Up-regulation of L- and non-L, non-N-type Ca^{2+} channels by basal and stimulated protein kinase C activation in insulin-secreting RINm5F cells

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Abstract **We studied the effect of protein kinase C (PKC) inhibition and activation on voltage-dependent Ca^{2+} channels in rat insulinoma RINm5F cells. PKC down-regulation by chronic (24 h) treatment with the PKC activator phorbol 12-myristate 13-acetate (PMA) reduced by about 60% the Ba^{2+} currents through L- and non-L, non-N-type high-voltage-activated Ca^{2+} channels, indicating that PKC tonically up-regulates the two main Ca^{2+} channel subtypes of RINm5F cells under basal conditions. Consistently, PKC activation by acute PMA application caused only a modest increase (average 23%) of Ba^{2+} currents in a minority of cells (24%). L- and non-L, non-N-type channels were differentially up-regulated by either basal or stimulated PKC activation. Acute up-regulation was predominant on L-type channels and caused an I/V shift of the Ba^{2+} currents in the hyperpolarizing direction. Non-L, non-N-type channels were less affected by acute PMA application, possibly reflecting a more effective tonic PKC up-regulatory action. Unexpectedly, the increase of Ba^{2+} currents during acute PMA application was followed by a progressive current decrease, which was also observed in isolation in another 24% of the cells and could be ascribed to PKC-induced ATP depletion, rather than to a direct effect of PKC on Ca^{2+} channels. We also provide evidence that PKC-mediated phosphorylation is not involved in the G-protein-mediated noradrenergic modulation of Ca^{2+} channels in RINm5F cells.**

Key words: Ca^{2+} channel; Protein kinase C; Channel phosphorylation; Noradrenaline; RINm5F cells; Insulinoma

1. Introduction

Insulin release from pancreatic β cells is a complex process regulated by changes of ion channel permeability, intracellular levels of cyclic nucleotides, phosphoinositide turnover, kinases and phosphatases activity [1]. Protein kinase C (PKC) is thought to play a critical role in the modulation of stimulus-secretion coupling, but the mechanisms of this action are still unclear. In rat insulinoma RINm5F cells, PKC activation promotes insulin release [2], possibly through a phosphorylation-mediated closure of ATP-regulated K^+ channels [3,4], which in turn induces membrane depolarization and Ca^{2+} entry [4,5]. Parallel to this mechanism, a PKC-mediated regulation of voltage-dependent L-type channels has also been proposed [6,7], but it remains to be elucidated whether other Ca^{2+} channel subtypes are also involved in the phenomenon. This is of importance since RINm5F cells do not express only L-type Ca^{2+} channels, but also N-type [8] and non-L, non-N-type (Q-like) channels [9]. Given that non-L, non-N-type channels are also implicated in insulin release from human β cells [10] and catecholamines secretion from chromaffin cells [11] and that they can be up-regulated by PKC activation in neurons [12,13], PKC-dependent phosphorylation of these channels in RINm5F cells may be relevant to the overall secretory activity of these cells.

Here we show that both L- and non-L, non-N-type Ca^{2+} channels are effectively up-regulated by PKC-dependent phosphorylation under basal conditions. In agreement with this, stimulation of PKC by acute application of phorbol ester causes only minor up-regulation of both channel subtypes, with a net prevalence of the L-type. Since PKC-induced phosphorylation has been proved to disrupt neurotransmitter inhibition on Ca^{2+} channels in different neurons and secretory cells [13–16], we also tested its direct involvement in the G-protein-mediated noradrenergic inhibition of Ca^{2+} channels [17]. The present findings exclude any relation between noradrenergic and PKC-mediated Ca^{2+} channels modulation, but underline the essential role of PKC-dependent phosphorylation in tonically maintaining voltage-dependent Ca^{2+} channels in a functional state.

