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Intracellular ADP Modulates the Ca^{2+} Release-activated Ca^{2+} Current in a Temperature- and Ca^{2+}-dependent Way*

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The rat basophilic cell line RBL-1 is known to express high levels of the Ca^{2+} current activated by store depletion, known as Ca^{2+} release-activated Ca^{2+} current (I_{CRAC}), the main Ca^{2+} influx pathway so far identified in nonexcitable cells. We show here that, as reported in other cell types, metabolic drugs strongly inhibit the Ca^{2+} influx operated by store depletion in RBL-1 cells also. We have tested the hypothesis that intracellular adenine and/or guanine nucleotide levels act as coupling factors between I_{CRAC} and cell metabolism. Using the whole cell configuration of the patch-clamp technique, we demonstrate that addition of ADP to the intracellular solution significantly reduces I_{CRAC} induced by inositol 1,4,5-trisphosphate. This phenomenon differs from other regulatory pathways of I_{CRAC}, since it is highly temperature-dependent, is observable only in the presence of low intracellular Ca^{2+} buffering capacity, and requires a cytosolic factor(s) which is rapidly lost during cell dialysis. Moreover, the inhibition is specific for ADP and is partially mimicked by ADPβS and AMP, but not by GDP or GTP.

Many receptor agonists convey their message to intracellular target processes by increasing the cytosolic concentration of ionized calcium, $[Ca^{2+}]_i$[1] (1, 2). This phenomenon is usually dependent on both Ca^{2+} release from intracellular stores and Ca^{2+} influx across the plasma membrane. Since the early 1980s, it was postulated that the increased permeability of the plasma membrane to Ca^{2+} was somehow linked to the depletion of internal Ca^{2+} pools, but this hypothesis became widely accepted only in the last few years (3–7). This influx pathway depends on a new class of Ca^{2+} channels, named "store-operated Ca^{2+} channels" or "SOCs" (8). Although much evidence demonstrates that activation of SOCs depends on the Ca^{2+} content of $InsP_3$-sensitive stores (5), the coupling between stored Ca^{2+} and channel activity is still largely obscure (6). The prototype of SOCs is the CRAC channel, initially described in rat mast cells (9). Channels with similar characteristics appear to be expressed in many different cell types (10–17). The main characteristics of CRAC are: (i) voltage independence of gating, (ii) low unitary conductance (<100 femtosiemens), and (iii) high Ca^{2+} selectivity (>1000:1 over monovalent cations) (9–11, 17–19). Among the various possible mechanisms suggested for CRAC activation, both a diffusible messenger released from empty stores, often referred to as CIF, for Ca^{2+} influx factor (20, 21), and direct coupling between the store and the plasma membrane channels have been considered (6–8). The modulation of CRAC activity is also a matter of debate. Small G proteins have been suggested to be involved either in the activation of CRAC itself or in the modulation of channel activity (22–25). Similarly, in some cell types, cGMP and CIF have been shown to up-regulate Ca^{2+} influx or currents activated in response to Ca^{2+} store depletion (8, 16, 26–28). Drugs acting on cytochrome P-450 or on intracellular kinases and/or phosphatases have also been shown to modulate CRAC-type currents (13, 29–32). The picture emerging from all these observations is rather complex, suggesting that although CRAC channels are subjected to multiple control mechanisms, either they are differently modulated in the various cell types or they are a heterogeneous class of channels with variable sensitivity and mechanism of activation-modulation.

We have recently shown that the energy level of the cell controls the influx of Ca^{2+} activated by store depletion in a variety of cell types (33). The interest of this observation is 2-fold: on the one hand, the effect of energy level appears as quite a general phenomenon, in as much as it was observed in cells as diverse as lymphocytes, hepatocytes, ascites tumor cells and epithelial-derived tumor cell lines; on the other, it may represent not only a general mechanism for modulating Ca^{2+} influx in physiological conditions, but also a safety device operative under pathological situations such as hypoxia, metabolic activation, etc. The mechanism through which the cell energy level inhibits Ca^{2+} influx and whether or not this inhibition concerns the CRAC channels is still unknown. In this contribution, by using the patch-clamp technique in the whole-cell configuration, we address this problem directly and test the effects of adenine and guanine nucleotides on the current sustained by CRAC channels. We demonstrate that ADP, but not ATP, GTP, or GDP, is a physiological modulator of I_{CRAC}. This modulation is rather complex, being highly sensitive to temperature and intracytosolic Ca^{2+} buffering and requiring a soluble factor(s) which is rapidly washed out during cell dialysis.

EXPERIMENTAL PROCEDURES

Cells—Rat basophilic leukemia cells (RBL-1, a gift from Dr. Reinhold Penner, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany) were cultured as described (22).

