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Two Types of Pharmacologically Distinct Ca^{2+} Currents with Voltage-dependent Similarities in Zona Fasciculata Cells Isolated from Bovine Adrenal Gland

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Abstract. Voltage-activated Ca^{2+} currents, in zona fasciculata cells isolated from calf adrenal gland, were characterized using perforated patch-clamp recording. In control solution (Ca^{2+}: 2.5 mM) a transient inward current was followed, in 40% of the cells, by a sustained one. In 20 mM Ba^{2+}, 61% of the cells displayed an inward current, which consisted of transient and sustained components. The other cells produced either a sustained or a transient inward current. These different patterns were dependent upon time in culture. Current-voltage relationships show that both the transient and sustained components activated, peaked and reversed at similar potentials: −40, 0 and +60 mV, respectively. The two components, fully inactivated at −10 mV, were separated by double-pulse protocols from different holding potentials where the transient component could be inactivated or reactivated. The decaying phase of the sustained component was fitted by a double exponential (time constants: 1.9 and 20 sec at +10 mV); that of the transient component was fitted by a single exponential (time constant: 19 msec at +10 mV). Steady-state activation and inactivation curves of the two components were superimposed. Their half activation and inactivation potentials were similar, about −15 and −34 mV, respectively. The sustained component was larger in Ba^{2+} than in Sr^{2+} and Ca^{2+}. Ni^{2+} (20 μM) selectively blocked the transient component while Cd^{2+} (10 μM) selectively blocked the sustained one. (±)Bay K 8644 (0.5 μM) increased the sustained component and nitrendipine (0.5–1 μM) blocked it selectively. The sustained component was inhibited by calciseptine (1 μM). Both components were unaffected by ω-conotoxin GVIA and MVIIC (0.5 μM). These results show that two distinct populations of Ca^{2+} channels coexist in this cell type. Although the voltage dependence of their activation and inactivation are comparable, these two components of the inward current are similar to T- and L-type currents described in other cells.

Key words: Adrenal cells — Ca^{2+} channel types — Voltage-dependence — Pharmacology

Introduction

Several classes of voltage-sensitive Ca^{2+} channels exist distinguished by their biophysical properties and their pharmacology. The most common types of Ca^{2+} channels described in neuronal and non-neuronal cells include T- and L-types which can coexist in the same cells. In response to positive voltage steps, the former elicits a transient inward current whereas the latter elicits a long-lasting inward current which either does not inactivate or inactivates very slowly. Other criteria also allow their identification. T-type Ca^{2+} channels activate at a more negative potential than L-type and the half-inactivation potential of the transient inward current is usually very negative, near the resting potential of the cells. While the pharmacology of T-type Ca^{2+} channels is poorly documented, except for their sensitivity to Ni^{2+}, L-type Ca^{2+} channels are selectively inhibited by different classes of organic compounds such as dihydropyridines (DHP), phenylalkylamines or benzodiazepines.

In endocrine cells, L-type Ca^{2+} channels are always expressed, occasionally with other types of Ca^{2+}- or Na^{+}-channels as in rat β-pancreatic cells (Ashcroft, Kelly & Smith, 1990) or bovine chromaffin cells (Fenwick, Marty & Neher, 1982). In cortical cells of the adrenal

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gland, voltage-sensitive Ca^{2+} channels have been described, mainly in rat and bovine adrenal zona glomerulosa (ZG) cells. While only voltage-sensitive T-type Ca^{2+} currents were characterized in rat ZG cells by Matsunaga et al. (1987*a*) and Quinn et al. (1992), other authors reported in the same preparation the coexistence of both T- and L-type Ca^{2+} currents (Varnai et al., 1995) and of an additional N-type Ca^{2+} current (Durroux, Gallo-Payet & Payet, 1988). In bovine ZG cells, Quinn et al. (1992) identified a T-type voltage-sensitive Ca^{2+} current that, from other works, could be coexpressed with an L-type Ca^{2+} current (Matsunaga et al., 1987*b;* Rossier et al., 1996*a*). Although the comparison of these different studies is complicated by differences in experimental conditions, it appears, as in other cell preparations, that the threshold potential of the T-type Ca^{2+} current is always more negative than that of the L-type and that the half-inactivation potential of the T-type current, when determined, is very negative (*see* for example Matsunaga et al., 1987*a*). In zona fasciculata (ZF) cells of rat adrenal gland, Barbara & Takeda (1995) have detailed biophysical and pharmacological properties of voltage-sensitive Ca^{2+} currents. In freshly isolated cells, the inward current was dominated by a T-type Ca^{2+} current that vanished with time in culture whereas a DHP-sensitive L-type Ca^{2+} current developed progressively. In these cells, half-activation and inactivation potentials of the T-type inward current were more negative than those of the L-type current. Another work performed on ZF cells isolated from bovine adrenal gland (Mlinar, Biagi & Enyeart, 1993*a*) reported the presence of a T-type Ca^{2+} current that in 10 mM external Ca^{2+} activated at −50 mV and was fully inactivated at about −30 mV. Furthermore, Coyne & Pinkey (1991) detected a DHP-sensitive L-type Ca^{2+} channel after incorporation of plasma membrane originating from bovine ZF cells into planar lipid bilayers.

The physiological relevance of these different components of inward Ca^{2+} current to the biological activity of adrenal cells is not completely understood. Although in adrenal ZG or ZF cells, Ca^{2+}-dependent action potentials were scarcely recorded in different ionic conditions (Matsunaga et al., 1987*a;* Barbara & Takeda, 1995; Bilbaut et al., 1996), an increase in intracellular Ca^{2+} concentration is required to trigger hormone steroidogenesis and secretion (Capponi et al., 1988). Sources of Ca^{2+} would be extra- and intracellular and one pathway by which Ca^{2+} could flow into adrenal cells might be through voltage-sensitive Ca^{2+} channels identified in these cell types (Yanagibashi, Kawamura & Hall, 1990; Enyeart, Mlinar & Enyeart, 1993; Rossier et al., 1996*b).*

In the present study, we described in ZF cells isolated from calf adrenal gland two distinct types of Ca^{2+} inward current, transient and sustained. These currents resemble the T- and L-type Ca^{2+} currents characterized in other cells in their kinetic and pharmacological properties while they differ by the similarity of their voltage-dependent steady-state activation and inactivation.

Materials and Methods

Cell Preparation

Isolated ZF cells were prepared according to the protocol described by Bilbaut et al. (1996). Briefly, adrenal glands originating from 4–6-month-old calves were sliced with a Stadie-Riggs microtome. Only the second slice (0.5 mm thick) was used for enzymatic dispersion by sequential trypsination as reported by Crozat, Penhoat & Saez (1986). The dissociation medium contained trypsin (Sigma, St. Louis, MO) at 0.125% in HAM F12/DMEM medium (1:1), gentamycin (20 μg/l), penicillin-streptomycin (100 U/ml), L-glutamin (5 mM) and $NaHCO_3$ (14 mM) buffered with HEPES (15 mM) at pH 7.4. Dispersed cells were washed and resuspended in culture medium containing HAM F12/DMEM (1:1), L-glutamin (5 mM), penicillin-streptomycin (100 U/ml), $NaHCO_3$ (14 mM) insulin (10 μg/ml), transferrin (10 μg/ml), and vitamin C (10^{-4} M) supplemented with fetal bovine serum (1%). The isolated cells were seeded at low density (4,000 to 6,000 cells/cm^2) in 35 mm petri dishes and cultured in a humidified incubator at 37°C and 5% CO_2 in air.

Solutions and Drug Preparation

The control external solution contained (in mM): NaCl, 135; KCl, 5; $CaCl_2$, 2.5; $MgCl_2$, 2; glucose, 10; HEPES, 10 at pH 7.2 buffered by NaOH. In Ba^{2+} solution, $BaCl_2$ (20 mM) was isosmotically substituted for NaCl in Ca^{2+}-free solution in which blockers of K^+ conductances, tetraethylammonium chloride (TEA, 20 mM) and 4-aminopyridine (4-AP, 5 mM) also replaced NaCl. 4-AP was prepared just before use and pH adjusted to 7.2 by HCl. Solutions containing Sr^{2+} or Ca^{2+} (20 mM) replacing Ba^{2+} were also used. Cd^{2+} (1–100 μM) and Ni^{2+} (10–100 μM) were added to the Ba^{2+} solution. Tetrodotoxin (TTX; Alomone Labs, Jerusalem, Israel) was used at a concentration of 2×10^{-6} M. Nitrendipine (Biomol, Plymouth Meeting, PA) and (±)Bay K 8644 (Calbiochem, La Jolla, CA) were prepared as stock solution in dimethylsulfoxide (DMSO) then kept at −20°C. When used, these DHP were protected from light. The final DMSO concentration (≤0.1%) did not affect the membrane properties of isolated cells. Calciseptine (Latoxan, Rosans, France), ω-conotoxin GVIA and MVIIC (Alomone Labs) were kept as stock solution at −20°C in distilled water. Serial dilutions of these drugs and toxins were made in Ba^{2+}-containing solution before use.

