Calcineurin Directs the Reciprocal Regulation of Calcium Entry Pathways in Nonexcitable Cells*

The reciprocal regulation of noncapacitative and capacitative (or store-operated) Ca\(^{2+}\) entry in nonexcitable cells (Mignen, O., Thompson, J. L., and Shuttleworth, T. J. (2001) J. Biol. Chem. 276, 35676–35683) represents a switching between two distinct Ca\(^{2+}\)-selective channels: the noncapacitative arachidonate-regulated Ca\(^{2+}\) channels (ARC channels) and the store-operated Ca\(^{2+}\) channels (SOC channels). This switch is directly associated with the change from oscillatory to sustained Ca\(^{2+}\) signals as agonist concentrations increase and involves a Ca\(^{2+}\)-dependent inhibition of the ARC channels. Here we show that this process is mediated via a calcineurin-dependent inhibition of the noncapacitative ARC channels. Pharmacological and molecular inhibition of calcineurin activity (using cyclosporin or the FK506 analogue ascomycin, and a transfected C-terminal domain of the calcineurin inhibitory protein CAIN, respectively) results in a complete reversal of the Ca\(^{2+}\)-dependent inhibition of the ARC channels. Agonist concentrations that result in oscillatory Ca\(^{2+}\) signals and specifically activate Ca\(^{2+}\) entry through the ARC channels fail to increase calcineurin activity. However, agonist concentrations that activate the store-operated Ca\(^{2+}\) channels and produce prolonged increases in cytosolic Ca\(^{2+}\) concentrations increase calcineurin activity. Thus, calcineurin is the key mediator of the reciprocal regulation of these co-existing channels, allowing each to play a unique and non-overlapping role in Ca\(^{2+}\) signaling.

Increases in the cytosolic concentration of Ca\(^{2+}\) ions ([Ca\(^{2+}\)]\(_i\)) represent the cellular signals generated by the actions of many hormones and neurotransmitters that operate through receptors coupled to phospholipase C (1, 2). A general feature of these Ca\(^{2+}\) signals is an enhanced entry of extracellular Ca\(^{2+}\). At high agonist concentrations, this entry is directly responsible for the resulting sustained, elevated cytosolic Ca\(^{2+}\) concentration, and for refilling of the intracellular stores following termination of the receptor activation. However, such sustained Ca\(^{2+}\) signals are not thought to be the major form of Ca\(^{2+}\) signal under physiological conditions, at least in most cells. Instead, the oscillatory Ca\(^{2+}\) signals generated by low agonist concentrations are considered to be more physiologically relevant, and here the principle role of the receptor-activated entry of Ca\(^{2+}\) is not to directly contribute to the changes in Ca\(^{2+}\), but to modulate the frequency of the oscillations (3–5).

Despite these very distinct roles in shaping the overall Ca\(^{2+}\) signal at different agonist concentrations, it was originally assumed that the entry of Ca\(^{2+}\) under both these conditions occurred via the ubiquitous capacitative or store-operated mechanism (6, 7). However in recent years evidence has accumulated indicating that, in many cell types, a novel noncapacitative pathway whose activation is dependent on arachidonic acid is responsible for the Ca\(^{2+}\) entry during oscillatory Ca\(^{2+}\) signals (8, 9). The channels involved in this pathway (ARC channels) have been characterized (10–12) and shown to be specifically activated at the low agonist concentrations associated with oscillatory Ca\(^{2+}\) signals, where they provide the predominant mode of Ca\(^{2+}\) entry (13). Only at higher agonist concentrations, when store depletion is more prolonged and profound, are the store-operated channels (e.g. CRAC channels) activated. Interestingly, it was found that the sustained elevated levels of [Ca\(^{2+}\)]\(_i\), that result from this activation of the store-operated channels act to turn-off the ARC channels (13). Thus, the specific mode of Ca\(^{2+}\) entry is switched at different agonist concentrations between the ARC channels and the store-operated channels, a phenomenon we have described as the “reciprocal regulation” of Ca\(^{2+}\) entry (13). The Ca\(^{2+}\)-dependent inactivation of the ARC channels that underlies this phenomenon is strictly dependent on an elevation in cytosolic Ca\(^{2+}\) per se, occurs at cytosolic Ca\(^{2+}\) concentrations only slightly above resting values, and is a slow process with maximal inhibition taking up to 2 min (13). This is precisely the situation that would be generated when store-operated channels are activated following the depletion of the intracellular Ca\(^{2+}\) stores by high concentrations of appropriate agonists. The result is that, despite the co-existence of these two parallel Ca\(^{2+}\) entry pathways in cells, they are not simply alternative, mutually redundant, routes of entry. The process of reciprocal regulation of Ca\(^{2+}\) entry results in the coordination of their activity in an agonist concentration-dependent manner that prevents their simultaneous activation, allowing each entry pathway to play a unique and non-overlapping role in Ca\(^{2+}\) signaling.