2. Materials and methods

2.1. Cell culture and solutions

Rat insulinoma RINm5F cells were cultured as previously described [8]. Experiments were carried out 3–7 days after plating. The external perfusion solution contained (mM): 125 NaCl, 10 $BaCl_2$, 1 $MgCl_2$, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 0.3 μM tetrodotoxin (pH 7.3 with CsOH). The internal pipette solution contained (mM): 110 CsCl, 30 tetraethylammonium chloride (TEACl), 2 $MgCl_2$, 10 ethylenebis (oxonitriol) tetraacetate (EGTA), 8 glucose, 10 HEPES and 4 Mg-ATP (pH 7.3 with CsOH). In some experiments 15 mM phosphocreatine (Sigma Chemical Co., St. Louis, MO, USA) and 0.3 mM guanosine-5'-triphosphate (GTP, Sigma Chemical Co.) were added to the internal solution. The bath Tyrode solution contained (mM): 150 NaCl, 4 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 glucose and 10 HEPES. Nifedipine was dissolved in ethanol 95%, diluted daily to 5 μM in the bath solution and applied under light protection. Chronic treatment with 3.2 μM ω-conotoxin (ω-CTx) GVIA (Bachem AG, Bubendorf, CH) was carried out by 20 min dish incubation in Tyrode's solution prior to recording. 4α- and 4β-phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma Chemical Co. and from ICN (Aurora, OH, USA), dissolved at 1.62 mM in DMSO and stored at −20°C. Final concentrations were 0.03–0.3 μM in the external solution for acute application and 1 μM in RPMI medium for 24 h PKC down-regulation [18]. Protein kinase C pseudosubstrate (PKCI(19–36)) was purchased from Bachem AG, dissolved in 0.5% acetic acid (20 mM) and stocked at −20°C. Final dilution to 5–20 μM in the internal solution was carried out daily. Noradrenaline bitartrate (Sigma Chemical Co.) was dissolved daily to 20 μM in the external solution. A multi-barrelled glass pipette was used for bath solution exchanges [8].

2.2. Electrophysiological measurements and analysis

Whole-cell currents were measured by a List EPC-7 patch-clamp amplifier and borosilicate glass electrodes of 3–6 MΩ resistance [8].

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Stimulation and acquisition were performed using a 12-bit A/D Tecmar Labmaster board operated by pClamp software (Axon Instruments, Forster City, CA). Current traces were sampled at digitizing rates of 10–40 kHz, filtered at 5–10 kHz and analyzed with AutesP program (Garching Innovation, Munich, Germany). Step depolarizations of variable amplitude (−40 to +90 mV) and duration (80 to 100 ms) were applied at 10–30 s intervals from a holding potential (V_h) of −90 mV. Capacitive transients and leakage currents were compensated electronically and by subtracting either Cd^{2+}-insensitive currents persisting after bath addition of 200 μM $CdCl_2$ or scaled P/4 hyperpolarizing pulses. Current amplitude was measured at the peak and divided by the cell capacitance to account for the cell size. Capacitance measurements were obtained from the C_{slow} setting after compensation. The time-dependent decay of Ba^{2+} currents in control conditions ('run-down') was measured in 4 cells stimulated for 15 min with 100-ms pulses to +10 mV repeated every 30 s. Averaged normalized current decays were fitted with a regression line, whose slope was taken as the standard run-down of Ba^{2+} currents (see Figs. 2A and 3). The delayed depression of Ca^{2+} currents induced by PMA application was estimated in a similar way and compared to the control run-down. Results were considered significant only when the slope of the PMA-induced decay differed by more than 3 times the standard deviation from the control run-down (see Fig. 3A). Data are expressed as means ± S.E.M. for n cells. All experiments were performed at room temperature (20–22°C). Statistical significance (P) was calculated using Student's t-test for unpaired data.

3. Results

In order to evaluate whether Ca^{2+} channels in RINm5F cells are tonically regulated by PKC-dependent phosphorylation we first compared control Ba^{2+} currents with currents of cells in which PKC activity was fully inactivated by 24 h incubation with 1 μM PMA [18]. As illustrated in Fig. 1A, Ba^{2+} currents in PKC down-regulated cells were smaller than in control cells, and showed almost no differences in both the activation-inactivation kinetics and in the voltage dependence of the current-voltage (I-V) relationship (Fig. 1B). In 27 down-regulated cells the mean peak current at 0/+10 mV was 18.8 ± 2.2 pA/pF, corresponding to 40.4% of the mean peak current measured in control conditions (46.5 ± 4.1 pA/pF, n = 34) (Fig. 1B inset). To exclude any non-specific effect of PMA on Ca^{2+} channels, Ba^{2+} currents were also recorded from cells incubated for 24 h with the PMA analogue 4α-PMA (1 μM), which is inactive with regard to PKC activation. The mean peak current after 4α-PMA pre-treatment differed by less than 1% from that recorded in control conditions (n = 12, not shown). Thus, Ca^{2+} channels appear already effectively up-regulated by PKC-dependent phosphorylation under basal conditions and the fraction of current lost in PKC-depleted cells may be taken as an indication of the degree of endogenous PKC-dependent up-regulation (~60%). Next, we tested which channel subtype was involved in this process by using saturating doses of ω-CTx-GVIA (3.2 μM) and nifedipine (5 μM) to selectively block N- and L-type channels in PKC down-regulated cells. As shown in Fig. 1C, non-N-type current (recorded in the presence of ω-CTx-GVIA), non-L, non-N-type current (recorded in the presence of ω-CTx-GVIA and nifedipine) and L-type current (estimated from the current block by nifedipine) were depressed to about the same percentage (46.19–53.88%, n = 20). Due to the modest contribution of the N-type channel to the total current in RINm5F cells (10–15%) [8,9], we were unable to evaluate the endogenous action of PKC on this Ca^{2+} channel subtype.

