear and the unitary conductance is 17 pS at −60 mV and 10 pS at −30 mV. The estimated zero-current voltage is +50 mV. (*C*) Upper panel: L-current recording at different voltages. Lower panel: Channel opening probability during individual sweep. N_t is the number of channels; p_o is the opening probability. (*D*) Channel activity recorded with a 90 mM $CaCl_2$ filled pipette. HP = 0 mV.

tion of nifedipine (Fig. 6*B*, trace *2* arrow). The B_3 channels are insensitive to nifedipine at the other potentials tested (−20 and 0 mV). Similar results have also been found with Bay K 8644 (potentials tested: −80, −60 and −30 mV). At a HP of −80 mV, the B_1 current is also insensitive to Bay K 8644 (1 μM) and to nifedipine (5 μM) since these dihydropyridines do not change the slope of cumulative opening probability (Fig. 6*B*, trace *1*). The same type of results have been obtained for the B_2 channel (*data not shown*).

B_1, B_2 and B_3 are Three Different Channels

Multiple states of conductance have previously been described for Ca^{2+} channels reconstituted in planar lipid bilayers (Coyne, Dagan & Levitan, 1987; Ma & Coronado, 1988). In order to determine if the B_1, the B_2 and the B_3 currents are activities of the same channel or of three different channels, we investigated more precisely patches with more than one type of amplitude.

Figure 7 illustrates results obtained on a patch that presented the B_1 and the B_2 currents. Panel *A* shows the whole amplitude histogram obtained from a 35-s long recording—at a HP of −70 mV— in which four different humps were observed. The first hump corresponds to the baseline level. The second hump corresponds to the B_1 current's amplitude, and the third and fourth humps to the B_2 and (B_1 + B_2) currents' amplitudes (*see also* the inset which represents the last two humps on a larger scale). Fitting the whole amplitude histogram is difficult, specially for the last two humps. To estimate precisely the occurrence of each type of events, four subfiles were created from the original recording file. As illustrated in the left part of Fig. 7, only one type

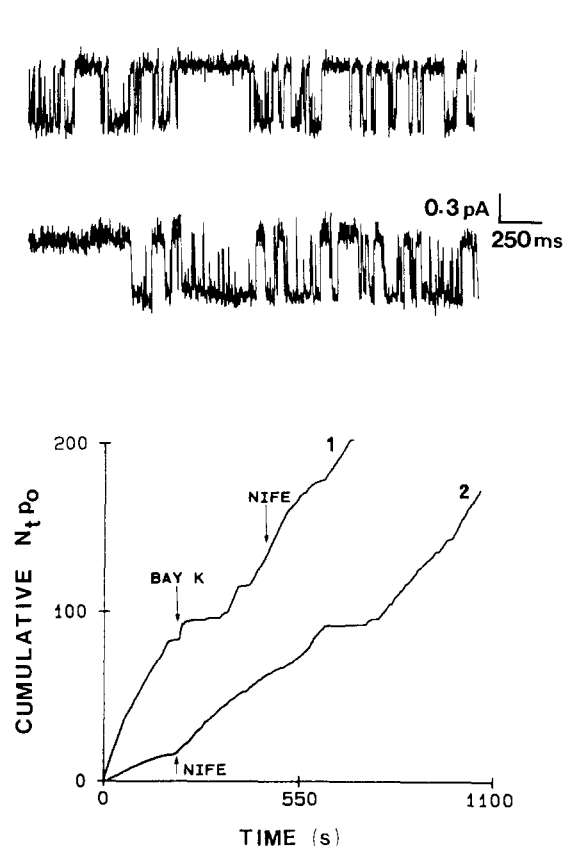

Fig. 6. (*A*) Effect of nifedipine (50 μM) on the B_3-type channel activity. Control (upper trace) and recorded after 15 min in the presence of nifedipine (lower trace). (*B*) Curve *1:* Evolution of the cumulative opening probability of the B_1 channel in the absence or the presence of Bay K 8644 (1 μM) and nifedipine (5 μM). HP = −80 mV. Curve *2:* Evolution of the cumulative opening probability of the B_3 channel in the absence or the presence of nifedipine (50 μM). HP = −40 mV.

of events (baseline, B_1, B_2, or B_1 + B_2 events) was taken into account in each subfile. The resulting amplitude histogram (right parts of panels *B–E*) clearly shows only one gaussian—corresponding to the type of events considered—which is easy to fit (smooth curves). The sum of the four gaussian curves of panels *B* (baseline events), *C* (B_1 events), *D* (B_2 events) and *E* (B_1 + B_2 events) was used to fit the whole amplitude histogram (panel *A*, smooth line). The "dissection" of the original recording file allowed us to estimate the probability of occurrence of each type of events.