$[Ca^{2+}]_i$ Measurements—Loading with fura-2/AM was performed as described (33). Briefly, cell suspensions (10^7/ml) or cells cultured on glass coverslips were incubated at 37 °C in RPMI 1640 containing 3%

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[1] The abbreviations used are: $[Ca^{2+}]_i$, cytosolic concentration of ionized Ca^{2+}; $InsP_3$, inositol 1,4,5-trisphosphate; CRAC, Ca^{2+} release-activated Ca^{2+} channel; I_{CRAC}, Ca^{2+} release-activated Ca^{2+} current; F, farad; fura-2/AM, fura-2 acetoxymethyl ester; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetatic acid; ADPβS, adenosine 5′-*O*-2-(thio)diphosphate; CFTR, cystic fibrosis transmembrane receptor.

fetal calf serum, 200 μM sulfinpyrazone and 2 μM fura-2/AM. After 30 min, the cells were washed and kept at room temperature until used. In experiments with cell suspensions, before each experiment, an aliquot of the cells (10^6 cells) was pelleted and resuspended in 2 ml of fresh medium. The standard external solution contained: 140 mM NaCl, 2.8 mM KCl, 2 mM $MgCl_2$, 10 mM Hepes (pH 7.4 at 25 °C or 37 °C); $CaCl_2$ and glucose concentrations varied according to the experiment. Fluorescence was measured as described (33). In the experiments where $[Ca^{2+}]_i$ and cell current were measured simultaneously, they were carried out with an inverted microscope (Axiovert100, Zeiss) equipped for epifluorescence and photometry (T.I.L.L. Photonics, Planegg, Germany). The light source was a xenon short arc lamp (75X-O Ushio Inc. Japan) and a diffraction grating mounted on a high speed scanner, providing monochromatic light at 360 and 380 nm wavelengths. Light from the lamp was directed through a quartz glass fiber to a gray filter (Oriel) before entering the microscope. To reduce photobleaching further, the monochromatic light was pulsed 10 ms at each wavelength, followed by 200 ms of dark. Light was deflected by a 420 dichroic mirror into the objective (Plan-Neofluar 40x, Zeiss). The emitted light was directed through a 450 nm cut-off filter (Oriel) to a photomultiplier tube (R928; Hamamatsu). To collect fluorescence from a single cell, a pin hole was placed in the image plane of the phototube. The fluorescence was calibrated in terms of $[Ca^{2+}]_i$ as described (9, 34), using a K_d for the Ca^{2+} fura-2 complex of 224 nM.

Current Measurements—Unless otherwise specified, patch-clamp experiments were performed in the tight-seal whole-cell configuration in a standard external solution containing: 140 mM NaCl, 2.8 mM KCl, 2 mM $MgCl_2$, 10 mM $CaCl_2$, 11 mM glucose, and 10 mM Hepes (pH 7.4 at 25 °C or 37 °C); 0.5 mM $BaCl_2$ was routinely added to block inwardly rectifying K^+ channels (19). Sylgard-coated patch pipettes had resistance between 2 and 4 MΩ after filling with the standard intracellular solution, which contained: 145 mM cesium-glutamate, 8 mM NaCl, 1 mM $MgCl_2$, and 10 mM Hepes (pH 7.2, 25 °C or 37 °C). Drugs were added by local pressure from a wide-tipped micropipette (5–10 μm). High resolution current recordings and fura-2 fluorescence were acquired by a computer-based patch-clamp amplifier system (EPC-9, HEKA, Lambrecht, Germany) controlled by the Pulse software (HEKA). All voltages were corrected for a liquid junction potential of 8 mV between external and internal solutions. High resolution currents were acquired at a sampling rate of 10 kHz, low-pass filtered at 2.3 kHz, and digitally filtered to 1 kHz for presentation. The holding current, the holding potential, the fura-2 fluorescence, and other parameters were synchronously recorded, at low resolution (2 Hz), by the X-Chart software (HEKA). Voltage ramps of 50-ms duration, from −100 to +100 mV, were delivered at 0.5 Hz from a holding potential of 0 mV. Capacitative currents were canceled before each voltage ramp using the automatic capacitance compensation of the EPC-9. Uncompensated series resistance was in the range of 5–12 MΩ and did not correlate with the inhibition of I_{CRAC}. For perforated-patch recordings, a fresh amphotericin B stock solution (60 mg/ml in dimethyl sulfoxide) was diluted 250-fold prior to use, in pipette solution containing 140 mM potassium glutamate, 8 mM NaCl, 1 mM $MgCl_2$, and 10 mM Hepes (pH 7.2 at 37 °C). Registration started when the access resistance was stable and below 50 MΩ.

Materials—Fura-2/AM was obtained from Molecular Probes; culture media and sera were from Technogenetics (Milano, Italy); $InsP_3$ was from Calbiochem; $GTPNa_2$ and $ADP\beta SLi_3$ were from Boehringer; ATPMg, $ADPK_2$, GDP-Tris, AMP, thapsigargin, and other chemicals were from Sigma.

Statistical Analysis—Results reported are average values ± S.E. (mean ± S.E.; n = number of cells from the same batch). Statistical significance was evaluated with the Student's t test.

