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Effects of dexamethasone on L-type calcium currents in the A7r5 smooth muscle-derived cell line

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Patch clamp experiments were used to characterize the effect of dexamethasone on calcium currents in A7r5 cells. Pretreatment for 48 h with 200 nM dexamethasone did not affect the single channel conductance, the voltage dependence of channel opening, or the voltage-dependent inactivation of L-type channels. However, dexamethasone caused an approximately 2-fold increase in the amplitude of L-type calcium currents in 5 out of 9 experiments, suggesting an increase in the number of active channels. The effect of dexamethasone appeared to be greatest on batches of cells with low control current density. The amplitude of T-type calcium current was not affected by dexamethasone.

Vascular smooth muscle; Calcium channel; A7r5 cell; Dihydropyridine; Dexamethasone; Cushing's syndrome

1. INTRODUCTION

Hypertension often accompanies Cushing's syndrome, which is caused by elevated levels of plasma glucocorticoids [1]. An anomalous reactivity to vascular agonists seems to underlie this hypertensive state since, in patients and animal models with this syndrome, lower doses of norepinephrine and angiotensin II are required to induce pressor responses [2].

Strong evidence suggests that the vascular tone is mediated by an steady calcium influx through L-type calcium channels [3]. Modulation of calcium channels by membrane potential or second messengers would affect calcium entry, and in turn, vascular peripheral resistance. This raises the possibility that the increased vascular resistance in Cushing's syndrome results from a glucocorticoid-induced increase in activity of L-type calcium channels. In support of this hypothesis, dexamethasone treatment increased both nefedipine-sensitive Ca^{45} influx and the number of high affinity binding sites for dihydropyridines, in the A7r5 vascular smooth muscle cell line [4]. An increase in channel activity could result from an increase in (a) the probability that a channel is open, (b) the single channel current, or (c) the number of functional channels. Binding of dihydropyridines (DHPs) and Ca^{45} flux experiments cannot distinguish between these possibilities. Moreover, interpretation of the results is complicated by the lack of a direct relationship between the number of DHP binding sites and the number of functional channels [5,6], and the voltage dependence of DHP binding [7]. Since changes in activity of voltage-dependent calcium channels could also be secondary to changes in membrane potential, measurements under voltage clamp conditions are necessary to understand mechanistically the basis of dexamethasone effects on L-type Ca^{2+} channels.

We have previously characterized the kinetics of activation and inactivation of L-type calcium channels in the A7r5 cell line using the patch-clamp technique [5,8–10]. Using the same methods, we have studied the effects of dexamethasone on calcium channel currents in this cell line. We find that dexamethasone does not affect the single channel conductance, the voltage dependence of activation, or inactivation of this channel. Instead, our results suggest that dexamethasone increases the number of active L-type calcium channels.

2. MATERIALS AND METHODS

2.1. *Cell preparation*

The A7r5 cell line, originally derived from embryonic rat aorta, was obtained from the American Type Culture Collection (ATCC). The two different batches of cells used were obtained from the ATCC in 3/88 and 8/92. Cells were grown as previously described [5]. Before the experiments, confluent cell layers were dispersed with trypsin, resuspended in Dulbecco's Modified Eagle's Medium containing 0.5% (by vol.) fetal calf serum, and plated on 35 mm dishes. After attachment of the cells to the dish, cytochalasin-D (1 μg/ml) was added to maintain a rounded morphology [5]. Experiments were carried out at room temperature (~ 22°C).

2.2. *Chemicals*

Dexamethasone (9α-fluoro-16α-methylprednisolone) (lots no. 129F-01295 and 62H0202; HEPES (*N*-[2-hydroxyethyl]-piperazine-*N'*-2-ethanesulfonic acid); NMG (*N*-methylglucamine); EGTA (ethyleneglycol-bis-(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid), TEA-Cl (tetraethylammonium chloride), $BaCl_2$, K glutamate, and MgATP were obtained from Sigma Chemical Co. (St. Louis, MO). CsCl and

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CsOH were obtained from Fisher (Pittsburgh, PA). (+)202-791 was a gift from Drs. D. Römer and E. Rissi (Sandoz, Basel, Switzerland).