Using a combination of pharmacological and molecular approaches, we now demonstrate that this Ca\(^{2+}\)-dependent effect is mediated via the calcium/calmodulin-dependent phosphatase calcineurin (protein phosphatase 2B). Moreover, we show
that calcineurin is specifically activated at the agonist concentrations associated with a sustained elevated Ca\(^{2+}\) signal, but not during oscillatory Ca\(^{2+}\) signals. This activation pattern explains how the ARC channels are turned off under the conditions where the store-operated channels are activated, thereby demonstrating the critical role of calcineurin in the reciprocal regulation of Ca\(^{2+}\) entry.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—HEK293 cells stably transfected with the human m3 muscarinic receptor (m3-HEK cells, generous gift from Dr. Craig Logsdon, University of Michigan) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum and antibiotics in a 5% CO\(_2\) incubator at 37 °C as previously described (9).Transient transfecions were carried out using the TransIT-TKO transfection reagent (Mirus, Madison, WI) in accordance with the manufacturer’s instructions.

**Electrophysiological Recordings**—Patch-clamp recordings of macroscopic whole-cell currents were performed using an Axopatch-1C patch-clamp amplifier (Axon Instruments, Foster City, CA) as previously described (10). The standard pipette (internal) solution contained (mM): cesium acetate 140, NaCl 10, MgCl\(_2\) 1.22, EGTA 5, HEPES 10 (pH 7.2) unless otherwise specified. CaCl\(_2\) was added to this solution to achieve the desired free Ca\(^{2+}\) concentration as computed with Maxchelator (14). The standard extracellular solution contained (mM): NaCl 140, MgCl\(_2\) 1.2, CaCl\(_2\) 10, CsCl 5, D-glucose 10, HEPES 10 (pH 7.4). Both MgCl\(_2\) and CaCl\(_2\) were omitted from this solution for the experiments involving divalent-free external solutions and the osmolarity (320 mOsm/liter) was maintained with additional D-glucose. Whole-cell currents were recorded using 250 ms voltage steps to −80 mV from a holding potential of 0 mV delivered every 2 s. Current-voltage relationships were recorded either by using 150 ms voltage ramps from −100 to +30 mV, or by pulsing to a series of potentials between −100 mV and +30 mV at 10 or 20 mV intervals. No significant difference was seen in the data obtained between these two methods. Data were sampled at 20 kHz during the voltage steps and at 5.5 kHz during the voltage ramps and digitally filtered off-line at 1 kHz. Initial current-voltage relationships obtained before activation of ARC currents, were averaged and used for leak subtraction of subsequent current recordings. Changes in the external (bath) solution were by perfusion of solution through the patch-clamp chamber (rate of -1.5 ml/min). All experiments were carried out at room temperature (20–22 °C). Data are presented as means ± S.E.

Single Cell Digital Imaging Microscopy—Cells transfected with the GFP-NFAT-(I) construct were visualized using a Nikon inverted epi-fluorescence microscope with a ×100 oil immersion objective. Illumination was via a TILL Polychrome IV monochromator and images were collected using a 12-bit interline charge coupled device camera (Sensicam), and TILL VisION software.

**Luciferase Assays**—calcineurin-dependent activation of NFAT was determined using a PathDetect system (Stratagene). This utilizes a construct consisting of the reporter gene linked to a basic promoter element (TATA box) and four tandem repeats of an NFAT-binding element. Cells were treated with the relevant concentration of agonist (carbachol) for 30 min, and activation of the transfected construct determined by luciferase assays after a further 5-h incubation using the manufacturer’s instructions. As a negative control, cells were transfected with the pCIS-CK plasmid, which does not contain any cis-acting DNA elements, and were incubated and assayed in an identical way.