RESULTS

Given that I_{CRAC} is relatively large and well characterized in rat mast cells and in the mast cell line RBL-1 (9, 18, 19, 22), we employed the latter model system to characterize the effect of metabolic inhibitors on this Ca^{2+} current. We first tested whether also in the rat cell line RBL-1 the Ca^{2+} entry induced by store depletion was sensitive to the treatment with mitochondrial poisons. Fig. 1 shows that oligomycin, a known inhibitor of the mitochondrial ATP synthase, caused a reduction of the $[Ca^{2+}]_i$ increases induced by treatment of the cells with the sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitor thapsigargin (36). In particular, Fig. 1*A* shows that oligomycin hardly affected $[Ca^{2+}]_i$ in high glucose medium, whereas it clearly reduced the steady state increase of $[Ca^{2+}]_i$ caused by

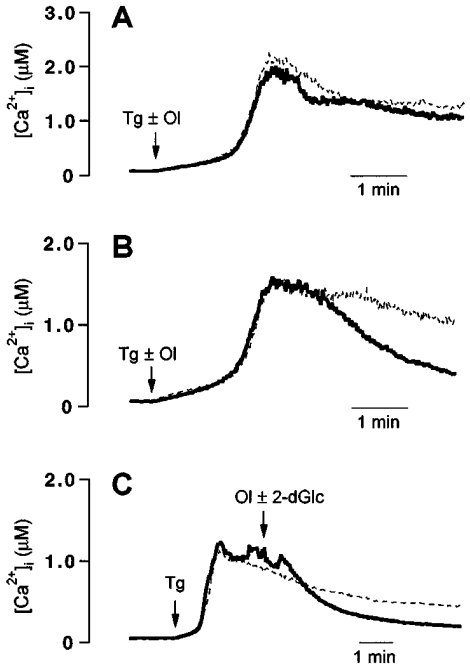

FIG. 1. **Effect of metabolic inhibitors in RBL-1 cells.** RBL-1 cells cultured on glass coverslips were loaded with fura-2/AM as described under "Experimental Procedures." Cells were bathed in standard solution containing 1 mM $CaCl_2$ and 11 mM (*A*), 0 mM (*B*), or 1 mM (*C*) glucose. *A*, and *B*, Ca^{2+} influx was activated by applying the same solutions containing 1 μM thapsigargin (*Tg*) in the presence (*continuous traces*) or absence (*dashed traces*) of 1.4 μM oligomycin (*Ol*). *Traces* are representative of 5 similar experiments. *C*, Ca^{2+} influx was activated by 1 μM thapsigargin (*Tg*) and during the Ca^{2+} plateau oligomycin was applied in the presence (*continuous trace*) or absence (*dashed trace*) of 10 mM 2-deoxyglucose (*2-dGlc*). Averaged responses of 10–12 cells representative of 3 similar experiments.

thapsigargin both in glucose-free medium (Fig. 1*B*) and in glucose medium but containing 10 mM 2-deoxyglucose (Fig. 1*C*). Thus, as observed in other cells, the effect of mitochondrial inhibitors is observed only if glycolysis is inhibited, indicating that the energy level rather than mitochondrial inhibition is the key factor regulating, directly or indirectly, Ca^{2+} influx induced by store depletion (33, 35). Other mitochondrial inhibitors, such as sodium azide, KCN, antimycin A, rotenone, and carbonyl cyanide *p*-fluoromethoxyphenylhydrazone exerted similar effects (not shown, and see Refs. 33 and 35).

The experiments described in Fig. 1 strongly suggest that the target of metabolic drugs is I_{CRAC} since the sustained increase of $[Ca^{2+}]_i$ caused by thapsigargin is due mainly to the activation of this current (37).

We hypothesized that the level of intracellular ADP or GDP, rather than that of ATP or GTP, is critical in modulating thapsigargin-induced Ca^{2+} influx (33). Considering that the patch-clamp technique in the whole-cell configuration gives access to the cell interior, we could test directly whether indeed the intracellular nucleotide level affects I_{CRAC}. In order to activate the current, $InsP_3$ was also included in the intracellular solution. Fig. 2*A* (*left panel*) shows that, shortly after establishing the whole-cell configuration, there is a rapid activation of an inward current which reached its maximum within about 1 min. The current-voltage relationship, as revealed by the voltage ramp from −100 to +100 mV, shown on the *right panel* of Fig. 2*A*, confirms that the current has the typical characteristics of I_{CRAC}, *e.g.* inward rectification and reversal potential above +50 mV. In control conditions (*dashed traces*), the pipette solution contained 2 mM ATP. Fig. 2*A* shows that, if the pipette contained 0.5 mM ADP in addition to ATP (continuous traces), neither the amplitude nor the kinetics of the current