2.3. *Steroid treatment*

Confluent cultures were exposed to 200 nM dexamethasone in 10% (by vol.) fetal calf serum for 48 h. 200 nM dexamethasone was shown to induce a maximal stimulation of Ca^{45} uptake in A7r5 cells after one day of exposure [4]. Where noted, before the addition of dexamethasone, fetal calf serum was treated with charcoal in order to remove contaminant cortisol [4]. Dexamethasone was added from a 20 mM stock solution dissolved in ethanol. The controls were cells treated with 10% fetal calf serum plus vehicle.

2.4. *Voltage clamp measurements*

Voltage clamp was established in the whole-cell and cell-attached configurations [11] using an Axopatch-1B patch clamp amplifier (Axon Instruments, Foster City, CA). Voltage commands were given and data were obtained using a Labmaster A-D converter with pClamp software (Axon Instruments) on an IBM AT-compatible microcomputer.

In whole-cell experiments, electrodes had series resistances of 2–5 MΩ. The standard extracellular solution contained (in mM): $BaCl_2$ 15, TEA-Cl 121, glucose 10, NMG-HEPES 10, pH 7.4. The standard intracellular solution contained (in mM): CsCl 130, MgATP 5, Cs-EGTA 10, Cs-HEPES 10, pH 7.2. During the whole cell recording, the capacity transient induced by a −10 mV step pulse was canceled by electronic compensation. The cell capacitance was read from the 'whole cell capacitance' dial on the Axopatch, following cancellation of the capacity transient. For the cell-attached configuration, electrodes of ~ 5 MΩ were made with borosilicate glass (World Precision Instruments, New Haven, CT) and coated with silicone rubber. The pipette solution contained (in mM) $BaCl_2$ 110 mM, NMG-HEPES 10 mM, pH 7.3. After seals were formed in a physiological salt solution, the cell membrane potential was zeroed with a bath solution containing (in mM): KGluconate 140, NMG-EGTA 5, NMG-HEPES 10, pH 7.3. 1 μM (+)202-791 was added to increase the resolution of single channel measurements.

3. RESULTS

Fig. 1A compares peak current densities in control vs. cells treated with 200 nM dexamethasone for 48 h. This represents a summary of all the experiments conducted using 9 different batches of cells. Each point represents the mean value from one cell preparation. Data points above the dashed line reflect up-regulation of calcium currents by dexamethasone, which was observed in 5 of 9 experiments. The effect on current density did not depend in an obvious way on the batch of cells used (indicated in Fig. 1 by different symbols). We attempted to decrease the experimental variability by pretreating the fetal calf serum with charcoal to remove steroids [4], but cell-cell and batch-batch variability did not decrease (open circles). The variability of the effect of dexamethasone could be partially accounted for by a variation in the basal level of calcium channel activity in the different experiments. This is shown in Fig. 1B, where the ratio of current densities is plotted as a function of the control current density. Four of five positive experiments had control current densities equal to or smaller than 10 pA/pF, and four of five experiments with such low current densities produced a positive result.

To understand the mechanism underlying the increase in current induced by dexamethasone in cells with low basal current levels, current-to-voltage relationships were examined for the three batches of cells where the current increased by a factor of 2.0 or larger (Fig. 2A). In A7r5 cells, with Ba^{2+} as the charge carrier, L-type calcium currents activate above −30 mV, reach a peak near +10 mV, and then decrease in amplitude as the driving force on Ba^{2+} decreases [5,8,12,13]. The shape of the current–voltage relation and the extrapolated reversal potential were not affected by dexamethasone (Fig. 2A). This suggests that the voltage dependence of channel gating and the permeation characteristics of the calcium channels were identical in control and treated cells. No change in cell capacitance was observed (Fig. 2, legend). Fig. 2B shows current–voltage relationships for batches of cells where no change in current was evident.