**RESULTS**

**Ca\(^{2+}\)**-dependent Inhibition of ARC Channels Does Not Require Permeating Ca\(^{2+}\) Ions and Is Acutely Sensitive to Ca\(^{2+}\) Concentrations in the Physiological Range—As noted above this reciprocal regulation, or more specifically the inhibition of ARC channels at high agonist concentrations, is a Ca\(^{2+}\)-dependent phenomenon. Although a Ca\(^{2+}\)-dependent inhibition of overall channel activity is seen in several types of Ca\(^{2+}\) channels, including the store-operated CRAC channels (15), these inhibitions generally reflect an effect of the Ca\(^{2+}\) ions passing through the channel and interacting with a site close to the inner face of the channel pore. As such, they operate within a relatively rapid time frame, typically of less than a second, and inactivation is directly related to the amount of Ca\(^{2+}\) current through the channel. Such fast inactivation by permeating Ca\(^{2+}\) ions is not seen in currents through ARC channels (10). Moreover, the Ca\(^{2+}\)-dependent inhibition described for ARC channels is still observed in divalent-free external media, when the channels are conducting monovalent cations (13). This feature proved useful as, in our previous studies, we were unable to fully quantify the Ca\(^{2+}\)-dependence of the inhibition of macroscopic ARC currents in their normal Ca\(^{2+}\)-conducting mode because of the very small current magnitudes seen at values approaching maximal inhibition (~0.2 pA/pF). We therefore determined the relevant macroscopic currents through the ARC channels in their monovalent permeant mode in the absence of external divalent ions, where the currents are ~40-fold larger. Although the overall Ca\(^{2+}\)-dependent inhibition of the currents appeared similar for both Ca\(^{2+}\)-conducting and monovalent-conducting modes, a concern was that complete removal of external divalent ions and/or the switch to the monovalent permeant mode might influence the Ca\(^{2+}\) sensitivity of the channels. By making further refinements to our patch-clamp setup, we have now succeeded in obtaining the full Ca\(^{2+}\)-dependent inhibition curve for the ARC channels in their normal Ca\(^{2+}\)-conducting state. HEK293 cells, stably transfected with the m3 muscarinic receptor (m3-HEK cells) were patch-clamped in the whole-cell configuration using internal (pipette) solutions containing buffered Ca\(^{2+}\) concentrations ranging from 0 to 400 nM. Macroscopic currents through the ARC channels were then induced by exogenous application of arachidonic acid (8 μM) and measured during 250 ms pulses to a potential of −80 mV from a holding potential of 0 mV. The data obtained confirm our previous findings (Fig. 1). Increasing the internal Ca\(^{2+}\) concentration to values greater than 100 nM markedly reduced the magnitude of the macroscopic Ca\(^{2+}\) currents through the ARC channels (Fig. 1, A and C), without affecting the basic features of the current (Fig. 1B). Maximal inhibition of ~65% was achieved at an internal Ca\(^{2+}\) concentration of 400 nM (Fig. 1C). An essentially identical inhibition was seen in the currents measured in divalent-free external media, when the channels are conducting monovalent cations (Fig. 1D). Clearly, as previously argued (13), the observed inhibition is independent of Ca\(^{2+}\) entering through the channels or of any resulting local domain of elevated Ca\(^{2+}\), and instead reflects an effect of the global level of cytosolic Ca\(^{2+}\).

**Effect of Calmodulin Inhibition on the Ca\(^{2+}\)**-dependent Inhibition of ARC Channels—We argued that the high sensitivity, yet indirect, inhibition of the ARC channels by internal Ca\(^{2+}\) suggested the possible involvement of calmodulin. In cells pre-incubated with the calmodulin inhibitor W7 (10 μM for 15–30 min), inward currents through the ARC channels measured at −80 mV with an internal Ca\(^{2+}\) concentration of 400 nM averaged 0.33 ± 0.04 pA/pF (n = 8), significantly larger than those seen in control cells under the same conditions (0.19 ± 0.02 pA/pF, n = 5) (Fig. 2A). In contrast, preincubation with W7 had no effect on the normal ARC currents recorded at 100 nM internal Ca\(^{2+}\) (0.47 ± 0.05 pA/pF versus 0.52 ± 0.04 pA/pF, n = 4 and 6, respectively). Preincubation with W7 therefore caused an ~40% reversal of the Ca\(^{2+}\)-dependent inhibition of ARC currents. Similar preincubation with another calmodulin inhibitor calmidazolium (10 μM) increased the magnitude of the ARC currents seen at 400 nM internal Ca\(^{2+}\) to 0.55 ± 0.05 pA/pF, a value not significantly different from the normal currents seen at 100 nM internal Ca\(^{2+}\) (Fig. 2A). These data indicate that calmidazolium induces an essentially complete reversal of the Ca\(^{2+}\)-dependent inhibition of ARC currents. However, it was noted that the resulting current/voltage relationship was markedly more linear than normally observed (Fig. 2B, open circles), raising concerns that calmidazolium...
may be inducing nonspecific effects. Nevertheless, the data did suggest a possible involvement of calmodulin in the Ca2+-dependent inhibition of the ARC channels.