Consistently with these data, we found that PKC activation by acute PMA application (30–300 nM) caused an average 23% increase of Ba^{2+} current amplitude in 24% of the tested cells (n = 50). The effect was PKC-mediated since it was never observed in the presence of the PKC-inhibitor PKCI(19–36) in the internal perfusate (25–50 μM, n = 9), in PKC down-regulated cells (n = 12) or using the inactive analogue 4α-PMA (n = 5). The time course of the Ba^{2+} current increase was similar in all the cells studied (Fig. 2A). The maximum was reached within 45 s, lasted 1–2 min and then declined with a slope steeper than the average Ba^{2+} current run-down (straight line in Fig. 2A) (see below). The channel activation-inactivation kinetics were never altered by PMA (Fig. 2B). In four cells, the current up-regulation was most evident between −30 and −10 mV, reaching maximal amplitude at around −15 mV (Fig. 2C). Thus, the I-V relationship associated with the up-regulated current was clearly shifted toward more negative potentials (dashed line in Fig. 2C), i.e. in the voltage region where L-type channels activate in RINm5F cells [8]. In 8 out of 12 cells the current increase was almost completely abolished by nifedipine (5 μM), confirming that the I-V shift was mainly due to L-type Ca^{2+} current up-regulation, rather than to a decrease of the voltage threshold for Ca^{2+} channel activation. In the two examples of Fig. 2D, the action of nifedipine was compared before (NIFE) and after acute application of PMA (NIFE + PMA). In one case (Fig. $2D_1$), the current amplitude recorded in the presence of PMA plus nifedipine was the same as in nifedipine alone, suggesting that the dihydropycidine (DHP) abolished both the PMA-induced current and the L-type current expressed by the cell. In the second case (Fig. $2D_2$), the L-type current prior to phorbol ester application was undetectable (compare traces C and NIFE), but nifedipine was nevertheless able to block the PMA-induced current. The up-regulating action of PKC was not limited to L-type channels. An example of PKC-induced current increase involving non-L, non-N-type channels is shown in Fig. 2E. The non-L, non-N-type current recorded from an ω-CTx-GVIA-treated cell exposed to nifedipine was increased following PMA application. Similar effects were observed in two other cells.

As mentioned above, PMA had also a depressing action that varied from cell to cell and occurred either overlapped to the Ba^{2+} current increase (Fig. 2A) or in isolation (Fig. 3A). In 12 out of 50 RINm5F cells the current decrease was significantly faster than the average Ba^{2+} current run-down (straight line in Fig. 3A, see Section 2) and caused no changes to the activation-inactivation kinetics (inset). As for the current up-regulation, the Ba^{2+} current decrease was very likely related to PKC activation, since it was not observed in 6 out of 7 down-regulated cells and in all the 4 control cells exposed to 4α-PMA. However, the current decrease induced by PMA could be largely prevented by adding a potent phosphate donor to the standard internal solution, such as 15 mM phosphocreatine (n = 8) (Fig. 3B). This suggests that Ba^{2+} current decrease induced by PMA was not due to a direct down-regulating action of PKC on Ca^{2+} channels but, very likely, to a shortening of intracellular ATP that may derive from an increased overall PKC-phosphorylating activity.