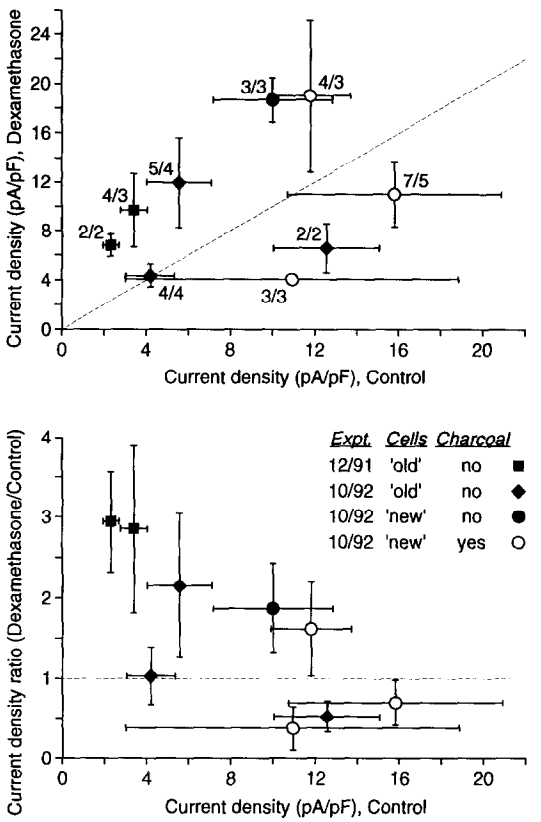

Fig. 1. The effect of dexamethasone on calcium current density. (A) Correlation between mean current levels in control and dexamethasone-treated cells. The numbers near each symbol indicate the number of dexamethasone-treated cells/number of control cells studied in each experiment. Values are mean ± S.E.; we note that S.E. values calculated for $n = 2$–3 may not be accurate. (B) Correlation between enhancement of current by dexamethasone (dex./control ratio) and the control current levels. For the dex./control ratio, the fractional SE (fSE = SE/mean) was calculated as $fSE_{dex/control} = (fSE^2_{dex} + fSE^2_{control})^{1/2}$.

Several lines of evidence indicate that the effect of dexamethasone is on L-type calcium currents, as opposed to the T-type currents that are occasionally ob-

served in A7r5 cells [12]. First, the potentiated currents were blocked by 1 μM nifedipine or 100 μM Cd^{2+}, whereas T-current is resistant to 1 μM nifedipine (not shown). Second, normal voltage dependence was observed for dexamethasone-treated cells when the membrane potential was held at −40 mV, which inactivates the rapid transient component of current (not shown). Third, dexamethasone had no effect at relatively negative voltages (−40 to −30 mV) (Fig. 2A), where T-type calcium channels can make a substantial contribution to the whole-cell current. Fourth, and most directly, the amount of current inactivating during a 70 ms depolarization to −20 mV (Fig. 2C) was unaffected by dexamethasone: the rapidly inactivating component was −0.36 ± 0.05 pA/pF in control (mean ± S.E., $n = 6$), compared to −0.35 ± 0.07 pA/pF in dexamethasone ($n = 7$). Measurement of T-current in this way is supported by the observation that L-currents in Ba^{2+} show little inactivation during pulses as long as 300 ms at −20 mV [8], while T-currents inactivate with a half time of 14 ms [12]. The sustained current could be contaminated slightly by T-current, which may not inactivate fully at negative membrane potentials [14]. However, since dexamethasone does not affect the transient component, it is unlikely that the hormone has a selective effect on a sustained component of T-current.