For the perforated patch recording, the ionic composition of the pipette filling solution was (in mM): Cs aspartate, 130; NaCl, 10; $MgCl_2$, 2; EGTA, 5; HEPES, 10 at pH 7.2 buffered by NaOH. Amphothericin B (Sigma, St. Louis, MO) at a concentration of 240 μg/ml was prepared as described by Rae et al. (1991): 6 mg of amphothericin B were solubilized in 100 μl of DMSO by sonication for a few seconds and 20 μl of this solution were then added to 5 ml of internal solution.

Electrophysiology

Voltage-clamp recordings were made in whole cell configuration using the perforated patch-recording method. Experiments were performed on isolated cells maintained in culture 24 to 72 hr after plating. A petri

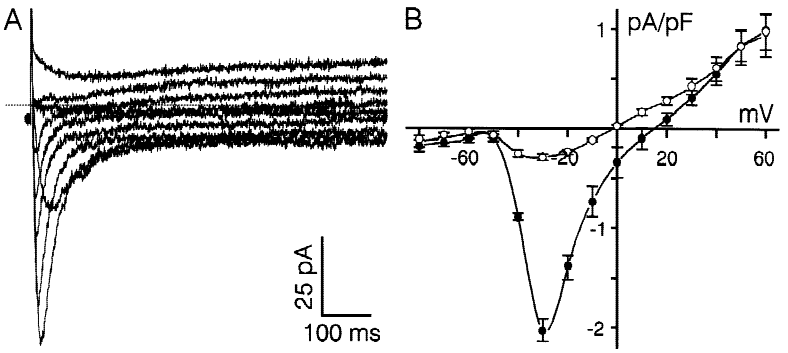

Fig. 1. (*A*) Inward currents evoked in 2.5 mM Ca^{2+} by test pulses ranging from −50 to +40 mV by 10 mV increments from a holding potential of −90 mV. The transient inward current is followed by a sustained inward component. In this and in the following recordings, dashed line indicates the zero current level. (*B*) *I/V* relationships of the transient (solid symbols) and sustained (open symbols) inward currents ($n = 5$).

dish was transferred from the incubator onto the stage of an inverted microscope and the culture medium was replaced by the control physiological solution. Further changes of external solution were then performed using a gravity perfusion system at a rate of 0.5 ml/min placed close to the cell (about 100 μm). Pipettes were pulled from thick-walled borosilicate glass (CG 150T–1.5 mm o.d., Clark Electromedical Instruments, Reading, GB) using a vertical puller (Kopf, Tujunga, CA) and were connected to the headstage of a patch-clamp amplifier RK 400 (Bio-Logic, Claix, France). For perforated patch recordings, the tip of the pipette was dipped into the pipette solution for a few seconds then the pipette was backfilled with the solution containing amphothericin B. Patch pipettes had a tip resistance of 2–4 MΩ in control solution. Partition of the cell membrane by amphothericin B was continuously monitored by applying 20 mV hyperpolarizing pulses every 30 sec from a holding potential of −90 mV. Experiments were started about 10 to 15 min after the seal was established when the increase of the transient capacitive current reached a steady-state value indicating a final series resistance of about 8 to 12 MΩ. Dialysis of the intracellular compartment through Cs^+-permeant amphotericin B pores was also monitored by applying episodic voltage pulses to +50 mV from a holding potential of −90 mV in order to control the blocking effect of this ion on the voltage-activated transient outward K^+ current described in this cell type (Bilbaut et al., 1996). Usually, this transient outward current was completely inhibited when the transient capacitive current was steady. The series resistance was not compensated because the voltage error introduced by the maximal activation of inward currents was estimated to be lower than 2%. Leak current was not subtracted except where indicated and capacitive transient current was not compensated. All experiments were performed at room temperature (20–25°C) on single cells of 15–20 μm diameter that adhered to the bottom of the petri dish.

Data Acquisition and Analysis

Pulse protocols were generated using the P-Clamp software (Axon Instruments, Burlingame, CA). Current signals were filtered at 0.3 kHz, digitized at 4 kHz with an analogue-to-digital converter (Labmaster TM 40, Scientific Solutions, Solon, OH) and stored on the hard disc of a computer. Holding current was continuously monitored on a pen recorder (Kipp & Zonen, Delft, the Netherlands). For data analysis, Bio-Logic softwares were used. For current-voltage (*I/V*) relationships, membrane currents are expressed in pA/pF. Results are presented as means ± SEM. Comparison of statistical data were considered as significantly different when *P* values obtained from *t*-test were lower than 0.05.

Results

Inward Currents in Physiological Ca^{2+} Concentration

When the external solution contained a physiological Ca^{2+} concentration (2.5 mM), a TTX-insensitive inward current was elicited in response to variable voltage pulses applied from a holding potential of −90 mV. In 60% of the cells (20/34), this membrane current was a transient inward current that decayed in a few hundred milliseconds. In the other cells, a tiny sustained component that did not show inactivation for 750 msec, prolonged the transient inward current as illustrated in Fig. 1*A*. Figure 1*B* shows *I/V* relationships ($n = 5$) of the early current at the peak (solid symbols), and the late membrane current at the end of a voltage pulse of 750 msec duration (open symbols). In these 5 cells, a sustained component of the inward current was present. The transient inward current was detected at about −50 mV, reached a maximum value at −30 mV and reversed at +16 mV (Fig. 1*B*, solid symbols). The sustained component (Fig. 1*B*, open symbols) was detected near −50 mV, peaked at −30 mV and reversed at 0 mV. Similar values were obtained when TEA (20 mM) and 4-AP (5 mM) were added to the control solution (6 cells). Averaged from 14 cells, maximum magnitude of the transient inward current was −42.5 ± 3 pA while that of the sustained component was −9 ± 1 pA. When the membrane potential was held at −30 mV, no voltage-dependent inward current could be recorded suggesting that it was fully inactivated at this potential.

Because the sustained component of the inward current was less than −10 pA and was detected in a similar range of potential as the transient inward current, the question remained whether this total inward current was due to the activity of one or of two distinct classes of ionic channels. Further experiments performed in Ba^{2+} solution revealed the presence of a large sustained component of the inward current in most of the cells. This led us to examine the characteristics of these current

