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Cytosolic Nucleotides Enhance the Tolbutamide Sensitivity of the ATP-Dependent K^+ Channel in Mouse Pancreatic B Cells by Their Combined Actions at Inhibitory and Stimulatory Receptors

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SUMMARY

In the plasma membrane of pancreatic B cells, a K^+ channel (K-ATP channel) has been identified that is regulated by cytoplasmic nucleotides. This channel is inhibited by sulfonylureas. We have previously shown that the potency of tolbutamide is much lower in excised membrane patches than in intact cells, unless the internal side of the membrane is exposed to the Mg^{2+} complex of ADP (MgADP). In the present study, the mechanism of this interactive control by sulfonylureas and nucleotides was examined using the inside-out configuration of the patch-clamp technique. When test solutions containing Mg^{2+} ions were applied, the opening activity of the K-ATP-channels was strongly stimulated by 2′-deoxyadenosine-5′-diphosphate (dADP) or GDP, slightly stimulated by ADP, and inhibited by adenosine-5′-*O*-(2-thiodiphosphate) (ADPβS) or adenylyl-imidodiphosphate (AMP-PNP). In the presence of Mg^{2+}, not only ADP but also its analogues dADP (1 mM) and ADPβS (0.1 mM) enhanced the potency of tolbutamide for channel inhibition; dADP at a low concentration (0.2 mM), GDP (0.2–1 mM), and AMP-PNP (0.2 mM) did not alter the potency of tolbutamide. The particular feature of the test solutions that enhanced the potency of tolbutamide was the presence of Mg^{2+}-bound and free nucleotides at channel-stimulating and channel-inhibiting concentrations, respectively. In the presence of Mg^{2+} and 0.2 mM dADP or 0.2–1 mM GDP, 0.2 mM AMP-PNP intensified the response to tolbutamide by serving as channel-inhibiting component. MgAMP-PNP did not stimulate the opening activity of the K-ATP channel. The sensitivity to tolbutamide that was enhanced by a submaximally effective ADP concentration was further increased by AMP-PNP or ATP but not by GDP. The sensitivity to the sulfonylurea analogue meglitinide was also enhanced by ADP. It is concluded that nucleotides inhibit and activate the K-ATP channel by interaction with two separate receptor sites at the cytoplasmic face of the B cell membrane. Effective inhibition of the channel openings by sulfonylureas results from the simultaneous occupation of both sites by appropriate nucleotides.

In the plasma membrane of pancreatic B cells, a K^+ channel has been identified that is closed when the B cells are exposed to glucose or other insulin-releasing fuels (1–3). The closure of this channel depolarizes the B cell to the threshold at which voltage-dependent Ca^{2+} channels open. The resultant increase in the cytosolic concentration of free Ca^{2+} ions stimulates insulin release (for review, see Ref. 4). It is believed that the cytosolic ATP/ADP ratio is the second messenger linking fuel metabolism and K^+ channel inhibition (4). Application of ATP or some other nucleotides to the cytoplasmic face of excised membrane patches of insulin-secreting cells decreases the opening activity of the characteristic K^+ channel (K-ATP channel) (2, 3, 5). This effect does not require the presence of Mg^{2+} and is not caused by protein phosphorylation (4, 5). In addition to the inhibitory site on the channel or an associated regulatory protein, there seem to exist at least two other sites by which cytosolic nucleotides control the activity of the K-ATP channel in B cells. 1) ATP prevents the rapid decline in channel activity observed in inside-out membrane patches in the absence of ATP (3, 6, 7). Because ATP cannot be replaced by nonhydrolyzable ATP analogues and requires the presence of Mg^{2+}, the Mg^{2+} complex of ATP (MgATP) might act by serving as substrate for protein kinases closely associated with the B cell plasma membrane. 2) In the presence of Mg^{2+}, but not in its absence, application of ADP or GDP to inside-out patches increases the opening activity of the K-ATP channel (3, 8–11). The mechanism of this effect is unclear.