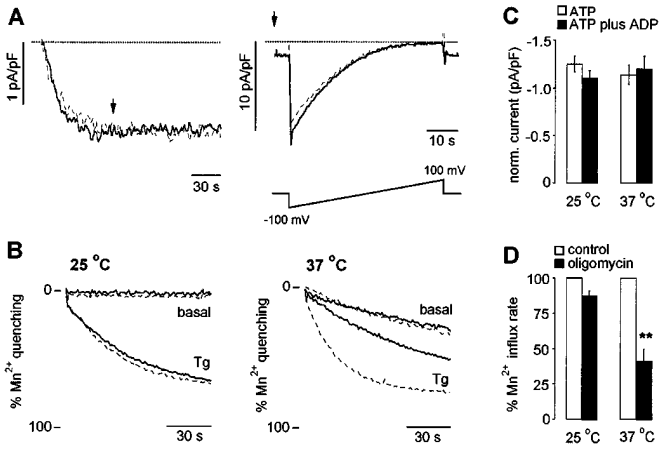

FIG. 2. **Effect of ADP and temperature on I_{CRAC} and capacitative Ca^{2+} influx.** *A*, *left*, kinetics of I_{CRAC} activation at 25 °C during perfusion with a standard pipette solution containing 10 mM cesium BAPTA, 50 μM $InsP_3$, 2 mM ATP with (*continuous traces*) or without (*dashed traces*) 0.5 mM ADP. Inward currents, recorded at 0 mV holding potential and sampled at 2 Hz, were normalized to the cell capacitance for comparison. *Right*, normalized current-voltage relationships derived from subtracting fast ramps, delivered after break-in (before activation of the current), from current responses at the time indicated by the *arrow*. The voltage-pulse protocol, delivered every 2 s, is schematically shown on the *bottom* of the same panel. *B*, cells loaded with fura-2/AM were suspended (10^6 cells/ml) in Ca^{2+}- and glucose-free external solution containing 0.2 mM EGTA. Cell suspensions were then challenged with 1 μM thapsigargin in the presence (*continuous traces*) or in the absence (*dashed traces*) of 1.4 μM oligomycin. After 3 min, 0.4 mM $MnCl_2$ was added to follow the activation of capacitative influx. In each panel, the slower traces represent the basal Mn^{2+} influx, recorded in the absence or in the presence of oligomycin. The vertical calibration on the *left side* represents the total Mn^{2+}-quenchable fura-2 fluorescence at 360 nm. The experiments shown on the *left panel* were carried out at 25 °C, those on the *right panel* at 37 °C. *C*, pooled data of normalized peak current, measured at 0 mV from current-voltage relationships, as shown in *A* (*right*). Cells, voltage-clamped at 25 or at 37 °C, were dialyzed with the above mentioned internal solution. For statistics, see Table I. *D*, the Mn^{2+} influx, induced by thapsigargin in the absence (control) or in the presence of oligomycin, was estimated from the initial rate of fluorescence quenching, after subtraction of the basal influx, and plotted as percentage of control. Pooled data are from 3 similar experiments (**, $p < 0.001$).

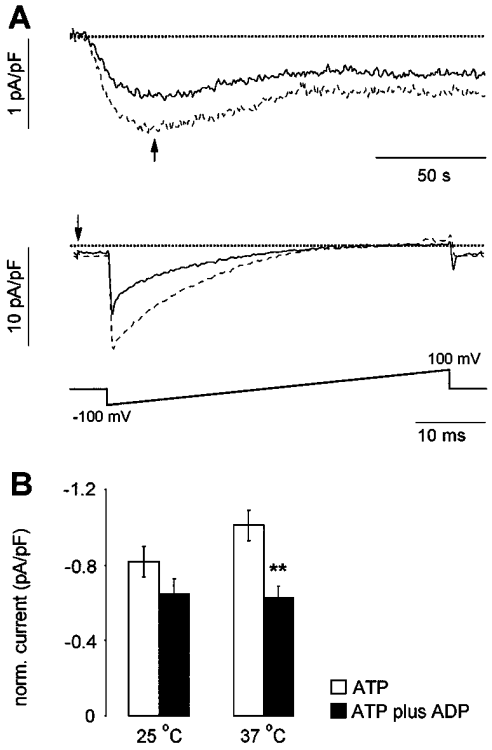

FIG. 3. **ADP inhibits I_{CRAC} at low internal Ca^{2+} buffering capacity.** *A*, *upper*, temporal pattern of I_{CRAC} activation at 37 °C during perfusion with a standard pipette solution containing 1.2 mM cesium EGTA, 50 μM $InsP_3$, 2 mM ATP with (*continuous traces*) or without (*dashed traces*) 0.5 mM ADP. Inward currents, recorded at 0 mV holding potential and sampled at 2 Hz, were normalized to the cell capacitance for comparison. *Lower*, normalized current-voltage relationships, obtained as described in Fig. 2*A* (*right panel*). *B*, pooled data of the normalized peak current at 0 mV, measured as described in Fig. 2*C*, with the above-mentioned internal solution (**, $P < 0.002$).

was affected. In addition, no significant effect on I_{CRAC} was detected if the same amount of GDP or GTP was added to the intracellular solution. Finally, none of the above-mentioned mitochondrial inhibitors had any direct effect on I_{CRAC}, as measured under these conditions (not shown).

The Effects of Temperature—The experimental conditions normally employed to study I_{CRAC} and those used to study Ca^{2+} influx with indicators differed in several aspects. Among these, the electrophysiological experiments are routinely carried out at room temperature, rather than at 37 °C, the intracellular perfusion medium is heavily buffered (with EGTA or BAPTA) to avoid Ca^{2+} inactivation by incoming Ca^{2+} (18), and small molecules are rapidly washed into the patch pipette upon establishing the whole-cell configuration (38). In order to understand whether the lack of effect of intracellular adenine (or guanine) nucleotides on I_{CRAC} was indeed due to different experimental conditions, a series of experiments was then performed. The effect of temperature was first tested in intact cells loaded with fura-2. Since the temperature affects also Ca^{2+} pumps and thus may complicate the interpretation, we used the Mn^{2+} quenching technique. Mn^{2+} is, in fact, a good Ca^{2+} surrogate since it is permeable through CRAC channels, but is not transported by either the plasma membrane or the intracellular Ca^{2+}-ATPases (39). Fig. 2*B* shows that while thapsigargin-induced Mn^{2+} influx is strongly inhibited by oligomycin at 37 °C (*right panel*), the drug had hardly any effect at 25 °C (*left panel*). Fig. 2*D* shows the mean effect of oligomycin on Mn^{2+} influx induced by thapsigargin in three similar experiments (mean ± S.E.). Similar results were obtained following a 10-min preincubation with this or other mitochondrial poisons, and when Ca^{2+} rather than Mn^{2+} was the permeant cation (not shown). Fig. 2*C* shows that when I_{CRAC} was tested under the conditions of the experiments reported in Fig. 2*A*, but at 37 °C, no difference was observed whether ATP or ATP plus ADP (or GDP) was included in the pipette solution. Finally, even at 37 °C, none of the mitochondrial inhibitors known to be effective in intact cells appreciably modified I_{CRAC} when applied during activation of the current in the whole-cell configuration (not shown and see below).