We also examined inactivation of L-type currents in control and dexamethasone-treated cells, using a double-pulse protocol [15]. Inactivation, produced during long prepulses (330 ms) to various voltages, was assayed by test pulses to +10 mV, from a holding potential of −40 mV. Using this protocol, inactivation increases monotonically with depolarization, and reaches a steady value at potentials more positive than +20 mV [8]. This voltage dependence was not modified by dexamethasone (not shown).

We further investigated the effect of dexamethasone at the single-channel level, using cell-attached patch clamp recording. Using (+)202-791 to enhance channel activity, L-type channels were observed in 7 of 20 (35%) patches from control cells, and 10 of 19 (53%) patches from dexamethasone-treated cells. This difference was not statistically significant (2 × 2 contingency table, [16]), although the data are consistent with a higher channel density per patch in dexamethasone treated cells (Fig. 3A). Since in these experiments we did not distinguish between batches of cells with high or low basal channel activity, the effect of dexamethasone may be underestimated. Dexamethasone did not affect the slope conductance of the channels (26 pS, Fig. 3B), consistent with the evidence from whole-cell recording (Fig. 2A) that calcium channel permeation is not affected.

4. DISCUSSION

From their experiments using Ca^{45} flux and DHP

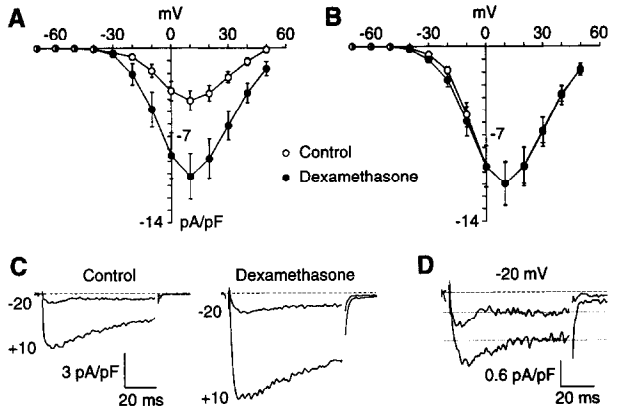

Fig. 2. Effects of dexamethasone on T- and L-type calcium currents. Current-to-voltage relationships from experiments where dexamethasone increased L-type calcium currents (A) and where no effect was noticed (B). The three batches of cells where the dex/control ratio was greater than 2.0 are included in Fig. 2A; other cells in Fig. 2B. The cell capacitance of control and treated cells in (A) was 30.2 ± 3.9 pF (mean ± S.E., $n = 9$) and 30.1 ± 3.6 pF ($n = 11$), respectively, and for experiments in (B) 32.2 ± 3.5 pF ($n = 19$) and 34.5 ± 3.8 pF ($n = 23$), respectively. Currents were elicited by 70 ms step pulses to different membrane potentials from a holding potential of −80 mV. Symbols are mean ± S.E. of 9 control and 11 treated cells in (A), and 20 control and 23 treated cells in (B). There was a significant difference ($P < 0.05$) between control and dexamethasone-treated cells at −20 to +50 mV in (A) by the ANOVA test. (C) Sample currents from a control cell (cell f1d16, whole cell capacitance 47.1 pF) and a dexamethasone-treated cell (cell d1d16, 33.5 pF). (D) Expanded view of the currents at −20 mV, from (C). The larger current was after treatment with dexamethasone. T-current was measured as the difference between peak current and the steady-state current at the end of the voltage step (dotted lines).

binding, Hayashi et al. [4,17] concluded that dexamethasone affects calcium entry through L-type Ca^{2+} channels in A7r5 cells. They further suggested that this effect requires the glucocorticoid receptor and protein synthesis, since it was blocked by the glucocorticoid antagonist RU 38486, cycloheximide, or actinomycin D. Thus, dexamethasone might act by increasing the synthesis of new calcium channels. In spite of this evidence, alternative or/and different effects of dexamethasone could not be excluded. For example, glucocorticoids might depolarize the cell membrane, which would increase Ca^{2+} entry through L-type calcium channels, and enhance binding of DHPs [5,7]. Such a possibility is not consistent with our results, since all the experiments were carried out under voltage clamp conditions.