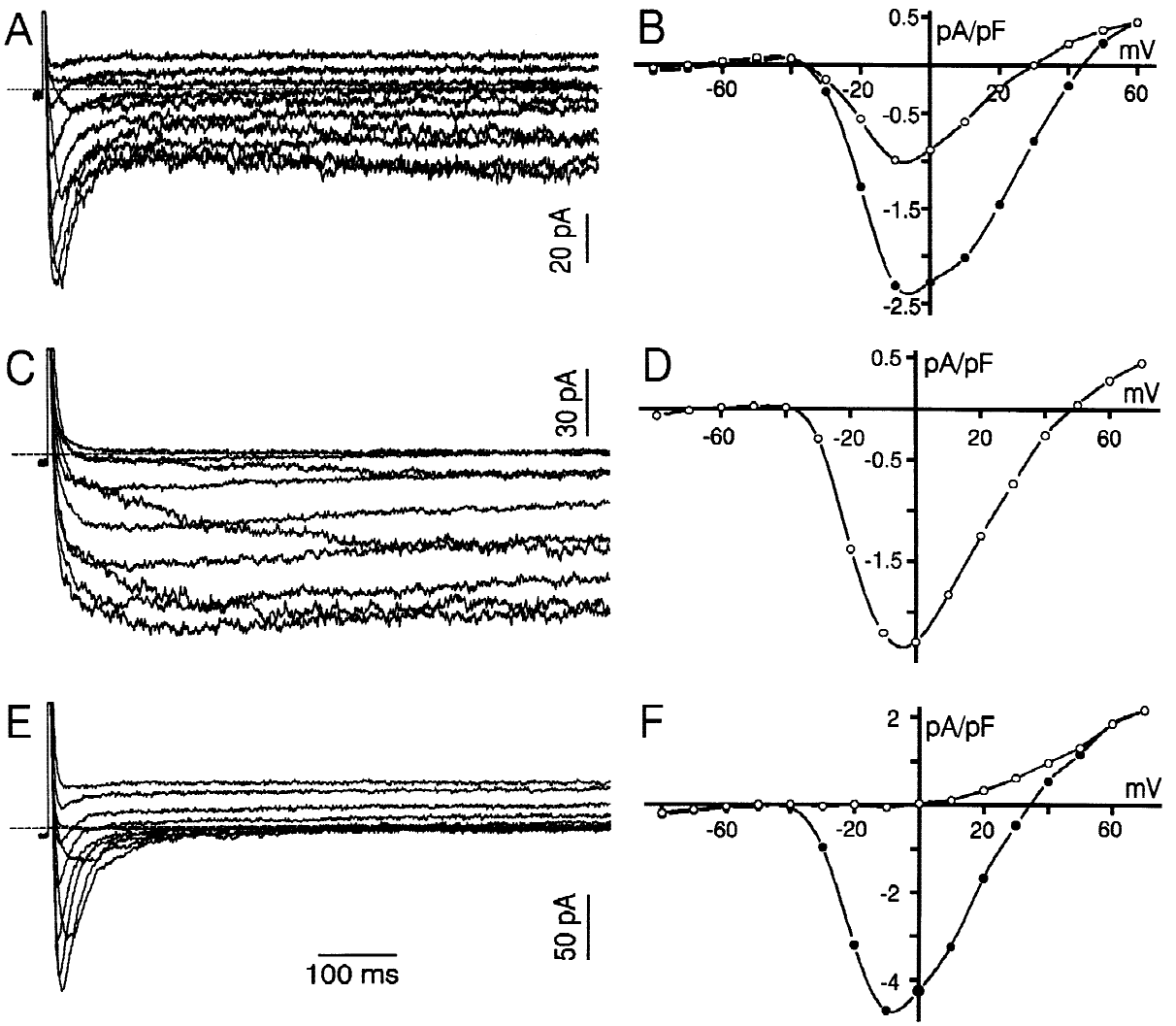

Fig. 2. Inward currents in Ba^{2+}-containing solutions (20 mM). (*A*) Family of membrane currents where a transient inward component is followed by a sustained component displaying no noticeable inactivation for 750 msec. (*B*) *I/V* relationships from the recording presented in Fig. 2*A*. Peak current (solid symbols) and sustained current (open symbols) are plotted against the membrane potential from −80 to +60 mV. Both components of the inward current are detected at a potential of −40 mV and peak at about −10 mV. Apparent reversal potential of the transient component is about 10 mV more positive than that of the sustained one. (*C*) Family of inward currents where the transient component is not observed. (*D*) *I/V* relationship of the sustained inward current shown in Fig. 2*C*. The membrane current was measured at the end of the voltage pulses. This curve is similar to that illustrated in Fig. 2*B* (open symbols). (*E*) Family of inward membrane current where only the transient component is observed. (*F*) *I/V* relationships of the peak (solid symbols) and late (open symbols) inward currents from the recording presented in Fig. 2*E*. The curve of the peak current is similar to that illustrated in Fig. 2*B* (solid symbols). Current traces in *A*, *C* and *E* were obtained from a holding potential of −90 mV for test pulses ranging from −40 to +50 mV by 10 mV increments. Interpulse duration: 45 sec.

components under Ba^{2+} conditions as detailed in the next sections.

Voltage-Activated Inward Currents in 20 mM Ba^{2+} Solution

In the external solution where Ba^{2+} (20 mM) was substituted for Ca^{2+} as the charge carrier, three different patterns of voltage-activated inward currents were recorded. In the majority of the cells (61%, 86/140), the inward current evoked by depolarizing steps of 750 msec duration consisted of a transient component followed by a sustained one (Fig. 2*A*). *I/V* relationships (Fig. 2*B*) established as defined above indicate that both components of the inward current activated at a similar potential (−40 mV), reached a maximum amplitude at about −10 mV while the apparent reversal potential of the transient and sustained components was +44 and +30 mV, respectively. In 29% of the cells (40/140), the initial transient

Table. Transient and sustained inward currents in culture over time

	Transient-type	Transient and sustained types	Sustained-type
24 hr	19%	70%	11%
(Total cells: 57)	(11/57)	(40/57)	(6/57)
48 hr	4%	59%	37%
(Total cells: 54)	(2/54)	(32/54)	(20/54)
72 hr	3%	48%	48%
(Total cells: 29)	(1/29)	(14/29)	(14/29)

The left column indicates the age of the cells in culture at time of experimentation. The other columns indicate in percent the different types of inward current recorded.

component was not observed (Fig. 2*C*). In these cells, inward current was totally dominated by a sustained current which activated at −40 mV, was maximal at about 0 mV and reversed at +46 mV as indicated by the *I/V* relationship shown in Fig. 2*D*. Lastly, in 10% of the cells (14/140), only a transient inward current was elicited (Fig. 2*E*). From the *I/V* relationships (Fig. 2*F*), threshold potential, maximum current and apparent reversal potential of the transient inward current (Fig. 2*F*, solid symbols) were −40, −10 and +34 mV, respectively. The late current (Fig. 2*F*, open symbols) was outward from a membrane potential more positive than 0 mV and intersected the *I/V* curve of the transient inward current at about +60 mV.

The reversal potential (E_{rev}) of these two components was determined by subtraction of the leak current from the total inward current. In cells where only the transient inward current was expressed, the leak current was estimated by measuring the membrane current at the end of the 750 msec test pulses. For the sustained inward current, the leak current was measured after this sustained component was blocked by 1 μM nitrendipine or 100 μM Cd^{2+} (*see* following section). Under these conditions, the reversal potential of the transient inward current was +61 ± 1.8 mV ($n = 10$) and that of the sustained component was +61.3 ± 1.9 mV ($n = 10$). These two values were not significantly different ($P > 0.05$).

The expression of these different kinds of inward currents was dependent upon time in culture. This is shown in the Table where the occurrence of transient inward current, preponderant in the early hours following cell dissociation, progressively diminished with time in culture. The sustained inward current was present in 81% of the cells 24 hr after plating and its appearance was maintained throughout the culture. Expressed in current density, the values 24 hr after plating, were 3 ± 0.3 pA/pF for the transient component ($n = 30$) and 2.5 ± 0.2 pA/pF for the sustained one ($n = 25$) while 48 hr later, these values were 1.7 ± 0.2 and 1.3 ± 0.2 pA/pF ($n = 16$), respectively. In these two situations, no evidence for a linear relation was found when the maximum current of both components was reported against the cell capacitance. The membrane capacitance was not significantly modified 24 and 48 hr after plating (33.5 ± 1.3 pF, $n = 30$ and 35.5 ± 1.2 pF, $n = 16$ with $P > 0.05$) indicating that the inward current progressively decreased with time in culture. This was confirmed by the fact that 3 days after plating, about 70% of cells failed to elicit inward currents and that usually, after 4 days, no inward current was detected.

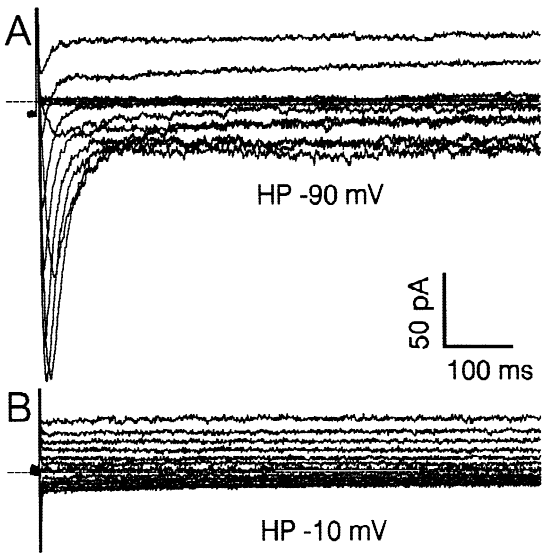

Fig. 3. (*A*) Transient and sustained inward currents elicited by voltage pulses of 750 msec duration from a control holding potential of −90 mV (HP −90 mV). Voltage steps were applied each 45 sec between −60 and +50 mV by 10 mV increment. (*B*) After the holding potential was made more positive (HP −10 mV), no voltage-sensitive Ba^{2+} current was observed in response to a stimulating protocol similar to that defined in *A*.