Calcineurin and the Ca2+-dependent Inhibition of ARC Channels; Effects of Cyclosporin A and Ascomycin—As it seemed that any effect of calmodulin was unlikely to be direct, given the slow response to elevated internal Ca2+ concentrations (13), we considered that this may reflect a calmodulin-dependent enzymatic effect, most likely either a phosphorylation or dephosphorylation. We reasoned that a phosphorylation reaction was unlikely because our standard internal (pipette) solution contained no ATP. We therefore focused on a possible action of a calmodulin-dependent phosphatase, the most obvious candidate being the Ca2+/calmodulin-dependent serine/threonine protein phosphatase calcineurin (protein phosphatase 2B). Pharmacological inhibition of calcineurin typically involves the use of the immunosuppressants cyclosporin A (CspA) and FK506 (16) which, in the presence of their corresponding intracellular immunophilins cyclophilin A and FKBP12, sterically prevent access of the substrate thereby blocking phosphatase activity (17, 18). Preincubation of cells in CspA (1 μM for 15–30 min) increased the magnitude of the inward ARC currents seen at 80 mV with 400 nM internal Ca2+ from 0.19 ± 0.02 pA/pF (n = 5) to 0.47 ± 0.05 pA/pF (n = 6), a value not significantly different from the normal currents observed with 100 nM internal Ca2+ (Fig. 3, A and C). Examination of the macroscopic current density over the entire range of internal Ca2+ from 0 to 400 nM Ca2+ demonstrated that this effect was specific to the Ca2+-dependent inhibition of the ARC channels as the currents at low internal Ca2+ (up to 100 nM) were unaffected by CspA (Fig. 3B). Similarly, preincubation of the cells with ascomycin (5 μM for 15–30 min), a close structural analog of FK506 (19), also completely reversed inhibition of ARC currents observed at 400 nM Ca2+ without affecting the currents seen at 100 nM Ca2+ (Fig. 3C). The increased current density through the ARC channels selectively observed at high internal Ca2+ concentrations (400 nM) following pretreatment with CspA or ascomycin occurred without changing the char-

![Graph A](image1.png)  
**Fig. 1. Internal Ca2+ inhibits currents through ARC channels.** A, representative traces showing the activation of inward Ca2+-selective currents through ARC channels following addition of 8 μM arachidonic acid (arrow). Currents were measured during 250-ms pulses to −80 mV from a holding potential of 0 mV. Examples are shown of experiments with an internal (pipette) solution containing 100 nM Ca2+ (filled circles), and 400 nM (open circles). B, mean (± S.E.) of the current-voltage relationships of the Ca2+-selective currents measured with internal Ca2+ concentrations of 100 nM (filled circles, n = 4), and 400 nM (open circles, n = 4). C, the effect of internal Ca2+ concentration on inward Ca2+-selective currents through ARC channels activated by 8 μM arachidonic acid. Values are mean ± S.E., n = 4–6. D, representative traces showing the inward monovalent currents through ARC channels activated by addition of 8 μM arachidonic acid, measured in a divalent-free external solution. Under these conditions, inward currents were carried by sodium ions. These currents were measured during 250 ms pulses to −80 mV from a holding potential of 0 mV. Examples are shown of experiments with an internal (pipette) solution containing 100 nM Ca2+ (filled circles), and 250 nM (open circles).
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characteristic inwardly rectifying nature, and high positive reversal potential, typical of normal ARC currents (Fig. 3D). Together then, these data using two chemically unrelated immunosuppressants demonstrate that the Ca$_{2^{+}}$-dependent inhibition of currents through the ARC channels is selectively reversed by inhibition of the Ca$_{2^{+}}$-CaM-dependent protein phosphatase calcineurin.