The Effect of Intracellular Ca^{2+} Buffers—In many of the Ca^{2+}-regulated processes, the cation itself is involved in a series of feedback inhibitory mechanisms. For example, voltage-operated Ca^{2+} channels of the L-type are known to be negatively regulated by the cytosolic Ca^{2+} concentration and so is I_{CRAC} (18, 19, 40). We thus hypothesized that the lack of effect of ATP/ADP or GTP/GDP levels on I_{CRAC}, under conditions which should mimic the situation of metabolic poisoning in intact cells, could be due to the "wash-in" of a high concentration of Ca^{2+} buffers. In other words, the possibility was considered that the effect of metabolic poisons on I_{CRAC} was somehow mediated through a sensitization to Ca^{2+} inhibition.

The experiments described in Fig. 3 were designed to test this possibility. The pipette solution contained 1.2 mM EGTA instead of 10 mM BAPTA. Thus, not only the absolute Ca^{2+} buffering capacity was reduced, but a "slow" buffer such as EGTA was substituted for a "fast" buffer such as BAPTA (18).

TABLE I
Kinetic parameters of I_{CRAC} activation

The control intracellular solution contained 2 mM ATP and 50 μM $InsP_3$, whereas in experiments with ADP, 0.5 mM ADP was added to the control solution.

Temperature	Buffer	Nucleotide	Delay	Time const.	0-mV current	−40-mV current	*n*
°C	*mM*		*s*	*s*	*pA/pF*	*pA/pF*	
37	EGTA, 1.2	Control	7.6 ± 1.5	8.5 ± 0.8	−1.09 ± 0.09	−2.97 ± 0.23	10
37	EGTA, 1.2	+ADP	8.4 ± 1.6	8.4 ± 0.9	−0.64 ± 0.08[a]	−1.88 ± 0.22[b]	9
25	EGTA, 1.2	Control	12.7 ± 2.5	16.6 ± 1.0	−0.84 ± 0.08	−2.56 ± 0.20	9
25	EGTA, 1.2	+ADP	18.1 ± 4.5	20.9 ± 4.9	−0.66 ± 0.08	−2.06 ± 0.22	10
37	BAPTA, 10	Control	3.3 ± 0.8	14.6 ± 1.5	−1.14 ± 0.10	−2.85 ± 0.25	11
37	BAPTA, 10	+ADP	3.8 ± 0.5	14.8 ± 3.5	−1.20 ± 0.10	−2.83 ± 0.25	8
25	BAPTA, 10	Control	5.9 ± 0.6	18.5 ± 1.9	−1.25 ± 0.08	−3.33 ± 0.23	13
25	BAPTA, 10	+ADP	7.2 ± 0.6	20.3 ± 1.7	−1.10 ± 0.08	−3.20 ± 0.21	11
37	EGTA, 10	Control	3.8 ± 0.7	6.3 ± 0.5	−1.27 ± 0.13	−3.43 ± 0.35	8
37	EGTA, 10	+ADP	3.0 ± 0.7	5.1 ± 0.6	−1.35 ± 0.14	−3.37 ± 0.34	5
37	BAPTA, 1.2	Control	8.5 ± 1.2	9.1 ± 1.4	−1.07 ± 0.11	−2.94 ± 0.30	7
37	BAPTA, 1.2	+ADP	10.3 ± 1.8	11.9 ± 1.4	−0.96 ± 0.09	−2.20 ± 0.38	11

[a] $p < 0.002$, Student's *t* test.
[b] $p < 0.01$, Student's *t* test.

As shown in Fig. 3*A*, at 37 °C and in the presence of 1.2 mM EGTA, addition of 0.5 mM ADP to the ATP-containing solution (*continuous traces*) significantly reduced the peak current, measured at a holding potential of 0 mV (*upper panel*), by an average factor of 42.0 ± 2.7% (mean ± S.E. of 3 similar experiments, 29 and 26 cells for each condition). The inhibition was observed also at −40 mV, when I_{CRAC} was measured during fast (50 ms) voltage ramps from −100 to +100 mV, delivered every 2 s from a holding potential of 0 mV (see Fig. 3*A*, *lower panel*, and Table I) or during long pulses (100 ms) to negative voltages (−100 mV, 55 ± 15% inhibition, $n = 7$). Fig. 3*B* shows the mean peak current at 0 mV, measured in the same batch of cells, at 25 and 37 °C, with 1.2 mM EGTA as the internal buffer.