Separation of the Two Components of the Inward Current

When T-type and L-type Ca^{2+} inward currents are coactivated, the simplest method used to separate them consists of changing the holding potential (Bean, 1985). This method could not be applied to our preparation because the threshold potential of both components of inward current was similar. However, when the membrane potential was set to −10 mV (Fig. 3*B*) instead of −90 mV (Fig. 3*A*), we observed that both the transient and the sustained inward currents failed to be produced in response to voltage steps of increased amplitude. This suggests that both components of the inward current were inactivated at this holding potential. Taking advantage of this fact and also of the rapid decay of the transient component, the following protocols were used to

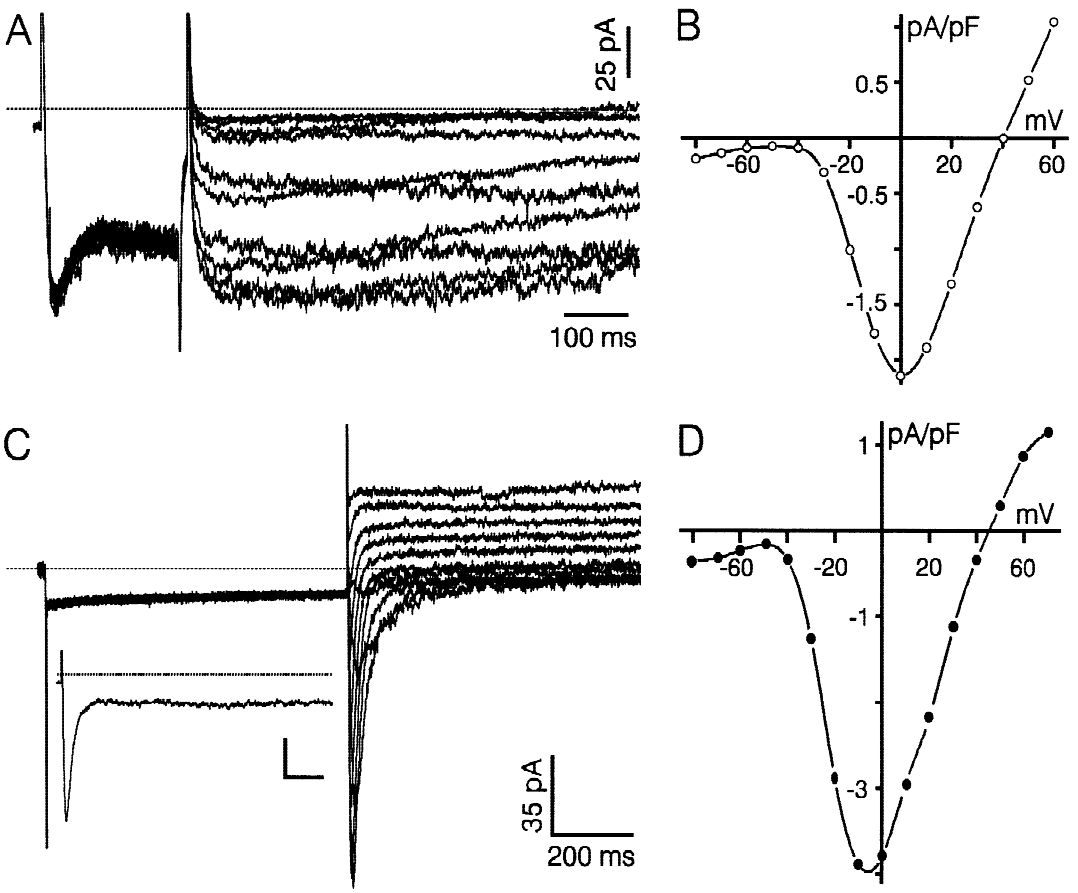

Fig. 4. (*A*) Suppression of the transient component by prepulses of 230 msec duration at −10 mV from a holding potential of −90 mV. Test pulses of variable amplitude elicit only a sustained inward current. (*B*) *I/V* relationship of the sustained component established by measuring the sustained current at the end of the test pulses. This curve is similar to that presented in Fig. 2*D*. (*C*) Suppression of the sustained component was obtained from a holding potential of −10 mV where the two components of the inward current were inactivated. Following prepulses of 750 msec duration at −80 mV, test pulses of variable amplitude reactivated only the transient component of the inward current. Inset: control current (holding potential: −90 mV; pulse: −10 mV) showing both transient and sustained components of the inward current (calibration: horizontal bar, 100 msec; vertical bar, 50 pA). (*D*) *I/V* relationship of the transient inward current measured at the peak. Compare with Fig. 2*F* (solid symbols).

separate the two components of the inward current. In the first protocol, the membrane was stepped by a depolarizing prepulse of 230 msec to a fixed potential of −10 mV from a holding potential of −90 mV. This prepulse was separated from a postpulse of variable amplitude by a brief interpulse of 15 msec duration resetting the membrane potential to −90 mV. As shown in Fig. 4*A*, only the sustained inward current was activated by the postpulses, the duration of the prepulse being long enough to inactivate the transient component. The *I/V* relationship of this sustained inward current (Fig. 4*B*) was similar to that reported in Fig. 2*D* obtained from a cell where only the sustained component was present. In the second protocol, the cell membrane was held at −10 mV to inactivate both components of the inward current. Then, the membrane was stepped to −80 mV during the 750 msec before variable postpulses were delivered to the cell. The result is illustrated in Fig. 4*C* where only the transient component of the inward current is elicited by the postpulses. This indicated that the duration of the repolarizing pulse was sufficient to reactivate the transient component but too short to reactivate the sustained one. The *I/V* relationship of the transient inward current obtained in such conditions (Fig. 4*D*) was very similar to that shown in Fig. 2*F* (solid symbols) recorded from a cell where only the transient component of the inward current was observed. However, in this experiment, the magnitude of the transient component obtained after reactivation from a holding potential of −10 mV was smaller by about 25% than that obtained in control conditions from a holding potential of −90 mV. This suggests, in agreement with the data reported by Mlinar et al. (1993*a*), that the duration of the prepulse might be too short to fully reactivate this current component. Indeed, the recovery from inactivation of transient Ca^{2+} currents in bovine ZF cells studied by Mlinar et al. (1993*a*) showed that the reactivation of this inward current was achieved in 5 sec from a holding potential of −80 mV

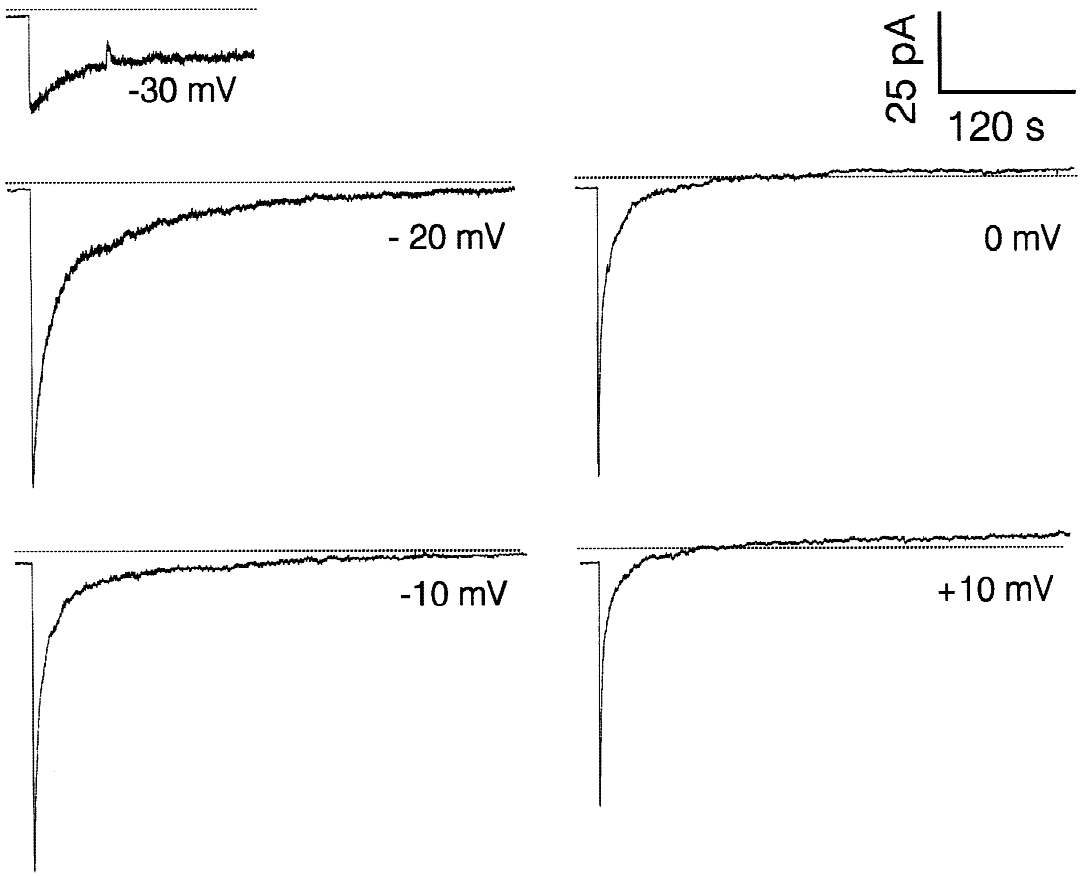

Fig. 5. Inactivation of the sustained component of the inward current obtained by depolarizations of 360 sec duration (except top trace) at different potentials as indicated on the right of each record. Holding potential: −90 mV. Interpulse duration: 180 sec.

and that after 1 sec at this potential, approximately 60% of the current was again available.