The last series of experiments was aimed at verifying whether PKC-dependent phosphorylation is involved in the G protein-mediated noradrenergic modulation of HVA Ca^{2+} channels. Noradrenaline (NA) inhibits HVA Ca^{2+} channels in RINm5F cells in a voltage-dependent and -independent man-

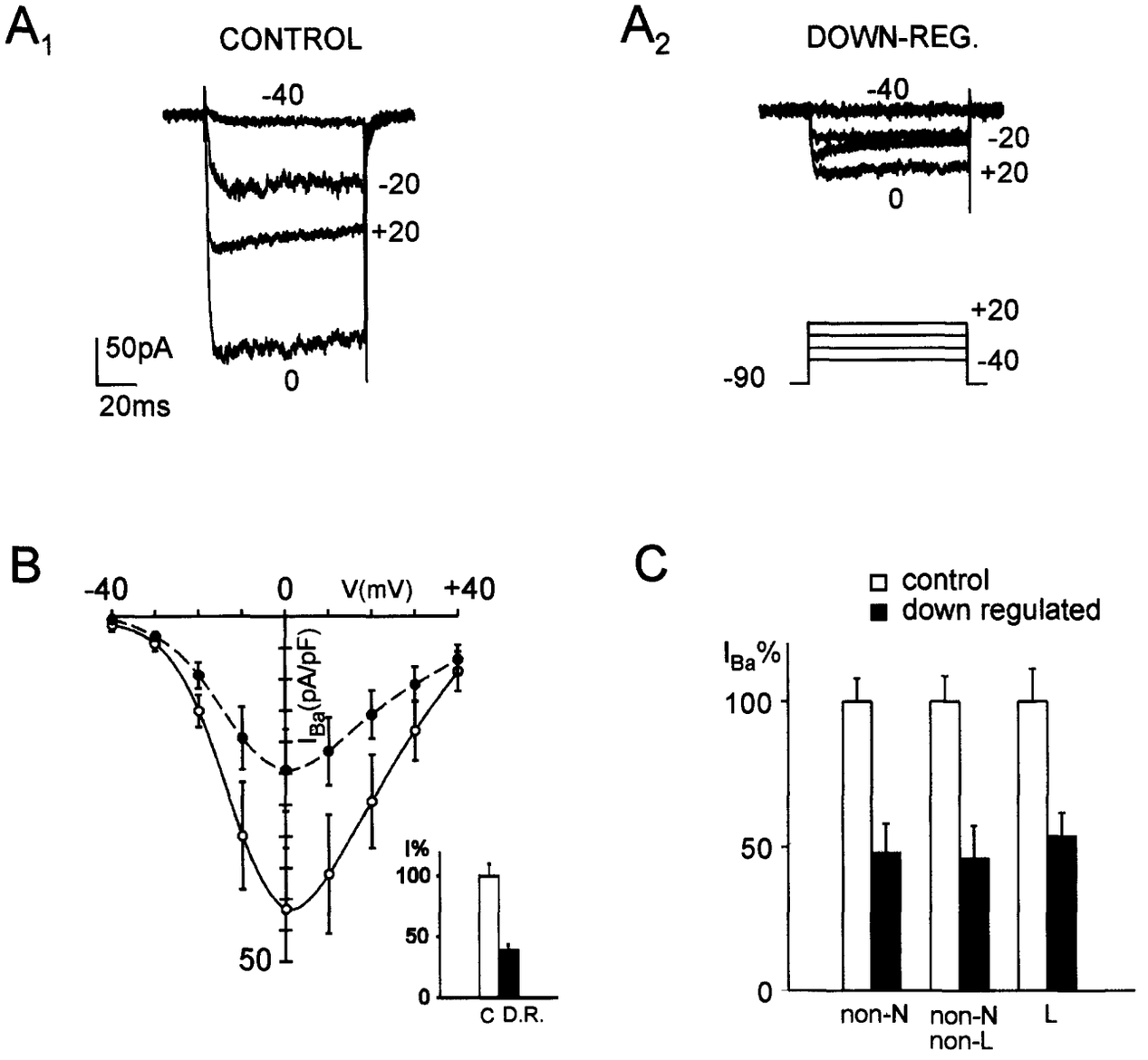

Fig. 1. Effects of PKC down-regulation on HVA Ba^{2+} currents in RINm5F cells. A: HVA Ba^{2+} currents recorded at potentials between −40 and +20 mV, with 20 mV step increments, from a control cell (A_1) and a PKC down-regulated cell (A_2) of equal capacitance (5 pF); V_h −90. B: Normalized peak current-voltage relationship for 10 control (empty circles) and 7 down-regulated cells (filled circles). Bars represent S.E.M. Inset: Averaged normalized peak Ba^{2+} current values for 34 control and 27 down-regulated cells. C: Averaged normalized peak Ba^{2+} current values for non-N, non-N, non-L- and L current components in 24 control and 20 down-regulated cells. All components appear equally depressed (48, 46.19 and 53.88% of control, respectively).