The observed phenomenon was largely independent of the ATP concentration since it occurred with solutions containing either 0, 0.5, or 2 mM ATP. Under these conditions, the normalized peak current in controls was −0.95 ± 0.05 ($n = 5$), −0.97 ± 0.08 ($n = 10$), and −1.09 ± 0.09 ($n = 9$) pA/pF respectively, whereas, the average I_{CRAC} inhibition induced by ADP was practically unchanged. These results support the hypothesis that the effect of metabolic poisons is not due to a reduction in the ATP level, but rather to an increase in the ADP concentration (33). In cells of similar size and access through the patch pipette, as monitored by membrane capacitance and series conductance, respectively, other current properties, such as the delay before current activation and the time constant to reach the peak current, did not change significantly in the presence of ADP (see Table I). However, as expected, a reduction of the Ca^{2+} buffering capacity altered the slow kinetics of I_{CRAC}. Thus, while with 10 mM BAPTA in the pipette the current hardly decreased from the peak level onwards, with 1.2 mM EGTA the current at steady-state was about 50% of the peak current, with or without ADP.

Finally, the data of Table I demonstrate that the effect of a reduced buffering capacity on I_{CRAC} inhibition by intracellular ADP can be appreciated only at the physiological temperature, since at 25 °C the ADP modulation was not statistically significant, even with 1.2 mM EGTA. The lack of inhibition by intracellular perfusion with ADP, in the presence of 10 mM BAPTA, could reflect a toxic effect of high BAPTA concentrations, rather than its ability to trap Ca^{2+}. This appears unlikely, since: (i) no effect of ADP was observed when the EGTA concentration was increased from 1.2 to 10 mM, while (ii) the effect of ADP became statistically insignificant when 1.2 mM EGTA was substituted with 1.2 mM BAPTA (see Table I).

The Wash-out Effect—In the experiment described above, I_{CRAC} was activated by including $InsP_3$ in the pipette. Due to the rapid diffusion of this latter compound, the current developed within 10–20 s after establishing the whole-cell configuration. I_{CRAC}, however, can be activated also by other experimental means, for example by first establishing the whole-cell configuration and then by depleting Ca^{2+} stores by photolysis of caged-$InsP_3$ or by addition of the Ca^{2+} ionophore ionomycin (9, 17, 22). However, even at 37 °C and in the presence of 1.2 mM EGTA, no effect of ADP could be observed when the current was activated by the addition of ionomycin 40–50 s after establishing the whole-cell configuration. The normalized peak current at 0 mV corresponded to −0.98 ± 0.01 pA/pF in controls ($n = 5$) and −1.02 ± 0.03 pA/pF in the presence of ADP ($n = 4$). To confirm that the washout of a small molecular weight component(s) was responsible for the lack of effect of ADP on I_{CRAC}, experiments with the "perforated-patch" technique were performed. Under these conditions, only small ions are allowed to freely diffuse across the patch membrane, while all other cellular components, including nucleotides, remain in the cell. If the cells were preloaded with a Ca^{2+} indicator, the effect of I_{CRAC} activation on $[Ca^{2+}]_i$ could be followed while clamping the cell voltage. Fig. 4*A* shows that, under these conditions, addition of a metabolic inhibitor such as KCN significantly reduced the $[Ca^{2+}]_i$ increases induced by thapsigargin. Furthermore, Fig. 4*B* shows that KCN reduced not only the $[Ca^{2+}]_i$ plateau but also the oscillations of $[Ca^{2+}]_i$ induced by fast changing the membrane potential from −40 to +40 mV. These oscillations of $[Ca^{2+}]_i$ are considered the hallmark of I_{CRAC} activation in voltage-clamped cells (4, 10, 39). Due to the low endogenous Ca^{2+} buffering capacity, the amplitude of I_{CRAC} was hardly measurable under these conditions.

Nucleotide Specificity—Taken together, the results described above clearly demonstrate the modulatory role of ADP on I_{CRAC}. The possibility that other nucleotides which are directly or indirectly linked to the ATP/ADP ratio could also affect the current was then investigated. The role of the nucleotides was tested under the conditions which had disclosed the ADP effect, *i.e.* 37 °C and 1.2 mM EGTA in the pipette. As shown in Fig. 5, in cells perfused with a solution containing ATP (2 mM) and the same amount of ADP, ADPβS, AMP, GDP, and GTP (0.5 mM)