From these observations, it appears that transient and sustained inward currents, when coactivated, can be separated by experimental protocols which take into account differences in their kinetics of inactivation and reactivation. This strongly suggests that the inward current in ZF cells of the calf adrenal gland is due to the activity of two distinct classes rather than a single class of Ca^{2+} channels.

Time- and Voltage-dependent Properties of the Inactivation of Current Components

The time dependence of inactivation of the sustained inward current component was analyzed from recordings obtained by stepping the cell membrane with variable voltage pulses of 360 sec duration. The interpulse duration was 180 sec and the holding potential set at −90 mV. A typical record obtained for 5 different test potentials is presented in Fig. 5. In this cell where the transient component of the inward current was absent, it can be seen that the sustained component inactivates very slowly. The decay of this component was well fitted by the sum of two exponential functions of the form: $I_m = A_0 + A_1(e^{-t/\tau f}) + A_2(e^{-t/\tau s})$ where A_0 was the offset current, A_1, τ_f and A_2, τ_s, were the maximal amplitude and the time constants of each exponential component. The analysis indicates that the two time constants are voltage-dependent (Fig. 6*A* and *B*). Their values reached a minimum for membrane potentials more positive than −10 mV, about 2 sec for the fast time constant (τ_f) and 20 sec for the slow one (τ_s).

The analysis of the decaying phase of the transient inward current was made from records where the sustained component was either not observed or was suppressed using the protocol defined above (*see* Fig. 4*C*). This decay was well fitted by a single exponential function of the form $I_m = A_0 + A_1(e^{-t/\tau})$, A_0, A_1 and τ having their usual meanings. The values of these time constants, similar to those reported by Mlinar et al. (1993*a*) were voltage-dependent with a minimum near 0 mV (Fig. 6*C*). For comparison, the time constant of inactivation of the transient inward component at +10 mV was in our preparation about 100 times smaller than the fast time constant of the sustained one.

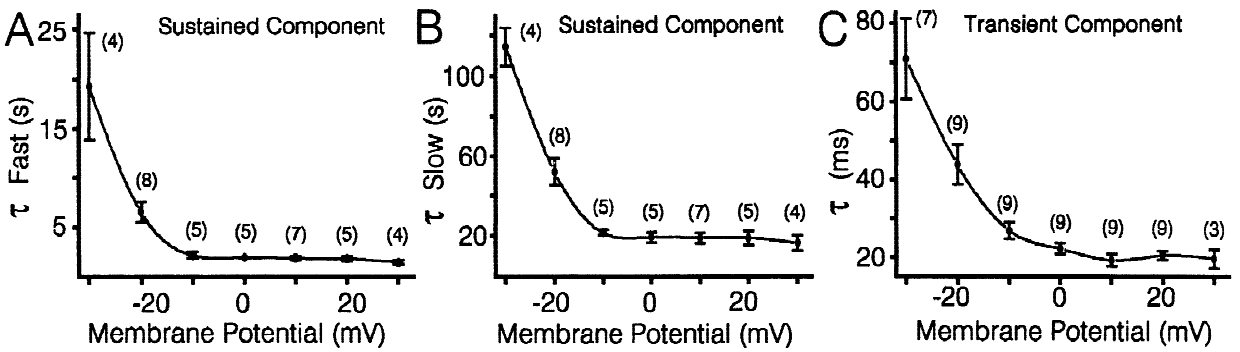

Fig. 6. (*A*) Fast time constant (τ_{Fast}) of the decaying phase of the sustained inward current plotted against the membrane potential. (*B*) Slow time constant (τ_{Slow}) of the decaying phase of the sustained inward current. (*C*) Time constant (τ) of the decaying phase of the transient inward current. Numbers above each symbols indicate (*n*). Note the difference in time scale.

The voltage dependence of the steady-state inactivation of the transient and sustained components of the inward current was studied using a double-pulse protocol where a variable conditioning potential was separated from a fixed test potential by a short pulse of about tens of msec at −90 mV. When the duration of the conditioning potential was 750 msec (Fig. 7*A*), the sustained component obtained for a test pulse to 0 mV did not display any inactivation over the entire potential range while the transient component disappeared. On the other hand, when the duration of the conditioning potential was increased to 180 sec (Fig. 7*B*), the two components of the inward current evoked by test pulses at 0 mV were fully inactivated. Steady-state inactivation curves of both components of the inward current, averaged from 4 different cells, were established by plotting the normalized current activated by test potentials against the values of the conditioning potential. The inactivation curves of the transient component of the inward current obtained using this protocol were similar to those constructed from recordings where only the transient inward current was observed. The inactivation curve of the sustained component was established by measuring the membrane current at the end of the test pulse of 600 msec duration that followed the conditioning potential of 180 sec. These conditions were made so that the sustained component could not be contaminated by the transient component that was fully inactivated in less than 200 msec at the test potential of 0 mV. As shown in Fig. 8, the two curves (solid line: transient component; dashed line: sustained component) are nearly superimposed. Fitted by a Boltzman function, the half-inactivation potentials of the transient and sustained inward current were −34.5 ± 0.4 mV and −33.5 ± 1.9 mV, respectively. The slope factor was 4.5 ± 0.4 for the transient component and 4.5 ± 0.14 for the sustained component. *t*-test analysis indicated that these different parameters were not significantly different with $P > 0.05$.

Voltage-dependent Properties of the Activation of Current Components

The voltage dependence of the steady-state activation was estimated by normalizing the membrane conductance calculated by division of the current peak by the driving force $E_m - E_{rev}$. To avoid the contamination of one component by the other, activation curves of the transient inward current were constructed from recordings where the sustained component was suppressed as shown in Fig. 4*C* or absent as illustrated in Fig. 2*E*. The activation curves of the sustained component were established by measuring the membrane current at the end of 750 msec test potentials. Figure 8 shows that these two curves, averaged from 4 different cells, superimposed perfectly. The mean half-activation potential and slope factor obtained from fitting of the Boltzman function was −16.3 ± 1.7 mV for the transient current and −15 ± 3.6 mV for the sustained current while the slope factor was 6.5 ± 0.5 and 6 ± 0.4, respectively. These two parameters were not significantly different ($P > 0.05$).

The steady-state activation and inactivation curves presented in Fig. 8 show that the threshold potential of the transient and sustained components, ranging from −50 to −40 mV, could not be clearly separated and that both components were fully inactivated at about −10 mV.

Selectivity

Figure 9*A*, representative of 5 cells, compares the effects of three divalent cations Ca^{2+}, Sr^{2+} and Ba^{2+} at a concentration of 20 mM on the magnitude of the inward current elicited by test pulses to 0 mV from a holding potential of −90 mV. In these cells, the inward current consisted of a transient component prolonged by a sustained one which was larger in Ba^{2+} than in Sr^{2+} and Ca^{2+} giving the following sequence $Ba^{2+} > Sr^{2+} > Ca^{2+}$.