Tolbutamide or other sulfonylureas block the K-ATP channel by direct interaction with the plasma membrane of insulin-secreting cells (12–20). Sulfonylureas are effective when applied to either the extracellular or the intracellular face of the mem-

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ABBREVIATIONS: K-ATP channel, ATP-dependent K^+ channel; dADP, 2′-deoxyadenosine-5′-diphosphate; AMP-PNP, adenylyl-imidodiphosphate; ADPβS, adenosine-5′-*O*-(2-thiodiphosphate); AMP-CP, α,β-methylene adenosine-5′-diphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

brane. In intact B cells the potency of tolbutamide for inhibition of the K-ATP channels is more than 10-fold higher than that in inside-out membrane patches exposed to nucleotide-free bath solutions (21, 22). However, application of the Mg^{2+} complex of ADP (MgADP) to the cytoplasmic side of excised B cell membranes strongly enhances the tolbutamide sensitivity of the membranes (21, 22). This finding suggests that cytosolic MgADP controls the tolbutamide sensitivity by interaction with a receptor site identical to the aforementioned site involved in MgADP-induced channel activation. In support of this idea, inhibition of K-ATP channel activity by ATP, ADP (in the absence of Mg^{2+}), or their nonhydrolyzable analogues AMP-PNP and AMP-CP is not accompanied by enhancement of the tolbutamide sensitivity (22).

The aim of the present investigation was to gain further insight into the mechanism whereby ADP and related nucleotides regulate the tolbutamide sensitivity of the K-ATP channels in B cells. We also have sought additional evidence that the channel-opening effect of MgADP is not due to interaction with the nucleotide receptor mediating channel closure. To address these issues, we performed patch-clamp experiments with inside-out membrane patches from pancreatic B cells. The tolbutamide sensitivity of the K-ATP channel was examined in the presence of nucleotides structurally related to ADP.

Materials and Methods

Isolation and culture of pancreatic B cells. Pancreatic B cells were isolated from male albino mice (NMRI, 11–15 weeks old, fed *ad libitum*) and cultured for 1–4 days as previously described (22), except that RPMI 1640 tissue culture medium (10 mM D-glucose) was used. The medium was supplemented with penicillin (100 units/ml), streptomycin (0.1 mg/ml), and fetal calf serum (10%).

Electrophysiological recording and analysis. The inside-out configuration of the patch-clamp technique (23) was used to record currents flowing through K-ATP channels, using a set-up previously described for our laboratory (22). All experiments were performed at room temperature (20–22°). The pipette potential was held at +50 mV. Inward membrane K^+ currents are indicated as downward deflections in all current traces. The bath was perfused continuously at 2 ml/min. The cytoplasmic face of the membrane patch was exposed to test pulses of bath solution (supplemented with or without test substances) for 10-sec intervals, alternating with 30-sec pulses of bath solution containing 1 mM ATP, applied by a microflow system (7). For periods of 2.5–3-min duration, the test pulses of bath solution contained various nucleotides at different concentrations and/or tolbutamide (30 or 100 μM) or meglitinide (5 μM). The mean of the amplitudes of the current responses (current amplitudes) during these periods was determined. The single-channel current amplitudes of the K-ATP channels were not changed by tolbutamide (30 or 100 μM), meglitinide (5 μM), or the tested nucleotide concentrations. Before and after application of tolbutamide- or meglitinide-containing bath solution, there were control periods during which the same solution was applied except that tolbutamide and meglitinide were omitted. The mean of the amplitudes of the current responses during the four pulses (10 sec) of bath solution before and after application of tolbutamide or meglitinide was set to 100% (control).

Recordings were made using an LM-EPC 7 patch-clamp amplifier (List Electronic, Darmstadt, Germany). Current signals were stored on magnetic tape (Store 4; Racal Recorders, Hythe, UK), at 1.875 inch/sec (bandwith, 0.5 kHz; −3-dB point), and in a microcomputer (Compaq 386/20) (digitization at 2 kHz), using an Axolab 1100 computer interface (Axon Instruments, Burlingame, CA). Analysis of data was performed with the program pCLAMP 5.5 (Axon Instruments). For the figures, taped data were replayed into a chart recorder (model 220; Gould, Cleveland, OH), which filtered the data at 140 Hz.