Although the above data indicate the involvement of calcineurin, this does not necessarily mean that such an action is direct. For example, it has been shown that calcineurin can activate protein phosphatase 1 (PP1) by dephosphorylation of the PP1 inhibitory peptide, inhibitor-1 (20) with the result that PP1 inhibits protein phosphatase 1 (PP1) by dephosphorylation of calcineurin, this does not necessarily mean that such an action is direct. For example, it has been shown that calcineurin can activate protein phosphatase 1 (PP1) by dephosphorylation of the PP1 inhibitory peptide, inhibitor-1 (20) with the result that PP1 inhibits PP1 by dephosphorylation of calcineurin, which binds calcineurin with high affinity (K$_{i}$ of ~450 nM) at a site distinct from the FK506/FKBP binding sites. Full length CAIN is a protein of ~240 kDa, but the calcineurin binding sites lie within a C-terminal domain encompassed by residues 1881–2173. Expression of this portion alone specifically inhibits the phosphatase activity of calcineurin (22, 23). This C-terminal calcineurin inhibitory domain was fused with GFP to create a GFP-c-CAIN fusion construct. Individual cells transfected with this construct were selected based on the appearance of green fluorescence and their currents compared with those recorded in cells transfected with a simple GFP construct alone. The magnitude of the inward ARC currents at –80 mV with an internal Ca$_{2^{+}}$ concentration of 400 nM in the GFP-c-CAIN transfected cells averaged 0.52 ± 0.04 pA/pF (n = 4) (Fig. 4, A, B, and D). This compares with values of 0.19 ± 0.02 pA/pF (n = 5) for control cells, and 0.23 ± 0.05 pA/pF (n = 4) for cells transfected with a control GFP construct (Fig. 4, C). In contrast, currents measured with an internal Ca$_{2^{+}}$ concentration of 100 nM were unaffected by transfection of the CAIN construct. Mean values were 0.49 ± 0.004 pA/pF (n = 4) for the GFP-c-CAIN transfected cells, compared with 0.52 ± 0.04 pA/pF (n = 6) for control cells, and 0.52 ± 0.03 (n = 3) for cells transfected with the control GFP construct (Fig. 4, A, C, and D). Overall, these data indicate that cells expressing the calcineurin-binding CAIN construct showed a complete reversal of the normal Ca$_{2^{+}}$-dependent inhibition of the ARC currents.