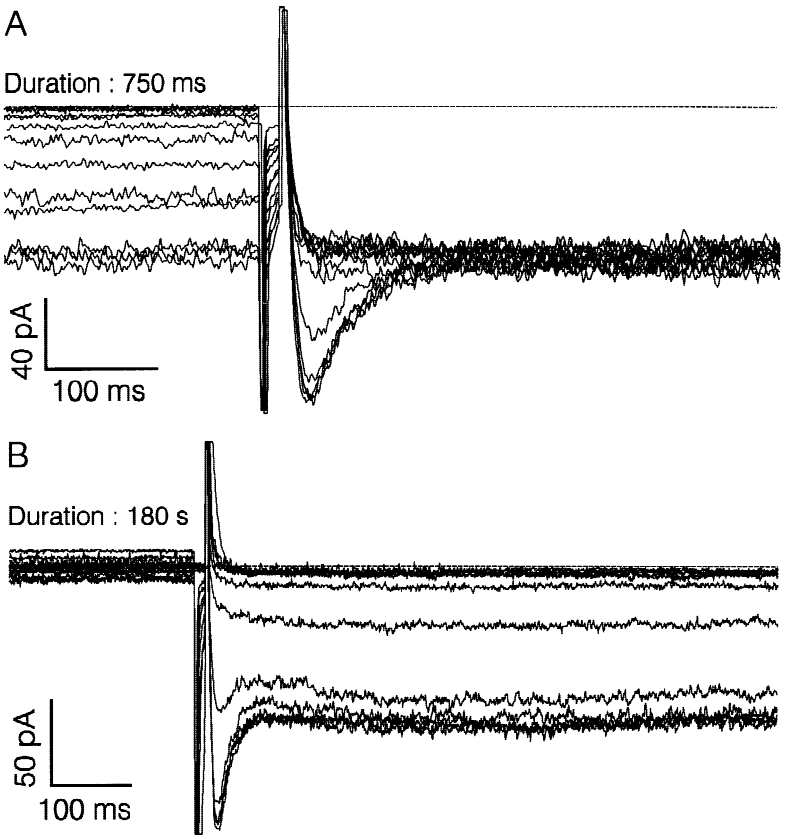

Fig. 7. (*A*) and (*B*) compares the inward current evoked by a double pulse protocol in two different situations. In *A*, test pulses are preceded by variable conditioning potential of 750 msec duration; only the transient component of the inward current was inactivated. In *B*, test pulses are preceded by variable conditioning potential of 180 sec duration; both transient and sustained components of the inward current were inactivated. Pulses were separated by 60 sec in (*A*) and 180 sec in (*B*). Note the difference in the magnitude of the membrane current at the end of conditioning potentials.

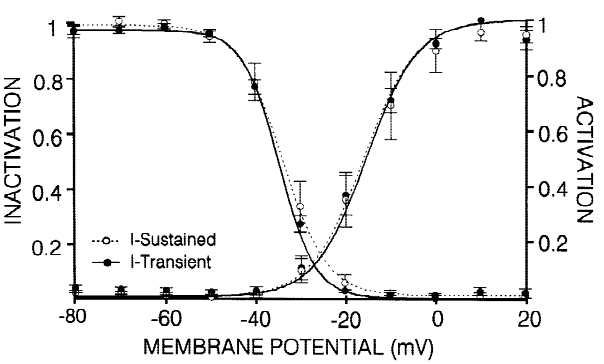

Fig. 8. Voltage dependence of steady-state activation and inactivation ($n = 4$). Experimental data were obtained as explained in the text and fitted by a Boltzman function. $V_{0.5}$ activation of transient (solid line) and sustained components (dashed line) is -16.3 ± 1.7 and -15 ± 3.6 mV, respectively. For steady-state inactivation $V_{0.5}$ is -34.5 ± 0.4 (transient component, solid line) and -33.5 ± 1.9 mV (sustained component, dashed line). Solid and open symbols correspond to experimental data for transient and sustained components of the inward current, respectively.

The magnitude of the transient component in Ba^{2+} and Sr^{2+} was similar and larger than that in Ca^{2+}. *I/V* relationships (Fig. 9*B*) of the sustained component of the inward current established from the experiment reported in Fig. 9*A* show that the threshold potentials were similar in these different solutions while the current peak in Sr^{2+} (open circles) and Ca^{2+} (triangles) was more positive than in Ba^{2+} (solid circles). Also, the apparent reversal potential of this component was more positive by 10 mV in Sr^{2+} than in Ba^{2+} and Ca^{2+}. To minimize the problem of current contamination of one component by the other during activation of the inward current, the effects of these divalent cations were tested on a cell where in 20 mM Ca^{2+}, only the transient component was observed. Compared to Ca^{2+}, the peak of the transient inward current was increased by 14% in Sr^{2+}- and 8% in Ba^{2+}-containing solution giving the following sequence $Sr^{2+} > Ba^{2+} \geq Ca^{2+}$ different from that obtained for the sustained component (*not illustrated*).

Pharmacology

Two inorganic blockers of Ca^{2+} channels, Ni^{2+} and Cd^{2+}, were tested on 6 and 7 different cells, respectively. Ni^{2+}, at low concentration, is known to act selectively on T-type channels while L-type channels are more sensitive to Cd^{2+}. A typical effect of Ni^{2+} is illustrated in Fig. 10*A*. In this cell where both components of current were present, only the transient component was affected by 20 μM Ni^{2+}, which at a concentration of 100 μM depressed also the sustained component. On the other hand, in a

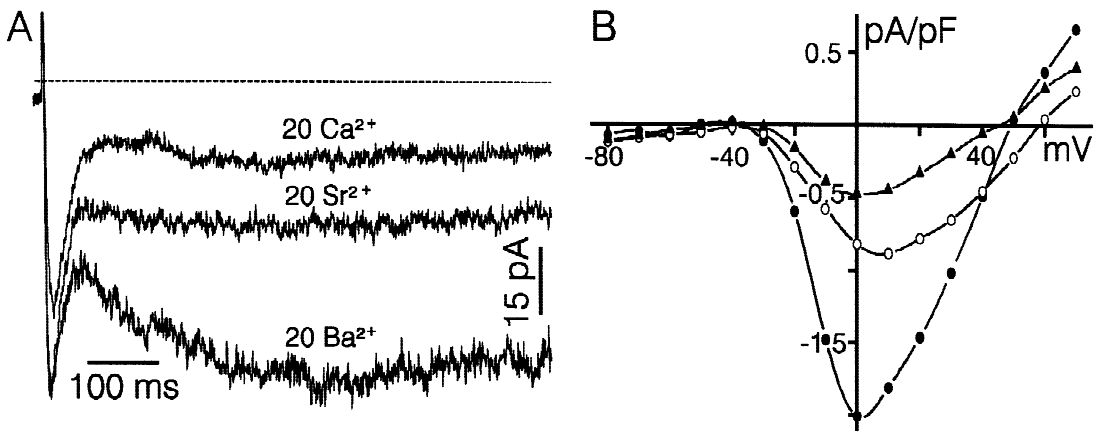

Fig. 9. (*A*) Inward current elicited by a test pulse to 0 mV from a holding potential of −90 mV in 20 mM Ca^{2+}, Sr^{2+} and Ba^{2+}. (*B*) *I/V* relationships of the sustained component of the inward current constructed from the recording presented in Fig. 9*A* in the presence of 20 mM Ca^{2+} (triangles), 20 mM Sr^{2+} (open circles) and 20 mM Ba^{2+} (solid circles).

cell where the inward current was dominated by a transient component (Fig. 10*B*), this was diminished by only 10% in the presence of 5 μM Cd^{2+} and by 14% in 10 μM Cd^{2+}. The late current also diminished with increasing Cd^{2+} concentration. The effect of Cd^{2+} on the sustained inward current was better observed on a cell where only this component was present (Fig. 10*C*). The membrane current was diminished by 47, 73 and 80% in the presence of 1, 5 and 10 μM Cd^{2+}, respectively. These effects were reversible. The sustained component of the inward current was fully inhibited when Cd^{2+} concentration was 50–100 μM.

Dihydropyridines are a class of organic Ca^{2+} channel agonists and antagonists known to selectively activate or inhibit L-type Ca^{2+} currents (Hess, Lansman & Tsien, 1984), while the T-type Ca^{2+} current is usually resistant to the effects of these drugs. In our preparation, (±)Bay K 8644, an agonist of L-type Ca^{2+} current, was a potent activator of the sustained component of the inward current (Fig. 11*A*, insert). At a concentration of 500 nM, it increased this component by a factor of 2.1 ($n = 4$). As illustrated by *I/V* relationships in Fig. 11*A*, in the presence of this drug, the threshold potential and the peak amplitude were shifted by 10 mV in the hyperpolarizing direction without modification of the apparent reversal potential. In a cell where the two components of the inward current were coactivated (Fig. 11*B*), Bay K 8644 increased the sustained component by a factor of 5.2 while the transient component was increased by a factor of 1.5. Because this agonist was not tested on a purely transient inward current, it was difficult to ascertain whether the increase of this current component was due to an effect of this drug on the channel activity or to the overlap of the two components during the time course of their activation.

A wide variety of dihydropyridine-based calcium antagonists are available and the one used in the present experiment was nitrendipine. Bean (1984) had reported that the effect of DHPs on L-type inward Ca^{2+} currents in cardiac muscle cells was voltage-dependent. Neither this property nor the effects of nitrendipine concentration on the inward current were examined on our preparation. However, when the concentration of this drug was lower than 200 nM, we found that from a very negative holding potential (−90 mV), the sustained but not the transient component of the inward current, elicited by test potentials to 0 mV, was inhibited to varying degrees. Complete block of the sustained current occurred at DHP concentrations ranging from 0.5 to 1 μM as shown in Fig. 11*C*. In this figure where the two components of the inward current were present, only the sustained component was fully inhibited by 1 μM nitrendipine. The peak of the transient component was also diminished by 15%. This could be attributed to an effect of nitrendipine on the channel activity or, more likely, corresponds to the fraction of the sustained component of the inward current that in control conditions overlapped with the transient component. Unfortunately, this drug was not tested on a purely transient inward current. The effects of nitrendipine were reversible at all concentrations. Figure 11*D* represents *I/V* relationships ($n = 4$) of the sustained component of the inward current before (solid symbols) and after (open symbols) exposure of cells to 1 μM nitrendipine. In the presence of drug, the membrane current shows a small inward inflection between −40 and −10 mV. For test potentials more positive than 0 mV, the membrane current is outward and intersected the control curve at +60 mV.