Dexamethasone might also increase calcium currents indirectly, by affecting the synthesis of components of second messenger pathways that can modulate L-type calcium channels. Supporting this speculation is the effect of glucocorticoids on several receptors, G-proteins, and kinases [17–24]. In this respect, a role for protein kinase C (PKC) in the stimulatory effect of dexamethasone on L-channels was suggested, as this gluco-

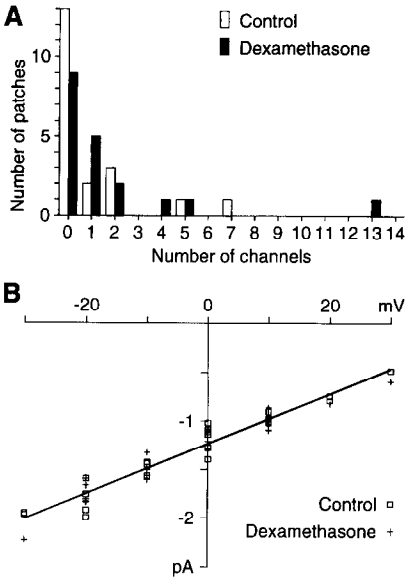

Fig. 3. Effects of dexamethasone on calcium channels recorded from cell-attached patches. (A) A histogram of the distribution of number of channels per patch. (B) Current–voltage relationship for single open channels. Each symbol indicates the mean value of openings from one patch. The lines were calculated by linear regression analysis.

corticoid increases the activity of both the cytosolic and membrane-bound fractions of PKC [17]. However, this interpretation must be taken with caution, as the effect of PKC on calcium channel activity is still controversial even under control conditions [5,12,25,26].

We approached the investigation of the reported modulatory effect of dexamethasone on calcium channels using patch clamp techniques. One difficulty is that L-type calcium currents in A7r5 cells are intrinsically variable, even under control conditions (2–31 pA/pF in the present study; see also [5,8]). Equal variability exists in the regulation of this current by β-adrenergic agonists [5], vasopressin [5,27], activators of PKC [5,12,25,26], and in the voltage-dependent inactivation of the current [8,13]. This variability could arise from uncontrolled factors in the culture medium, and may reflect the well-described phenotypic instability of vascular smooth muscle in cell culture.

In spite of the variability, we found a tendency for dexamethasone to increase L-type calcium currents in cells that had low current levels under control conditions. One interesting possibility is that the mechanism activated by dexamethasone was already active in preparations with higher basal current levels. When the increase in whole cell current was observed, there was no effect on the single channel conductance, on the shape of the current–voltage relationship, or on inactivation. These results argue against an effect of dexamethasone on the single-channel current or on the probability that a channel is open, since either effect should change the shape of the current–voltage relationship. The most natural explanation of an increase in p(open) for a voltage-dependent channel is a shift in the activation curve, so that channels open at more negative voltages, which would shift the peak of the current–voltage curve. It is conceivable that p(open) could be increased by the same factor at every voltage, but a 2-fold increase in p(open) would require that p(open) be less than 0.5 in control conditions, even at extreme positive voltages. In contrast, an increase in the number of active channels would naturally predict an increase in current at all voltages, without effect on the shape of the current–voltage relationship, as we observe. We cannot rigorously rule out an effect on p(open), but our results strongly suggest that dexamethasone acts by increasing the number of calcium channels ready to open upon depolarization. Interestingly, up-regulation of calcium currents by glucocorticoids has been reported recently in hippocampal neurons [28]. In contrast to the potentiating effect of dexamethasone on T-currents in PC12 cells [29], we found that T-current activity was not affected in A7r5 cells after treatment with dexamethasone.

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