Calcineurin and the Activity of Store-operated Channels—Although we have demonstrated that inhibition of calcineurin is effective in reversing the Ca$_{2^{+}}$-dependent inhibition of currents through ARC channels, it was important to determine whether this effect was specific to these conductances. Most relevant was the question of whether calcineurin activity also influenced currents through the endogenous store-operated Ca$_{2^{+}}$-selective (SOC) currents in the m3-HEK cells (10). These currents display most of the characteristic features of the classic CRAC channels first described in mast cells and Jurkat lymphocytes (24, 25). In particular, they are highly Ca$_{2^{+}}$ selective, show marked inward rectification, very positive reversal potentials, inhibition by La$^{3+}$ and by 2-APB and, most importantly their activation is entirely dependent on depletion of internal Ca$_{2^{+}}$ stores (10). To examine the effects of calcineurin
Fig. 3. Calcineurin inhibitors reverse the Ca^{2+}-dependent inhibition of ARC channels. A, representative traces comparing the activation of inward Ca^{2+}-selective currents through ARC channels by addition of 8 μM arachidonic acid (arrow) in cells treated with CspA (1 μM for 15–30 min) with internal (pipette) solutions containing 100 nM Ca^{2+} (open circles) and 400 nM Ca^{2+} (filled circles). B, the effect of internal Ca^{2+} concentration on inward Ca^{2+}-selective currents through ARC channels activated by 8 μM arachidonic acid in control cells (filled circles, see Fig. 1), and in cells pretreated with 1 μM CspA (open circles). Values mean ± S.E., n = 4–6 for the CspA-treated cells. C, magnitude of the inward Ca^{2+}-selective currents through ARC channels following addition of 8 μM arachidonic acid with internal (pipette) solutions containing Ca^{2+} at 100 nM and 400 nM as indicated. Values (mean ± S.E.) are shown for control cells (filled columns, n = 6 and 5, respectively), and in cells preincubated for 15–30 min in 1 μM CspA (cross-hatched columns, n = 6), or 5 μM ascomycin (open columns, n = 9 and 10, respectively). D, mean (±S.E.) of the current-voltage relationships of the Ca^{2+}-selective currents measured with internal Ca^{2+} concentration of 400 nM for control cells (filled circles, n = 4), CspA-treated cells (open triangles, n = 4), and ascomycin-treated cells (open circles, n = 10). E, mean (±S.E.) of the current-voltage relationships of the Ca^{2+}-selective currents measured with internal Ca^{2+} concentration of 400 nM for control cells (filled circles, n = 4), and for cells exposed to 0.1 μM tautomycin via the pipette (open circles, n = 5).
activity on these conductances, we included adenophostin (2 μM) in the pipette solution. Previous studies have shown this high affinity agonist for the InsP3 receptor to be particularly effective at depleting agonist-sensitive internal Ca$^{2+}$ stores and maximally activating store-operated conductances including CRAC (26, 27). Preincubation of the cells with either CspA or ascomycin (1 and 5 μM, respectively, for 15–30 min) resulted in inward currents through the SOC channels measured at $80 \text{ mV}$ with 400 nM internal Ca$^{2+}$ of 0.38 ± 0.03 pA/pF ($n = 12$) and 0.41 ± 0.03 ($n = 5$) respectively. These values indicate that the immunosuppressants induced an ~35% reduction in the currents through the SOC channels measured at 400 nM internal Ca$^{2+}$. However, it is uncertain whether this reflects a calcineurin-dependent effect as a similar reduction in SOC currents was observed with 5 μM ascomycin when measured with 100 nM internal Ca$^{2+}$ (0.57 ± 0.04 pA/pF, $n = 7$, versus 0.43 ± 0.03 pA/pF, $n = 17$, for controls and ascomycin-treated cells, respectively). At only 100 nM internal Ca$^{2+}$, we would predict that activation of calcineurin would be minimal, suggesting that the observed effects of these drugs on currents through SOC channels are unlikely to be related to calcineurin activity. This was confirmed by experiments examining the SOC currents activated by 2 μM adenophostin in cells overexpressing the calcineurin-binding GFP-c-CAIN construct. In these, expression of the GFP-c-CAIN construct had no effect on the magnitude of the mean inward current through the SOC channels at $80 \text{ mV}$ with an internal Ca$^{2+}$ concentration of 400 nM (0.55 ± 0.04 pA/pF, $n = 4$, versus 0.61 ± 0.02 pA/pF, $n = 5$, for control cells under the same conditions). Consistent with this, Glitsch et al. (28) have reported that currents through the CRAC channels in RBL-1 cells are unaffected by pre-exposure to 5 μM CspA. Based on these data, we conclude that calcineurin activity has a specific influence on the ARC channels, and does not affect the magnitude of the current density through SOC channels.

Calcineurin Activity at Different Agonist Concentrations—
The above data demonstrate that the Ca$^{2+}$-dependent inhibition of the ARC channels is mediated by calcineurin. It is our contention that the activation of calcineurin occurs subsequent to the generation of an elevated, sustained Ca$^{2+}$ signal result-

![Image](http://www.jbc.org/40093)
FIG. 5. Effect of agonist concentration on calcineurin activation. A, the calcineurin-dependent nuclear translocation of NFAT occurs at a high agonist concentration (when store-operated Ca$^{2+}$ entry predominates), but not at a low concentration when ARC-mediated Ca$^{2+}$ entry predominates. The fluorescence images of a representative cell expressing the GFP-NFAT4-n construct show the distribution of the GFP-tagged NFAT construct in an individual cell at zero time (i), after 30 min of exposure to 0.5 μM carbachol (ii), and after a subsequent 15-min exposure to 10 μM carbachol (iii). B, the effect of carbachol at different concentrations on the calcineurin-dependent, NFAT-mediated expression of a luciferase reporter gene. Cells transfected with the negative control pCIS-CK construct (filled columns) or the NFAT PathDetect construct (open columns) were exposed to either 0.5 or 10 μM carbachol, and assayed for luciferase expression 3 h later. Data are expressed as the percent carbachol-induced stimulation of luciferase expression (mean ± S.E., n = 5 and 6 for 0.5 μM and 10 μM carbachol, respectively).