ner, distinguishable on the basis of the response to strong depolarizing pre-pulses [8,17]. In order to ascertain whether NA-induced inhibition was due to an antagonistic action on basal PKC-induced Ca^{2+} channel up-regulation, we studied the effects of NA with a two-pulse protocol (a test pulse to 0 mV alone or preceded by a pre-pulse to +90 mV) in PKC-down-regulated ($n=7$) and in control cells ($n=7$). As shown in Fig. 4A, both voltage-dependent and -independent inhibition were preserved in PKC-depleted cells. The strong current depression induced by NA (vertical arrow on trace c, Fig. 4A, right) was partially recovered by the pre-pulse (cf. horizontal arrow on trace d) indicating the persistence of both a voltage-dependent inhibition (trace d minus c) and a voltage-independent inhibition non-recovered by voltage (trace b minus d). Analogous results were obtained in two cells internally perfused with 50 μM PKCI(19–36) (not shown). Finally, we compared in the same cell the effects of NA applied before (Fig. 4B, top) or during acute application of PMA (Fig. 4B, bottom). The inhibitory action of NA was not significantly affected by PMA in any of the cells tested ($n=6$), suggesting that the PKC action and the G-protein-mediated noradrenergic Ca^{2+} channel inhibition are independent processes [17].

4. Discussion

Ca^{2+} channels in RINm5F cells are up-regulated by PKC activation in an effective way. Most interestingly, PKC action is not confined to the L-type channel, as usually believed [6,7],