If independence is assumed, the probability of simultaneously observing the B_1 current and the B_2 current ($p_{o(1+2)}$) is equal to the probability of observing the B_1 current (p_{o1}) multiplied by the probability of observing the B_2 current (p_{o2}). In this experiment, p_{o1} and p_{o2} were respectively equal to 0.58 and to 0.071. We expected a $p_{o(1+2)}$ of 0.041 and the experimental value was 0.041. We can then conclude that B_1 and B_2 are two independent channels.

Moreover, in a batch where five B_2 channels were detected, no B_1 or B_3 currents could be seen, suggesting that the B_2 current and the B_1 and B_3 currents are not interrelated states of one channel but are three different channels (*data not shown*). Among all the patches tested, the three currents are distributed at random: the B_2 current and the B_3 current have been observed four times each independently and twice together. The B_1 current has also been observed alone or associated with the B_2 and/or the B_3 current.

Discussion

In the present study, we have identified for the first time three types of background calcium permeable channels (named B_1, B_2 and B_3) in adrenal glomerulosa cells. It is clear that these currents are cationic currents since the reversal potentials for the three currents are greater than 0 mV. The reversal potential of the chloride anion cannot be greater than −13 mV since we used 110 mM $BaCl_2$ as pipette solution and 130 mM KCl in the bath medium. These results indicate that the background channels are permeable to calcium and to barium.

It is obvious that the three background channels reported here constitute a family which greatly differs from the T and N, and the L channels previously described (Durroux et al., 1988). Contrary to the T, the N, and the L channels, no inactivation gating of the background channels can be detected. Moreover, the B_1, B_2 and B_3 currents were observed at all the holding potentials tested, and these activities cannot be explained by a window current of a T, L, or an N channel, considering their limited voltage range activation. The Table summarizes the characteristics of the background channels compared to the L channel.

Our results clearly indicate that the B_1 and the B_2 currents are independent since the probability of $p_{o(1+2)}$ is equal to $p_{o1} \times p_{o2}$. Moreover the currents are distributed at random; no types were preferentially found together and some patches turn out to exhibit only one type of event. When patches exhibited more than one conductance, superimposition of the two types of events can be seen. All these reasons suggest that the currents correspond to the activities of three different channels.

Recently, background calcium channels have been described in rat ventricular myocytes (Coulombe et al., 1988), in cardiac membrane (Rosenberg et al., 1988) and in myotubes (Fong et al., 1990). The