To examine this, we investigated the effect of stimulation of the m3-HEK cells with different concentrations of the muscarinic agonist carbachol on the activity of calcineurin in vivo. We used two different approaches, both of which are based on the calcineurin-dependent dephosphorylation of the cytoplasmic components of the NFAT transcription complex, and their subsequent activation (29). The first approach involved the microscopic examination of the distribution of a transfected GFP-tagged N-terminus NFAT construct (GFP-NFAT4-n) (30). Under resting conditions, NFAT is phosphorylated and restricted to the cytosol. Activation of the transcription factor involves a calcineurin-dependent dephosphorylation, exposure of nuclear localization sequences, and the consequent translocation of the dephosphorylated NFAT to the nucleus (31). Studies have shown that the first 351 residues of (NFAT4-n) encompass the calcineurin-dependent dephosphorylation site and is sufficient for calcium-dependent nuclear translocation (30, 32). Fusion of GFP to this NFAT-n construct allows the calcineurin-dependent translocation of the construct to the nucleus to be followed in real time. As illustrated (Fig. 5A), examination of m3-HEK cells transfected with the GFP-NFAT4-n construct showed a predominantly cytosolic localization. Exposure of the cell to 0.5 μM carbachol, a concentration that almost maximally activates the ARC channels (13), failed to induce a significant change in the cytosolic localization of the GFP-NFAT4-n even after 30 min. However, subsequent exposure to a higher concentration of carbachol (10 μM) induced the translocation of the GFP-NFAT4-n that was clear after only 15 min. Importantly we have previously reported that, at this concentration, Ca$^{2+}$ entry is predominantly via the store-operated Ca$^{2+}$-selective channels and the contribution of the ARC channels is negligible (13).

To obtain a more readily quantifiable estimate of the relative activation of calcineurin, we measured the carbachol-activated transcription of a luciferase reporter gene driven by a synthetic NFAT-dependent promoter. Using this approach, a 30 min exposure to 10 μM carbachol resulted in an approximate 2.5-fold stimulation in NFAT-dependent luciferase expression (Fig. 5B). In contrast, similar exposure to 0.5 μM carbachol failed to increase NFAT-dependent luciferase expression above background levels (not significantly different from values obtained using a pCIS-CK negative control construct, p = 0.09). Clearly then, under conditions of agonist stimulation when the entry of Ca$^{2+}$ is essentially exclusively via the ARC channels (0.5 μM carbachol), the activation of calcineurin is negligible. The Ca$^{2+}$ signal under these conditions typically takes the form of repeated oscillations, and the data show that neither this, nor the Ca$^{2+}$ entering via the ARC channels, is effective in inducing a significant activation of calcineurin. In contrast, there is a significant activation of calcineurin at 10 μM carbachol, when we know the entry of Ca$^{2+}$ is via the store-operated Ca$^{2+}$ channels and entry via the ARC channels is inhibited (13).

DISCUSSION

The results presented here demonstrate that the previously described Ca$^{2+}$-dependent inhibition of currents through ARC channels that underlies the reciprocal regulation of Ca$^{2+}$ entry pathways in nonexcitable cells, is mediated via the action of calcineurin. The observed steep relationship between cytosolic Ca$^{2+}$ and channel inhibition is entirely consistent with the known highly cooperative activation properties of the enzyme (Hill coefficient = 2.8–3), which reflect the Ca$^{2+}$-dependent binding of calmodulin to the A subunit (33). This enables the enzyme to respond to narrow Ca$^{2+}$ thresholds following stimulation, consistent with the observed effects on current magnitude. Furthermore, we have demonstrated that, while the high agonist concentrations that induce an entry of Ca$^{2+}$ via store-operated Ca$^{2+}$ channels are associated with a significant activation of calcineurin, agonist concentrations that give rise to an entry of Ca$^{2+}$ exclusively via the ARC channels fail to increase calcineurin activity. We have previously shown that the cytosolic Ca$^{2+}$ signal under these latter conditions typically takes
Calcineurin is a widely distributed serine/threonine protein phosphatase that is involved in the regulation of a diverse range of cellular responses including lymphocyte activation, neuronal and muscle development, neurotransmitter release, morphogenesis of vertebrate heart valves, memory and learning, and certain examples of Ca$^{2+}$-dependent cell death (31, 35). Calcineurin has also been implicated in the modulation of the activity of certain ion channels including NMDA receptor channels (37, 38), and L-type Ca$^{2+}$ channels, at least in some cell types (39, 40). In these cases the activation of calcineurin is generally dependent on the Ca$^{2+}$ flux through the channel, which then modulates the activity of the same channel in an autoregulatory manner. However, a rather different mode of action of calcineurin is suggested by the data presented here. These show that the activation of the phosphatase is independent of Ca$^{2+}$ flux through the ARC channels and is, instead, dependent on a sustained elevation in cytosolic Ca$^{2+}$ concentration. In the intact cell, the sustained elevation in cytosolic Ca$^{2+}$ concentration required for ARC channel inhibition, results from the activation of SOC channels following the depletion of the stores at high agonist concentrations. Consistent with this, the Ca$^{2+}$ signals associated with entry through store-operated channels are known to be particularly effective activators of calcineurin. Moreover, certain mutant strains of Jurkat lymphocytes selected for their inability to promote the calcineurin-mediated, NFAT-dependent expression of a toxin gene were found to be specifically defective for store-operated entry (41, 42). During the reciprocal regulation of Ca$^{2+}$ entry the calcineurin thus activated acts, not to feedback on the Ca$^{2+}$ entry, the ARC channels. As yet, it is uncertain whether it is the ARC channels themselves that are dephosphorylated or whether some kind of intermediate regulatory molecule is involved. The fact that the calcineurin-dependent dephosphorylation is insensitive to Ca$^{2+}$ entering the channel suggests that at least some component of the overall pathway (e.g. calmodulin, calcineurin, or putative regulatory protein) is probably not directly associated with the channel.