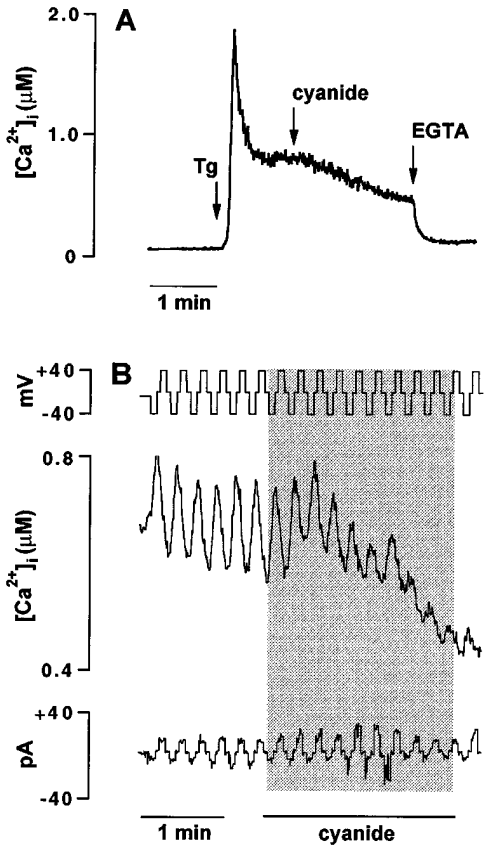

FIG. 4. **Metabolic inhibitors are effective in perforated-patch conditions.** *A*, a single cell, loaded with fura-2/AM, perfused at 37 °C with a standard external solution containing 1 mM $CaCl_2$ and 11 mM glucose, was voltage-clamped at −40 mV in the perforated cell configuration as described under "Experimental Procedures." Ca^{2+} influx was induced by local application of 1 μM thapsigargin (*Tg*). After establishing of the Ca^{2+} plateau phase, the external solution was switched to the same one but containing 1 mM KCN (cyanide) in the absence of glucose. *B*, conditions as in *A*. Once the Ca^{2+} plateau was established, the cell was alternatively clamped at −40, 0, and +40 mV to induce typical $[Ca^{2+}]_i$ oscillations driven by the membrane potential. Traces are representative of 3 similar experiments.

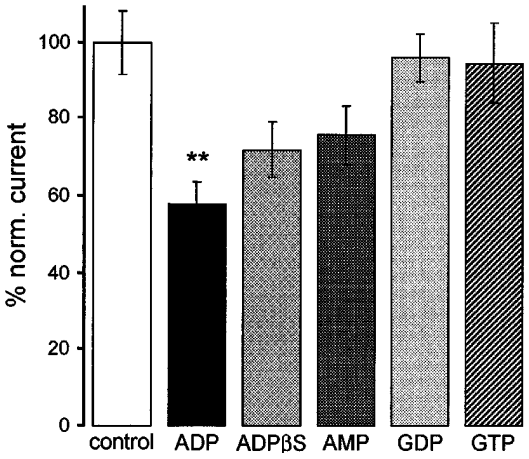

FIG. 5. **Effect of nucleotides on I_{CRAC}.** Cells were voltage-clamped at 37 °C in the whole-cell configuration as described in Fig. 3. The standard internal solution contained 1.2 mM cesium EGTA, 50 μM $InsP_3$, 2 mM ATP in the absence (control, $n = 16$) or presence of 0.5 mM ADP ($n = 13$), ADPβS ($n = 11$), AMP ($n = 8$), GDP ($n = 10$), or GTP ($n = 4$). The normalized peak current at 0 mV, measured as described in Fig. 2*C*, is shown as percentage of control (**, $P < 0.001$).

respectively, only the adenine nucleotides mimicked the ADP effect, although with reduced efficacy.

The sensitivity to adenine nucleotide is known to be a characteristic of metabolism-regulated K^+ channels which have been widely characterized in either pharmacological or molecular terms (41). We thus investigated whether drugs such as tolbutamide (100 μM) or diazoxide (100 μM), which are known to affect K^+ channels, could also affect Ca^{2+} influx through CRAC channels. None of these drugs affected either the Ca^{2+} influx induced by store depletion (measured in intact cells with Ca^{2+} indicators) or the current induced by $InsP_3$ (measured by the patch-clamp technique, not shown).

Kinetic Parameters of I_{CRAC} Activation—A closer inspection of Table I reveals also new properties of I_{CRAC}. At 37 °C and low Ca^{2+} buffering capacity, the current amplitude increased by a factor of about 1.3, and the time constant to maximal current was twice as short. This acceleration in current developing similarly occurred in the presence of both high and low EGTA, whereas it was reduced at high BAPTA concentration.

DISCUSSION

The selective Ca^{2+} current, named I_{CRAC}, activated by store depletion and originally described in rat mast cells and human T lymphoma cells, has been shown more recently to be expressed in many eukaryotic cell types (9–17). Whether or not CRAC or CRAC-like channels are a homogeneous group of proteins and whether they represent the mammalian homologues of the *Drosophila* cation channels, the *trp/trpl* proteins, is under intense investigation (42–44). It is clear, however, that, as originally shown in Jurkat T cells (Ref. 10), many physiological stimuli, in addition to store-depleting drugs, also activate I_{CRAC} or I_{CRAC}-like currents (14, 17, 37, 45). The physiological relevance of I_{CRAC} has been strengthened by the discovery of its absence in lymphocytes from a patient with a primary immunodeficiency associated with a defective T cell proliferation (46). As for other key Ca^{2+} channels, it is predictable that CRAC channels also are under strict and multiple control mechanisms. Surprisingly, up to now, little is known, not only about the molecular mechanism of activation of this current, but also on its modulation by physiological stimuli. Several drugs, in fact, have been shown to affect I_{CRAC} or, more generally, Ca^{2+} influx activated by store depletion, but very few examples of physiological modulation of this current have been reported so far (30–33, 40). From this point of view, the demonstration that Ca^{2+} influx induced by depleting $InsP_3$-sensitive Ca^{2+} stores is modulated by the energy level of the cells appears of major interest since it is found in many different cell types and because of its pathophysiological implications (33).