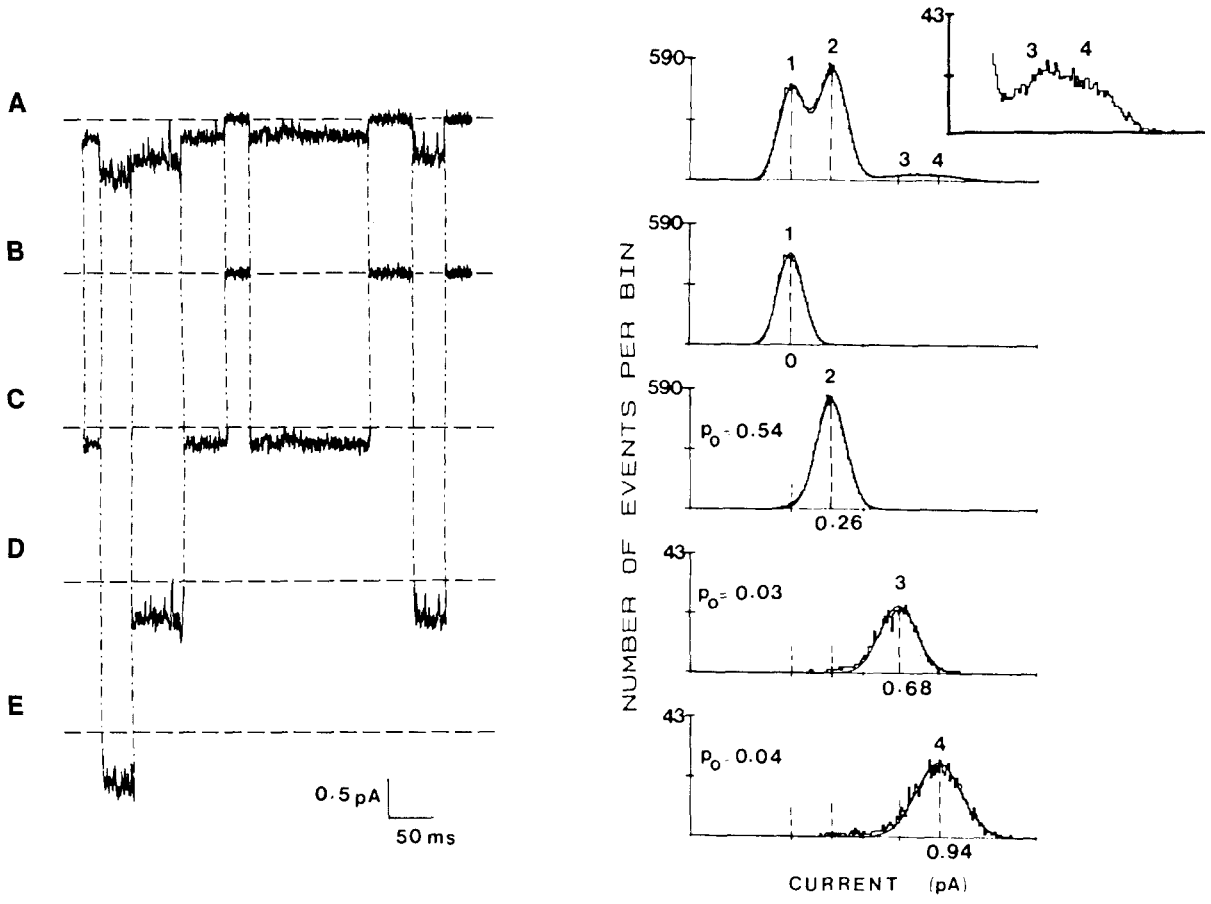

Fig. 7. Amplitude histogram obtained from a patch containing the B_1 and the B_2 currents. The membrane potential is −70 mV. (*A*) Trace of current presenting the four types of events and amplitude histogram of the full recording. Three humps can clearly be distinguished. The inset is a magnification of the third hump. The third hump corresponds to the B_2 current and to the superimposition of the B_1 and B_2 currents. The smooth line is the fit of the histogram. It results from the fit of the four gaussians corresponding to each level of the amplitude after they had been separated from the total distribution (lower panels). (*B*) Separation of the baseline events and the amplitude histogram corresponding to the baseline events file. (*C*) Separation of the baseline events and the amplitude histogram corresponding to the level of the B_1 current (i = 0.26 pA). The estimated p_o normalized to the total amplitude histogram (*A*) is 0.54. (*D*) Separation of the baseline events and the amplitude histogram corresponding to the level of the B_2-type current (i = 0.63 pA). p_o is 0.03. (*E*) Amplitude histogram corresponding to the level of the superimposition of the B_1 and B_2 currents. The amplitude is 0.94 pA. The p_o is 0.04. The signal was sampled at 3000 Hz and filtered at 1000 Hz.