Chemicals and solutions. Tolbutamide, 8-hydroxyquinoline, *N*-ethylmorpholine, and Na_2dADP were obtained from Sigma (St. Louis, MO). Na_2ATP, K-ADP, Li_4AMP-PNP, Li_2GDP, and Li_3ADPβS were from Boehringer (Mannheim, Germany). All other chemicals were obtained from the sources described elsewhere (20). The solution at the cytoplasmic side of the membrane (bath solution) contained (in mM) 140 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 EGTA, and 5 HEPES (titrated to pH 7.15 with KOH) (free $[Ca^{2+}]$ = 0.05 μM). Unless stated otherwise in Results, the free Mg^{2+} concentration was held close to 0.7 mM by addition of appropriate amounts of $MgCl_2$ to the nucleotide-containing solutions. The required amounts of $MgCl_2$ and the composition of the solutions for pH 7.15 were calculated with a computer program (24). The stability constants detailed below were used instead of the constants of the program. After addition of 1 mM Na_2ATP, the bath solution was also used for filling the U-shaped polythene capillary of the microflow system (7). The pH of all solutions was controlled after addition of tolbutamide, meglitinide, or any nucleotides and was readjusted to 7.15 if necessary. The pipette solution contained (in mM) 146 KCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, and 10 HEPES (titrated to pH 7.40 with KOH).

Stability constants. The logarithms of the absolute stability constants (log *K*) for binding of H^+, Ca^{2+}, and Mg^{2+} to EGTA, ATP, or ADP (except for formation of $MgADP^-$) were taken from Refs. 25, 26, or 27, respectively. The corresponding log *K* values for AMP-PNP were calculated from the values for ATP (26) and the differences between the values for ATP and AMP-PNP (28, 29). Because the published log *K* values were measured at 0.1 M ionic strength, all log *K* values were corrected for the ionic strength of the bath solution (0.17 M) (30). Ionic strength was expressed in terms of ionic equivalents rather than formal ionic strength. The log *K* values for all proton-ligand complexes were corrected by 0.126, in order to include the proton activity coefficient appropriate for the bath solution (31).

The log *K* value for the first protonation (5.2) (32) was the only log *K* value for ADPβS found in the literature. Therefore, we measured the stability constant for $MgADP\beta S^-$ using the spectral changes accompanying the binding of Mg^{2+} to 8-hydroxyquinoline (33). For comparison, the $MgADP^-$ complex was studied under identical experimental conditions. In place of the other stability constants for ADPβS, the corresponding values for ADP were used as maximum values, because the replacement of an oxygen by sulfur destabilizes the complexes (34). The error is negligible in the calculation of the composition of our bath solutions.

Spectral measurements (at 360 nm and 22°) were carried out in a 1-cm light-path cuvette containing 3 ml of a solution with 0.2 mM 8-hydroxyquinoline, 150 mM KCl, and 50 mM *N*-ethylmorpholine (titrated to pH 8.0 with HCl). Titration of 8-hydroxyquinoline with $MgCl_2$, in the absence and presence of ADPβS (1–3 mM) or ADP (1–3 mM), and analysis were carried out as described (33). The log *K* value for binding of Mg^{2+} to completely deprotonated ADPβS or ADP was found to be 2.47 or 3.16, respectively. These values are the means of three separate determinations and have been used without corrections.

The nature of the ribose or the purine base moiety is generally believed to have little to do with the binding of metal ions to nucleoside di- and triphosphates (<5 mM) (35, 36). No significant binding of Mg^{2+} to the ribose and base moieties of ADP or ATP occurs (37). Therefore, the log *K* values for ADP were also used for dADP and GDP.

Treatment of results. Values are presented as mean ± standard error. Significances were calculated by the two-tailed *U* test of Wilcoxon and of Mann, and Whitney and by the two-tailed matched-pairs signed rank test of Wilcoxon. $p < 0.05$ was considered significant. Relations between nucleotide concentrations and inhibitory effects were analyzed by fitting the function

$$E = 1 - \frac{[A]^n}{EC_{50}^n + [A]^n}$$

to the experimental data by a nonlinear least-squares routine, with E = normalized effect in the presence of test substance, $[A]$ = concentra-

tion of test substance, EC_{50} = half-maximally effective concentration, and n = slope parameter (Hill coefficient).