The present findings not only demonstrate directly that I_{CRAC} is the target through which metabolic poisons inhibit Ca^{2+} influx activated by store depletion, but they also allow the mechanism of this inhibition to be defined more precisely. In particular, among the different hypotheses suggested in order to explain the effect of metabolic stress, we can now indicate ADP, instead of GDP, as the main factor coupling energy depletion to I_{CRAC} inhibition. At the same time, we can exclude that changes in ATP concentration, within a physiological range, can have a direct modulatory role on the current. In fact, within the first 30 s of intracellular perfusion, varying the ATP concentrations, from 0 to 2 mM, did not modify significantly the current activated by $InsP_3$. Although under these conditions the equilibrium between the pipette solution and the cytosol is not complete, we can reasonably assume that the cytosolic ATP concentration qualitatively reflects that of the intracellular perfusion buffer. These data obviously do not exclude that intracellular ATP may affect I_{CRAC} indirectly, by acting on either store-refilling or through kinases/phosphates (30–32). However, the affinity of the SERCAs and of the kinases for the nucleotide are in the μM range and it is unlikely that the ATP

concentration can decrease to such low levels under physiological conditions.

Since inhibition induced by ADP is detectable only at the beginning of cell perfusion when equilibration is not yet complete, the effective concentration of ADP remains unknown. Nonetheless, the physiological intracellular concentration of free ADP is very low, between 30 and 50 μM, and the concentration routinely employed in this study (0.5 mM) is large enough to reflect the 5–10 times changes that occur under metabolic stress (33).

Modulation by adenine nucleotides is not a peculiarity of CRAC channels. Several plasma membrane and intracellular channels are known to be sensitive to ATP and/or ADP. Among them, the ATP-dependent K^+ channels (K_{ATP}) expressed in pancreatic β cells, cardiac myocytes, and neurons, and the CFTR Cl^- channels expressed in many epithelial cells are the best characterized (41, 47). However, the effect of ADP on I_{CRAC} is quite different from that of ATP or ADP on the other channels. For example, the control of CFTR or K_{ATP} channels by adenine nucleotides is observed also in the excised inside-out configuration of the patch-clamp technique, while that of ADP on I_{CRAC} is lost after a few tens of seconds of intracellular dialysis. Indeed, a direct binding of the nucleotides to the channels themselves, or to closely associated membrane proteins, has been demonstrated in the case of CFTR and K_{ATP} channels, respectively. In the case of CRAC channels, our data suggest a more indirect mechanism of regulation by adenine nucleotides.

Another unique feature of I_{CRAC} inhibition by ADP is its temperature dependence, a characteristic which distinguishes it from all other mechanisms of I_{CRAC} modulation. A Q_{10} factor, ranging from 3 to 5, suggests the involvement of a high energy barrier in this inhibitory pathway. The concomitant requirement for a low Ca^{2+} buffering capacity indicates that Ca^{2+} is also involved in the process. In this respect, both the amount of Ca^{2+} buffering and its kinetic characteristics appear relevant. The simplest interpretation of these findings is that the ADP effect results from an increase of the known inhibitory effects of Ca^{2+} on I_{CRAC} (18, 31, 40). However, unlike the fast inactivation, discussed in detail by Zweifach and Lewis (40), that induced by ADP is quantitatively similar at different voltages and it is abolished not only by buffering with high and low BAPTA, but also with high EGTA, suggesting that the Ca^{2+} binding sites underlying ADP modulation are farther from the channel mouth than those linked to fast inactivation (40).

Finally, the need to study I_{CRAC} at physiological temperature has unraveled other important characteristics of this current. In particular, at 37 °C, the amplitude of the current is only slightly increased with respect to 25 °C, while the time constant to reach the peak current is significantly shorter. A weak temperature dependence of current amplitude is not unusual among ion channels. This was, however, not easily predictable in the case of I_{CRAC}, a current which is thought to be activated by an intracellular second messenger, synthesized or released upon depletion of intracellular Ca^{2+} stores (13, 20). In turn, this finding suggests that either the messenger(s) activating the current is produced (or released) via a process weakly sensitive to temperature and/or that its steady-state concentration, even at 25 °C, is sufficient to activate the current maximally. The decrease in time constant at higher temperature could be in part attributed to a faster rate of production of the activatory messenger, but a role for Ca^{2+} on this parameter may be suggested by the slowdown effect exerted by BAPTA at higher concentration. This problem is presently under investigation. Last, but not least, increasing the temperature from 25 °C to 37 °C also reduced the delay before current activation, about twice as short. No striking difference was observed when comparing equal concentrations of EGTA and BAPTA; however, the higher the buffer concentration, the shortest the delay, suggesting that Ca^{2+} ions exert an inhibitory action at some step during current recruitment. In the presence of ADP, the delay and the time constant were not significantly modified.

In summary, we have shown here that the energy status of the cell directly modulates I_{CRAC} through a Ca^{2+}- and temperature-dependent mechanism. This modulation depends primarily, or exclusively, on the intracellular ADP level. Our findings represent a further example of the key role played by this adenine nucleotide in coupling cell metabolism to the signal transduction pathways at the plasma membrane level.

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Intracellular ADP Modulates the Ca Release-activated Ca Current in a Temperature- and Ca-dependent Way

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