Finally, toxins known to preferentially block different types of Ca^{2+} channels were tested on our preparation. Calciseptine (1 μM), an inhibitor of L-type Ca^{2+} channels (de Weille et al., 1991), blocked between 80 and 100% (3 cells) of the sustained component of the inward current (*not illustrated*). On the other hand, this

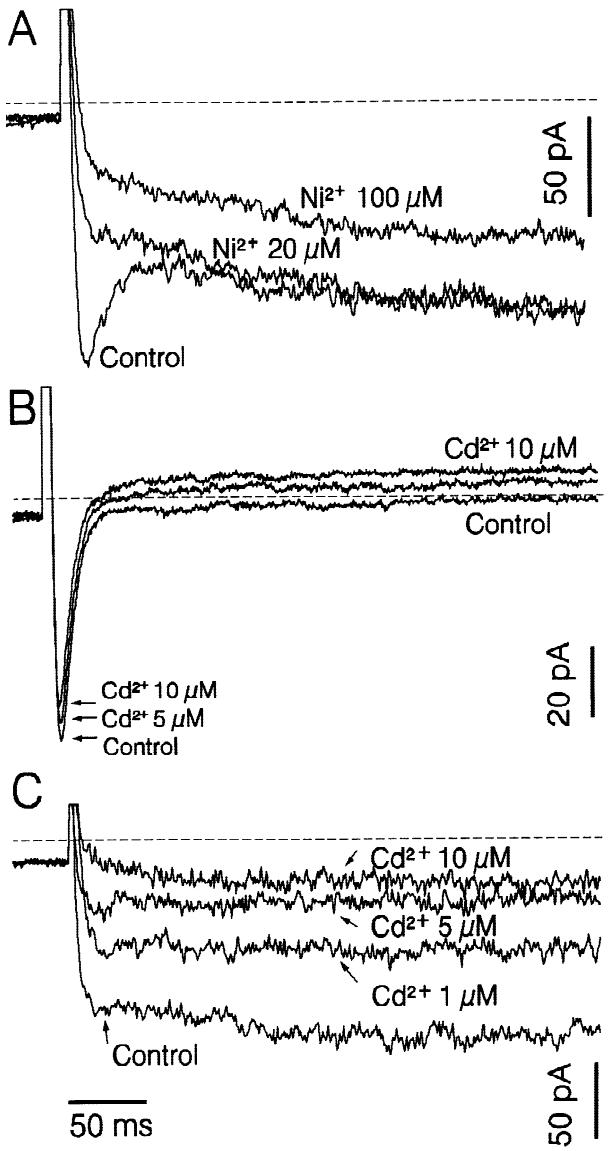

Fig. 10. (*A*) Effect of Ni^{2+} on the inward current activated at 0 mV from a holding potential of −90 mV. Only the transient component was inhibited by 20 μM Ni^{2+}. At 100 μM, the sustained component was also diminished. (*B*) Effect of Cd^{2+} (5 and 10 μM) on an inward current where the transient component was dominant. Test pulse: 0 mV. Holding potential: −90 mV. (*C*) Effect of Cd^{2+} (1, 5 and 10 μM) on an inward current where the sustained component was dominant. Compare with Fig. 10*B*. Test pulse: 0 mV. Holding potential: −90 mV.

toxin used at a similar concentration was without effect on a cell where only the transient component was activated. Other toxins, ω-conotoxin GVIA (Olivera et al., 1985) that inhibits N-type Ca^{2+} channels and ω-conotoxin MVIIC (Hillyard et al., 1992) that inhibits P/Q-type Ca^{2+} channels do not produce noticeable effects on the two components of the inward current after 5 min of exposure at a concentration of 500 nM (5 and 3 cells, respectively).

Discussion

We described here the existence of voltage-sensitive transient and sustained Ca^{2+} currents in ZF cells isolated from calf adrenal gland. These two current components, revealed in the presence of Ba^{2+}, displayed similar voltage-dependent activation and inactivation but could be separated on the basis of their inactivation kinetics and their pharmacological properties thus giving definitive evidence for the coexistence of two distinct types of Ca^{2+} channels in this cell type.

Sustained Inward Current

From the present results, the sustained component of the inward current is sensitive to low concentration of DHP agonist and antagonist, is blocked by low Cd^{2+} concentration and by the toxin calciseptin but is insensitive to different ω-conotoxins. Such a pharmacological profile led us to conclude that this current component results from the activity of ionic channels belonging to the class of L-type Ca^{2+} channels. This is strengthened by the fact that this current component was greater with Ba^{2+} as the charge carrier than with Sr^{2+} and Ca^{2+}. Because the reversal potential of these different ionic currents was not precisely determined in the present work, we did not attempt to estimate their permeability ratios.

The activation potential of this L-type inward current was around −55 mV in 2.5 mM Ca^{2+} and around −40 mV when external Ca^{2+} was replaced by 20 mM Ba^{2+}, this difference being likely due to the screening of surface charges. Such values are rather negative but a survey of the voltage-dependent characteristics of activation of L-type currents in different preparations reveals their heterogeneity. Threshold potentials, although often positive of −20 mV, can be as negative as −50 mV even in the presence of a high divalent cation concentration (Plant, 1988; Ganitkevich & Isenberg, 1990). In this work, we show that this sustained component of the inward current inactivates very slowly according to a bi-exponential function whose time constants were voltage-dependent for test potentials more negative than −10 mV. Although little data are available on the time- and voltage-dependence of inactivation of L-type currents, it appears that in other cell types, such as smooth muscle cells (Nilius, Kitamura & Kuriyama, 1994), the decaying phase of this type of Ca^{2+} current also obeys a biexponential function, with fast and slow time constants. From Nilius et al. (1994) this channel inactivation process would involve the transition between several inactivated states.

Transient Inward Current

The transient component of the inward current in bovine ZF cells inactivated rapidly and was principally sensitive