B_1 current shares some similarities with the B-type current recently described by Rosenberg et al. (1988) in a bilayer system in terms of: (i) voltage range activation, (ii) zero-current voltage, (iii) p_o-voltage relationships, (iv) kinetics, and (v) insensitivity to dihydropyridines. Nevertheless, some differences do exist between the B_1 and the B channels: the conductance we report, when measured between −40 and 0 mV, is smaller (4 pS) than that of the B channel (greater than 7 pS). This discrepancy may be explained firstly by the difference in the solution used since we did not use Na^+ and secondly by the weak selectivity of the B channel for Ba^{2+} and Na^+ (Rosenberg et al., 1988). Moreover, the greatest open time constant at a HP of −60 mV, reported by Rosenberg et al., is higher than 300 ms, while it is close to 60 ms in our study. This may be due to the different cut-off frequencies used (100 *vs.* 1000 Hz).

The B_2 and the B_3 channels have a distinct behavior since their activities may be stopped for several minutes. Such a behavior has recently been reported for background calcium channel present in cardiac cells (Coulombe et al., 1989). However, the conductances reported by Coulombe and collaborators are much greater than those found by us, and the pharmacology is different since dihydropyridines activate the background calcium channel in cardiac cells.

Finally, the three currents—B_1, B_2 and B_3 currents—have also been observed in bovine glomeru-

Table. Comparison of the background channels and the L channel

	Background channels			L channel
	B_1 channel	B_2 channel	B_3 channel	
Conductance (110 mM Ba^{2+})	4–7 pS	6 pS	10–17 pS	18 pS
(Voltage range used, mV)	(−40, −70) (−30 to +10)	(−100 to −20)		(−60, −20)
Observation voltage range	−90 to −20 −30 to +10	−100 to −20		−80 to 0
Maximum p_o voltage (mV)	−70 mV	n.d.	n.d.	> +50
Dihydropyridine sensitivity	No	No	No	Yes
Greatest open time constant (ms[a])	>60 ms	n.d.	n.d.	7.3
(Voltage used)	(−70 mV)			(−20 mV)

n.d.: not determined.
[a] With 5 μM Bay K 8644.

losa cells. However, more experiments have to be done to determine whether the frequency with which each channel appears is statistically different from what we observed in rat glomerulosa cells.

Conclusion

This study demonstrates the presence of three types of background calcium-permeable channels—the B_1, the B_2, and the B_3 channels—in rat and bovine adrenal glomerulosa cells. These currents may be important at several levels. Firstly, these calcium currents and more precisely the B_1 current may be very important at resting potential since the driving-force and the opening probability of the B_1 channel are very high at resting potential. The B_1 channel is less important when the cells are depolarized because of the low opening probability, of the low driving-force and also because other types of calcium channels may be open.

These currents may play a role in the resting potential and may explain why the membrane potential depolarized by a 45-mV/10-fold increase in K^+ concentration, whereas the Nersnt equation predicts a value of 59 mV (Quinn, Cornwall & Williams, 1987*b*). Moreover, these background calcium-permeable channels can be responsible for the background calcium influx which has been demonstrated in glomerulosa cells at resting potentials close to −80 mV. Contrary to the T, N, and L channels which are not activated at that range of potentials, the B_1, B_2, and B_3 channels can be responsible for this basal calcium influx. Furthermore, a close correlation was demonstrated between the cytosolic calcium concentration and the membrane potential. Indeed, Capponi et al. (1984) showed that increasing the external potassium up to 10 mM induced an increase of the cytosolic calcium, whereas for higher concentrations of potassium, the calcium concentration began to decrease. According to Quinn et al. (1987*b*), for cells bathed in a 10 mM potassium medium, the membrane potential is around −65 mV, a potential close to the value where the p_o of the B_1 channel is maximum. It should also be stressed that, to our knowledge, glomerulosa cells are the only cells that show such a behavior for the calcium concentration when extracellular potassium concentration is increased. Finally, the insensitivity of the background calcium-permeable channels to the dihydropyridines can be linked to the lack of effect of nifedipine on the basal secretion of aldosterone. From these studies, we can postulate that these background currents are involved in the maintaining of basal calcium influx and aldosterone secretion. These channels may also be implicated in responses induced by factors that provoked hyperpolarization as has been suggested for the action of EGF in human A431 carcinoma cell line (Peppelenbosch, Terlooren & de Laat 1991). Nevertheless, more experiments have to be done before we can conclude on the definitive role of these channels in glomerulosa cells.

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