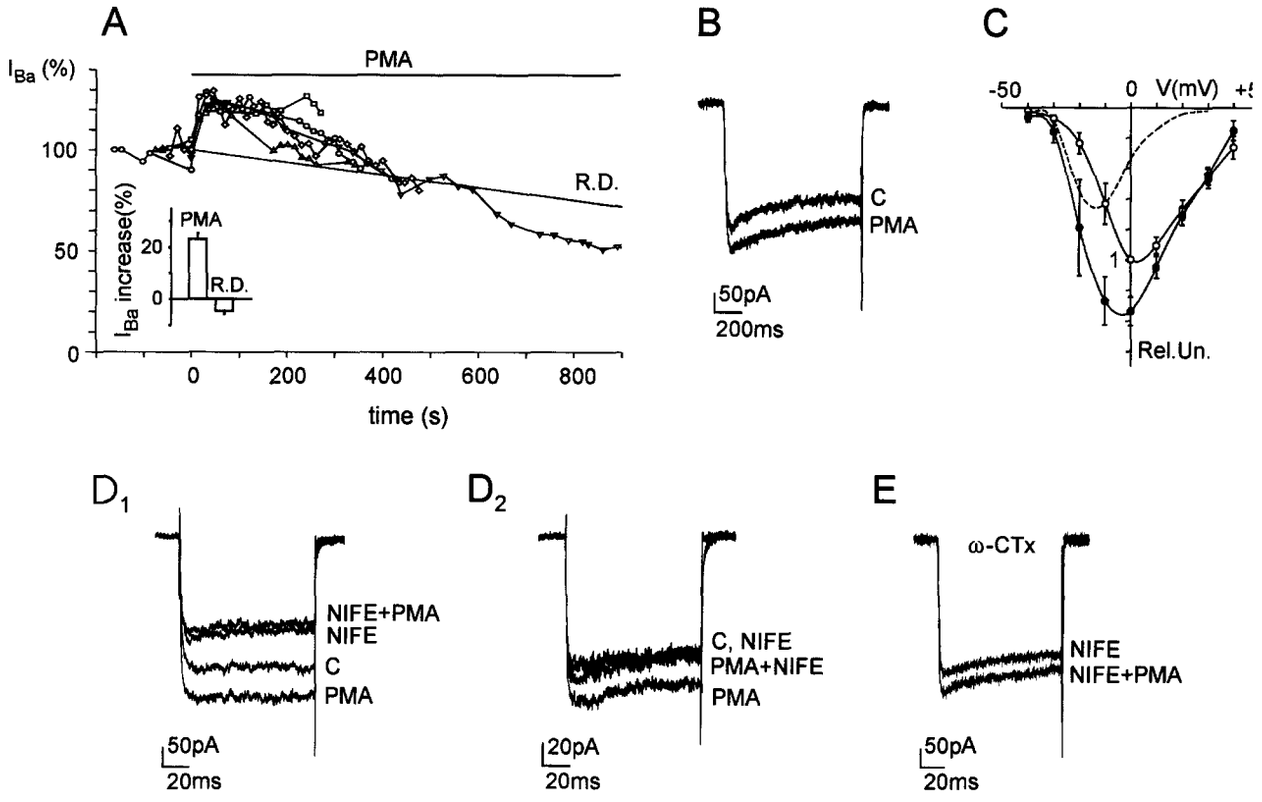

Fig. 2. Enhancement of HVA Ba^{2+} currents by PMA in RINm5F cells. A: Time-course of HVA Ba^{2+} current amplitude during 300 nM PMA perfusion in 5 selected cells. Each dot represents the peak current at −10 or 0 mV. Recordings are synchronized at the beginning of PMA perfusion (bar); their amplitude is normalized at the control value of the trace preceding PMA application. R.D. marks the regression line of 4 control cells in which the run-down was studied (see Section 2). Inset: Maximal PMA-induced Ba^{2+} current increase in 12 cells compared with the corresponding run-down in 4 control cells. B: Current traces recorded at 0 mV from a cell bathed in 10 mM Ba^{2+}, in control conditions (upper trace) and during PMA application (lower trace). V_h −90 mV. C: Normalized peak current-voltage relationship from 4 cells in control conditions (empty circles) and during application of 300 nM PMA (filled circles). Note the more negative peak of the enhanced current (difference between the two curves, dotted line). D: Current traces recorded at −10 mV (D_1) and in a second cell at +10 mV (D_2) in control conditions (C), in the presence of 5 μM nifedipine (NIFE), in the presence of 300 nM PMA (PMA) and of both PMA and nifedipine (PMA+NIFE). E: Same as in D, but in an ω-Ctx-GVIA-treated cell. V_{test} +10 mV; V_h −90 mV.

but also involves the non-L, non-N-type (Q-like) channel which is responsible for a large proportion of Ba^{2+} currents in these cells [9]. This up-regulation is tonically active, as revealed by the strong current reduction in PKC-down-regulated cells, and targets both L- and non-L, non-N-type Ca^{2+} channels. Despite this common feature, however, the two channels are differently up-regulated by stimulated PKC activation. Acute cell exposure to PMA causes Ba^{2+} current increases predominantly in a voltage range in which L-type channels are preferentially activated, and this effect is greatly reduced or even abolished by DHP antagonists. In some cells, PKC stimulation even recruits L-type currents that were undetectable under basal conditions (Fig. 2D). This is in line with observations in hippocampal neurons, where L-type channel activity in cell-attached patches is strongly enhanced by phorbol esters [19]. On the other hand, PKC stimulation causes only a minor increase of the ω-CTx-GVIA- and DHP-resistant current in a small percentage of cells (Fig. 2E), suggesting that the non-L, non-N-type channel is already maximally up-regulated by PKC in a tonic way. A parallel up-regulation of both L- and non-L-type (N and P/Q) channels has been shown in frog sympathetic neurons [20] and rat hippocampal pyramidal neurons [12].

Taken all together our findings are in good agreement with previous reports on the action of PKC on rodent β cells and β-cell lines. For instance, in mouse β-cells the down-regulation of PKC activity causes a 50% reduction of Ca^{2+} current amplitude [21], suggesting that tonic up-regulation of Ca^{2+} channels may be a common property of insulin-secreting cells. In RINm5F cells, the PKC activator didecanoylglycerol (DiC_{10}, 12.5 μM) causes an increased open channel probability in cell-attached patches [7], while PMA (10 nM) enhances by 80% the whole-cell Ca^{2+} currents during acute application [6]. In these experiments, however, PMA was found to have no effect when applied for 48 h to down-regulate PKC. This might reflect a much lower level of endogenous PKC activity, due to different recording conditions (5 h rather than 3–7 days after plating, as in our case). An increased Ca^{2+} channel activity due to PKC-enhanced channel phosphorylation is also in line with a recent report, in which the phosphatase inhibitor okadaic acid was shown to increase the Ca^{2+} current of RINm5F cell at rather negative voltages [22]. This reinforces the concept that L-type channels may be effectively up-regulated beyond the basal level, either by stimulating PKC-dependent phosphorylation or by inhibiting phosphatase activity.