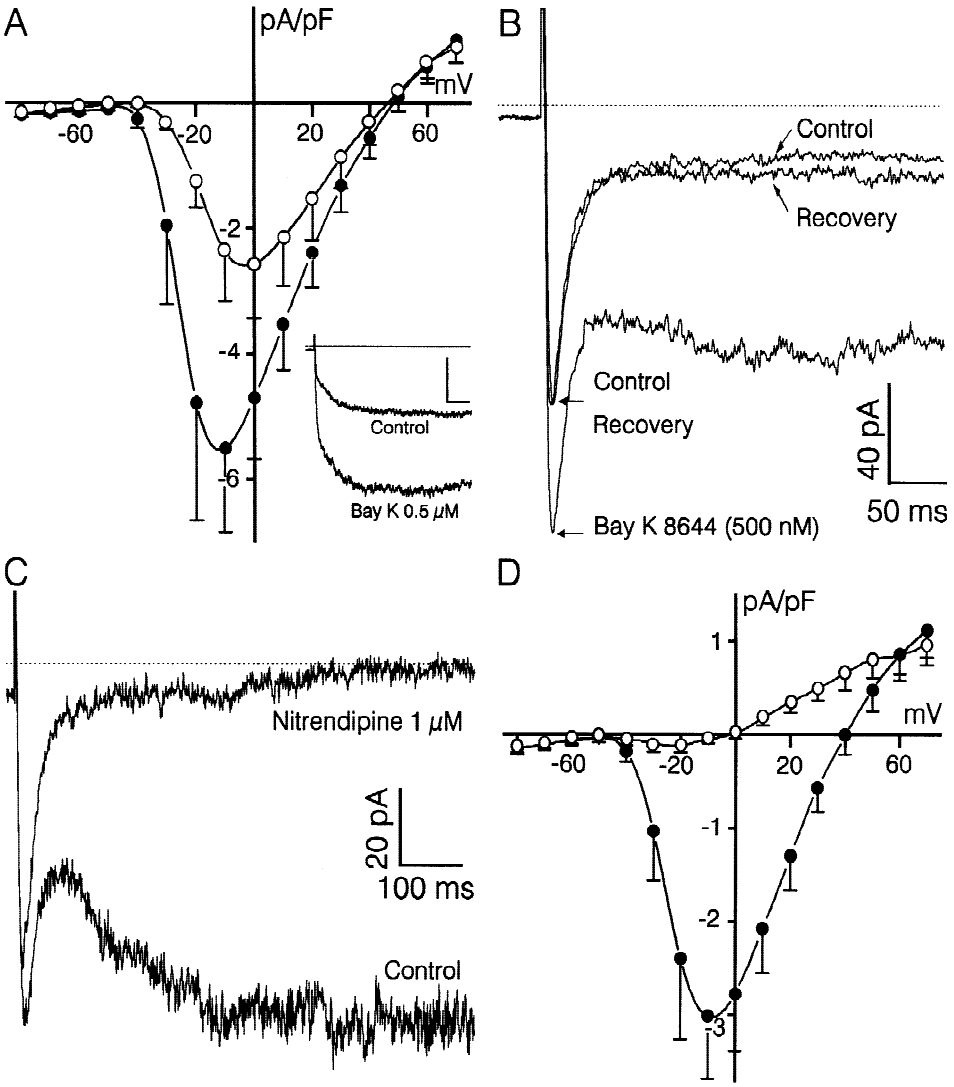

Fig. 11. (*A*) *I/V* relationships ($n = 4$) showing the effects of Bay K on the sustained component of the inward current (open circles: control; solid circles: Bay K). Maximum current and threshold potential were shifted towards a hyperpolarizing potential. Inset: current trace at 0 mV from a holding potential of −90 mV in the absence and in the presence of Bay K (calibration: horizontal bar, 100 msec; vertical bar, 100 pA). (*B*) Reversible effect of Bay K on an inward current displaying transient and sustained components. (*C*) Effect of 1 μM nitrendipine on both components of the inward current elicited at 0 mV from a holding potential of −90 mV. (*D*) *I/V* relationships ($n = 5$) of the membrane current measured at the end of the voltage pulses in control conditions (solid symbols) and in the presence of 1 μM nitrendipine (open symbols).

to a weak concentration of Ni^{2+}. This component of inward current was nearly unaffected when external Ca^{2+} was equimolarly replaced by Sr^{2+} or Ba^{2+}. These different characteristics led us to propose that this inward current results from the activity of a class of Ca^{2+} channels belonging to T-type. Although T-type Ca^{2+} channels constitute a heterogeneous class of ionic channels (Huguenard, 1996) that from Randall (1998) would need a subclassification, the transient component of the inward current of calf ZF cells activates and inactivates in a potential range more positive than that usually determined for typical T-type currents. Therefore, we cannot exclude that this transient inward current was related to the activity of another class of Ca^{2+} channels, the E class Ca^{2+} channels (Soong et al., 1993) corresponding to so-called R-type current (Zhang et al., 1993). Compared to T-type currents, R-type currents activate at more positive potentials, exhibit a transient waveform, are blocked by weak Ni^{2+} concentration and have permeation properties nearly similar to T-type with Sr^{2+} more permeant than Ba^{2+} and Ca^{2+} (Bourinet et al., 1996). Further experiments using other approaches would be necessary to decide between these two possibilities.

This transient inward current is very similar to the T-type Ca^{2+} current described by Mlinar et al. (1993*a*) on the same endocrine cells, except that, in the present

study, the threshold potential (−40 mV) and half-inactivation potential (−34.5 mV) were more positive by 10–15 mV. This discrepancy cannot be strictly ascribed to a surface charge effect due to differences in the ionic composition of external solution (10 mM Ca^{2+} in Mlinar's work *vs.* 20 mM Ba^{2+} in the present study) since in the two preparations the half-activation potential calculated from the normalized activation curves was comparable (around −16 mV).

A singular feature resulting from this work was that the steady-state activation and inactivation of transient and sustained inward currents of calf adrenal ZF cells have a similar voltage dependence raising the question of their functional significance in the membrane activities of this cell type. Assuming that the transient component of the inward current reported here is a T-type current, the authors agree that this class of ionic channels may be involved in repetitive firing of the cell membrane (Huguenard, 1996) since these channels are known to activate at a more negative potential than L-type channels. Consequently, such a mechanism cannot be proposed to explain the repetitive membrane activities observed in various ionic conditions in calf ZF cells (Bilbaut et al., 1996) and a better understanding of this phenomenon would be reached by modeling the different ionic currents identified in this cell type, namely, transient and sustained inward Ca^{2+} currents and A-type outward current (Bilbaut et al., 1996).

Differential Expression of Transient and Sustained Inward Currents

As pointed out in the introduction of the present work, L-type Ca^{2+} currents were not always detected in rat or bovine adrenal cortical cells. This can be due to the well-known lability of this type of inward current which is subject to "run down" processes during the experiments performed using standard patch recording (broken membrane). In bovine adrenal ZF cells examined by this method Mlinar et al., (1993*a*) detected L-type inward currents with Ca^{2+} (10 mM) as the charge carrier in only 2% of cells, while from our results performed using the perforated patch recording method, 40% of cells produced sizeable sustained Ca^{2+} current with 2.5 mM Ca^{2+} in the bath. Such a difference may depend not only on the recording method used, but also on the charge carrier since in the presence of Ba^{2+} (20 mM) we found that 90% of calf adrenal ZF cells expressed an L-type inward current. Another explanation is that, while in the present experiments adrenal ZF cells were obtained from calves of 4–6-months-old, Mlinar et al. (1993*a*) worked on adrenal ZF cells originating from steers of 1–3-years-old. In these conditions, changes in the expression of Ca^{2+} current cannot be excluded since such events have been reported during the postnatal development of cardiomyocytes (Osaka & Joyner, 1991) and skeletal muscle cells (Beam & Knudson, 1988). In these two cell types, while T-type current is preponderant during the embryonic stages, it diminishes or disappears during the postnatal life in parallel with the appearance of L-type Ca^{2+} current.

The expression of these two components of inward current was dependent on time of culture. This process affected principally the transient component, which was preponderant in the early stage of culture then progressively disappeared with the time. Barbara & Takeda (1995) observed in rat adrenal ZF cells the appearance of a sustained inward current during culture, while in calf adrenal ZF cells this type of inward current was present 24 hr after plating and most often coactivated with a transient component. We do not know if this sustained component was already present in freshly isolated cells or if it appeared during the time separating the plating from the first experiments. Modulation of the expression of T- and L-type Ca^{2+} current with time in culture has also been reported in other preparations such as vascular smooth muscle cells (Akaike et al., 1989; Richard et al., 1992), neuronal cells (Yaari, Hamon & Lux, 1987) and cardiomyocytes (Gomez et al., 1994).

Ca^{2+} Currents and Secretion

The involvement of voltage-sensitive Ca^{2+} channels in adrenocorticotropin hormone (ACTH)-induced steroidogenesis was reported in bovine adrenal ZF cells. From Yanagibashi, Kawamura & Hall (1990) corticoid secretion is blocked by antagonists of Ca^{2+} channels such as nifedipine and PY 108-068. From Enyeart et al. (1993), the secretory response, correlated with a membrane depolarization (Mlinar, Biagi & Enyeart, 1993*b*), is inhibited by different blockers of T-type Ca^{2+} channels. For these authors, Ca^{2+} would flow into the cell through T-type Ca^{2+} channels which activate in a potential range corresponding to the window current. Recently, Chorvatova et al. (1998) have shown in calf adrenal ZF cells that the stimulation by angiotensin II, from a holding potential of −10 mV, induced a long-lasting transient outward current due to the activity of both $Cl_{Ca^{2+}}$ and apamin-sensitive $K_{Ca^{2+}}$ channels. This outward current was dependent on extracellular Ca^{2+} but was not modified in the presence of strong concentration of DHP antagonist, indicating that Ca^{2+} flow did not occur through the voltage-dependent Ca^{2+} channels. The present results, establishing that T- and L-type Ca^{2+} currents are fully inactivated at the holding potential of −10 mV, confirm the conclusions of Chorvatova et al. (1998) whose study was carried out at this holding potential. In agreement with other works performed on ZG cells (Burnay et al., 1994; Rohacs et al., 1994), hormonal stimulation can activate an entry of Ca^{2+} by voltage-insensitive Ca^{2+} channels,

such as store-operated Ca^{2+} channels, although in ZF cells, some participation of voltage-sensitive Ca^{2+} channels in this mechanism cannot be excluded in physiological conditions.

To conclude, this study demonstrates the presence of two types of voltage-sensitive Ca^{2+} channels, transient and sustained, in calf adrenal ZF cells. Their activities in the physiological function of this cell type are still to be determined.

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