The data presented in this report demonstrate an entirely novel function of calcineurin in that they represent the first example of a calcineurin-dependent inhibition of a Ca$^{2+}$ entry channel in nonexcitable cells. Moreover, they illustrate an example of a calcineurin-dependent inhibition of a Ca$^{2+}$ channel that is mediated not by the channel itself, but indirectly as a result of the activity of another coexisting Ca$^{2+}$ channel. Physiologically, the process of reciprocal regulation of Ca$^{2+}$ entry results in the agonist-dependent activation of these two coexisting channels to be coordinated in a way that prevents their simultaneous activation (13). This, in turn, allows each entry pathway to play a unique and non-overlapping role in Ca$^{2+}$ signaling. At low agonist concentrations, Ca$^{2+}$ entry is predominantly via the ARC channels where the main effect of such entry is to modulate the frequency of the induced Ca$^{2+}$ oscillations. Because of the phenomenon of reciprocal regulation, Ca$^{2+}$ entry at high agonist concentrations is switched to the store-operated channels (e.g. CRAC channels) and its main effect is to modulate the amplitude of the sustained elevated cytosolic Ca$^{2+}$ concentration (13). In addition to these effects on the overall Ca$^{2+}$ signal, the targeting of specific subsets of effectors to these two channel types could result in the activation of distinct responses in the cell in a uniquely agonist concentration-dependent manner. Such direct effects will be essentially independent of the nature and magnitude of the overall Ca$^{2+}$ signal, and therefore raise the potential of yet another layer of complexity and versatility in the types of response that changes in agonist concentration can induce in the cell. Clearly, these two co-existing channel types (SOC or CRAC channels, and ARC channels) are not simply alternative, mutually redundant, routes of entry. They not only have different modes of activation, but they also serve different functions in the overall control of Ca$^{2+}$ signals and their downstream responses. Finally, it should be noted that the actions of the immunosuppressants in reversing the Ca$^{2+}$-dependent inhibition of ARC channels would imply that the presence of these drugs would severely impair the normal reciprocal regulation of Ca$^{2+}$ entry pathways. As this regulatory process normally results in a switching between ARC-mediated and SOC-mediated Ca$^{2+}$ entry as agonist concentrations change, the presence of the immunosuppressants will result in the entry of Ca$^{2+}$ via these two pathways becoming additive at higher agonist concentrations. This would be especially true in cells which express only low levels of calcineurin (i.e. non-neuronal/muscle cells) which are, consequently, considerably more sensitive to the effects of the immunosuppressants (35). Given this, it seems conceivable that such a phenomenon may contribute to the many known toxic/malignant effects of these drugs which, to date, have severely limited their therapeutic usefulness (36, 43).

Acknowledgments—We thank Pauline Leadlay for technical assistance, Dr. Frank McKeon (Harvard Medical School) for the GFP-NFAT4-n construct, and Dr. Jianjie Ma (UMDNJ) for the GFP-c-CAIN construct.

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