A complication in our study was the existence of two contrasting effects on Ca^{2+} channels evoked by PKC activation:

a fast up-regulation and a delayed Ca^{2+} current depression (Fig. 2A). We do not have a clear explanation for the origin of the latter effect, which could also be observed in isolation and was too fast to be confused with the slow and unspecific run-down of Ca^{2+} channels occurring during cell dialysis. However, since the delayed Ba^{2+} current depression was largely prevented by adding high doses of creatine phosphate to the intracellular medium, the phenomenon could be ascribed to the decreased level of ATP rather than to the direct effect of PKC on Ca^{2+} channels. ATP depletion may be just the consequence of the increased metabolic needs resulting from the enhanced PKC activity toward the many available phosphorylation sites inside the cell. This point, however, deserves further investigation.

The final question we addressed was whether there was a link between the PKC-dependent phosphorylation and the

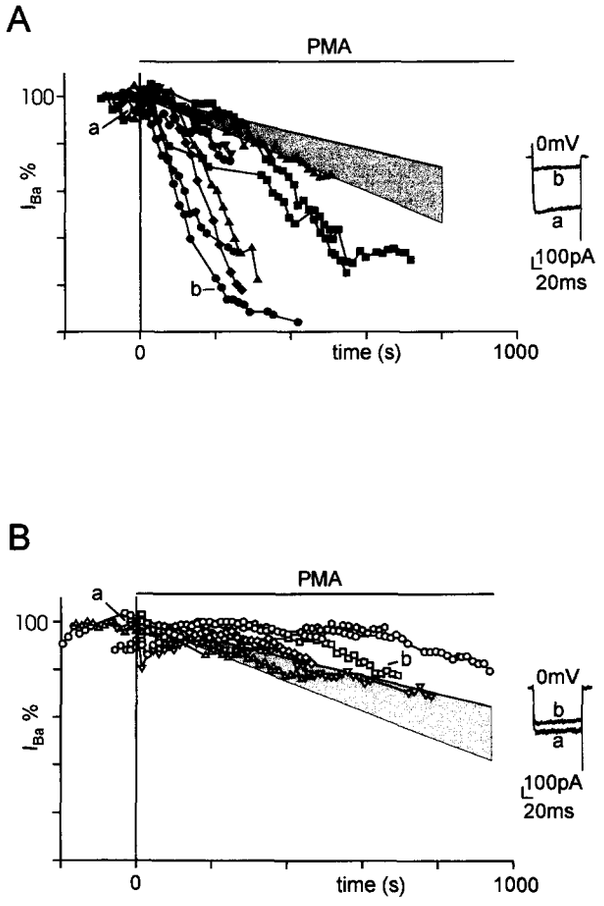

Fig. 3. Depression of HVA Ba^{2+} currents by PMA. Time-course of HVA Ba^{2+} current amplitude during application of 300 nM PMA, in the absence of phosphocreatine ($n=9$, filled symbols in panel A) and in the presence of 15 mM phosphocreatine in the internal solution ($n=7$, empty symbols in panel B). Different symbols correspond to different cells. Synchronization and normalization of the time courses as in Fig. 2A. The straight thick line is the averaged regression line obtained from 4 control cells, taken as the standard current run-down. The slope of current decay in PMA-treated cells is considered significantly different from that of run-down only when falling below the shaded area, representing 3 times the S.D. of the control run-down ($1.87 \pm 0.72\%$ min^{-1} for panel A and $2.25 \pm 0.4\%$ min^{-1} for panel B). Insets: HVA Ba^{2+} currents recorded in control conditions (a) and after exposure to 300 nM PMA (b) at the time indicated in the diagram. V_{test} 0 mV; V_h −90 mV.

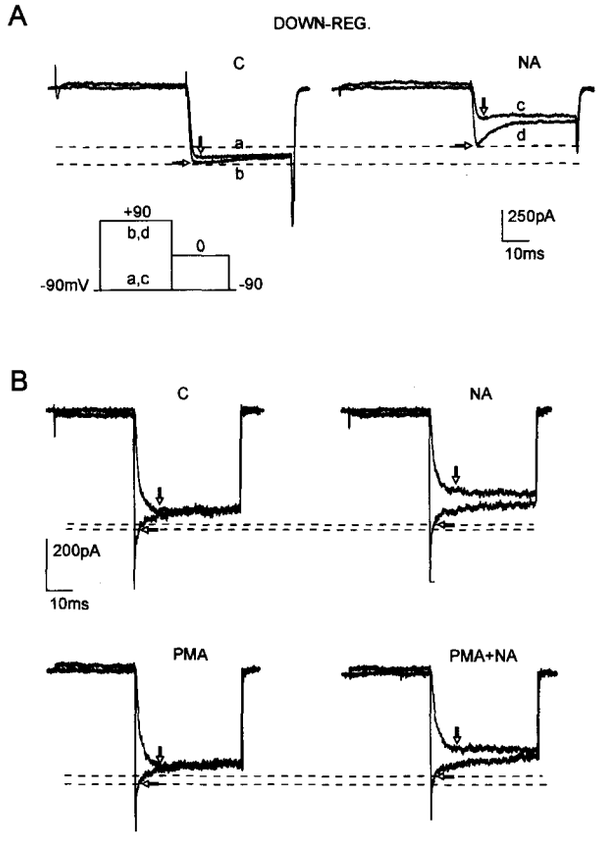

Fig. 4. Interactions between NA and PKC modulation of HVA Ca channels. A: Effects of 10 μM NA in a down-regulated cell. A test pulse to 0 mV was delivered alone (a, c) or preceded by a strong depolarization to +90 mV (b, d). Vertical arrows indicate the current peak in control conditions and the corresponding time in the trace with NA. In control conditions (left) the two traces virtually overlap, while the inhibition induced by NA (vertical arrow on the right) is partly recovered by the pre-pulse (horizontal arrow). Dotted lines help visualization of the current levels marked by horizontal arrows. B: Same as in A, in a control cell before (top panels) and during acute 300 nM PMA application (bottom panels). Horizontal arrows measure the pre-pulse-induced current facilitation 2 ms after the onset of the test pulse. NA induces about the same V-dependent and V-independent Ba^{2+} current depression before and during PMA perfusion, as evidenced by the dotted lines. V_h −90 mV; V_{test} 0 mV.

α_2-adrenergic Ca^{2+} channel inhibition [8,17], with PKC activation possibly interfering with the G-protein-mediated, membrane-delimited mechanism. Support to this hypothesis comes from studies in central and peripheral neurons [12,13,15,23], where PKC activation is shown to increase Ca^{2+} currents by removing the Ca^{2+} channels inhibition induced by activated G proteins either tonically or through neurotransmitter receptors. We found, however, that this is not the case. Neither disruption of the tonic PKC phosphorylation in down-regulated cells, nor acute PKC stimulation affected noradrenaline inhibition in RINm5F cells, in either its voltage-dependent or voltage-independent components. The same occurs in frog sympathetic neurons in which N- and L-type channels appear up-regulated by PKC activators, but the action is uncoupled from the Ca^{2+} channel inhibition induced by 100 μM intracellular GTP-γ-S [20]. These findings suggest that PKC-induced phosphorylation and noradrenergic inhibition of Ca^{2+} channels are independent processes in RINm5F cells, support-

ing the view that PKC phosphorylation may follow different pathways and target different sites depending on the Ca^{2+} channels, G proteins and membrane receptors expressed by the cells (cf. [15,23]).

In conclusion, we believe that the PKC-dependent Ca^{2+} channel activation described here provide complementary data to previously proposed schemes in which PKC activity has been implicated in the regulation of Ca^{2+} influxes in response to insulin secretagogues. Like secretagogues, activation of PKC causes the closure of ATP-regulated K^+ channels [4] with consequent cell depolarization and Ca^{2+} entry through voltage-dependent Ca^{2+} channels. Alternatively, our findings suggest that PKC-dependent phosphorylation of Ca^{2+} channels may support insulin release by directly up-regulating L- and non-L-type channels, possibly without interfering with other Ca^{2+} channel inhibitory mechanisms. The high level of PKC-mediated Ca^{2+} channels up-regulation under basal conditions suggests that the maintenance of L-type and Q-like channels in a proper functional state might represent both a permissive factor in the regulation of insulin release and the possible target of modulatory events inducing protein phosphatases activation [22,24] and/or PKC inhibition.

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