Results

We have previously observed that cytosolic MgADP strongly enhances the sensitivity to tolbutamide of the K-ATP channels in B cell membranes (21, 22). To test whether the typical sulfonylurea receptor is involved in these effects, we performed experiments with meglitinide (HB 699). Meglitinide is a benzoic acid derivative that is structurally related to glyburide and, like the sulfonylureas, binds to the sulfonylurea receptor, inhibits the K-ATP channel activity, and stimulates insulin secretion (16, 20, 38). The example in Fig. 1 illustrates that the sensitivity to meglitinide, too, was enhanced by ADP in the presence of Mg^{2+}. In six similar experiments, the current amplitude (expressed as percentage of control) was 44.5 ± 4.0% in the presence of meglitinide (5 μM) and 19.1 ± 2.5% in the presence of both meglitinide (5 μM) and ADP (0.1 mM). These mean values were significantly different.

Enhancement of the potency of tolbutamide or meglitinide was induced by MgADP in the presence of 0.7 mM free Mg^{2+}. Therefore, we wanted to know whether this effect of MgADP depends on the concentration of free Mg^{2+}. Table 1 shows that increasing concentrations of free Mg^{2+} (0.32–1.52 mM) did not alter the tolbutamide sensitivity of excised inside-out B cell membranes exposed to a maximally effective concentration of total ADP (1 mM) (22).

A number of nucleotides structurally related to ADP were used to gain insight into the requirements of the receptor controlling the tolbutamide sensitivity of the K-ATP channel. The role of the ribose moiety was examined with dADP. The example in Fig. 2 demonstrates that 0.2 mM dADP enhanced the channel activity by 82% (compare Fig. 2, *traces B* and *C*). At this concentration, dADP did not alter the inhibitory effect of tolbutamide on the K-ATP channel (Fig. 2; Table 2, series A). However, 1 mM dADP increased both the channel activity (by 264%) (Fig. 3) and the channel-blocking potency of tolbutamide (Table 2, series B). The role of the base moiety was tested with GDP. In accordance with previous results (21), GDP (0.2 and 1 mM) did not affect the tolbutamide sensitivity of the K-ATP channel (Table 2, series C and D), even though GDP activated the K-ATP channel as much as dADP (compare

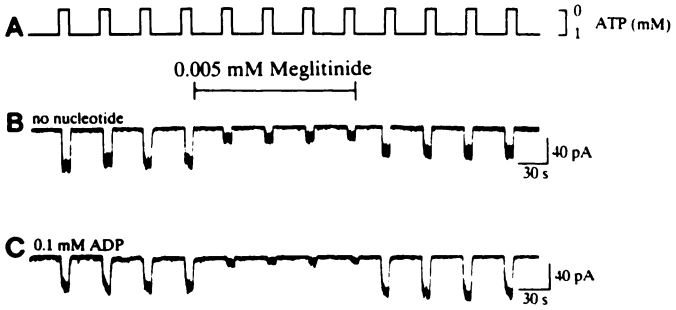

Fig. 1. Effect of ADP on meglitinide-induced inhibition of K-ATP channels in an inside-out patch of a mouse pancreatic B cell. *Trace A*, schematic protocol of solution exchange at the inside of the patch. ATP (1 mM) was present at the low level and absent at the high level of this trace. *Traces B* and *C*, current traces are shown in the sequence of recording during the experiment. The inside-out patch was exposed to no ADP (*trace B*) or 0.1 mM ADP (*trace C*) during the pulses of ATP-free bath solutions, which were also supplemented with meglitinide (5 μM) as indicated by the *horizontal bar above traces B and C*. In this experiment, meglitinide inhibited the channel activity by 64% (*trace B*) and 88% (*trace C*).

TABLE 1

Effects of ADP on tolbutamide-induced inhibition of K-ATP channel activity in excised inside-out patches from mouse B cells

Each group consisted of six experiments using different membrane patches and testing the effects of tolbutamide (0.03 mM) in the presence of the indicated concentrations of total Mg^{2+} (Mg_T), free Mg^{2+} (Mg_F), free ADP (ADP_F), and Mg^{2+} complex of ADP (MgADP) (for experimental design, see Fig. 1C). The control (100%) was the mean of the amplitudes of the current responses during the four pulses (10 sec) of the corresponding bath solution before and after application of tolbutamide. Results are presented as mean ± standard error.

Group	Test substances				n	Current amplitude in the presence of tolbutamide
	Mg_T	Mg_F	ADP_F	MgADP		
	mM					% of control
A	2.60	1.52	0.35	0.65	6	17.4 ± 3.6
B	1.40	0.72	0.54	0.46	6	19.5 ± 2.4
C	0.70	0.32	0.72	0.28	6	17.6 ± 4.1

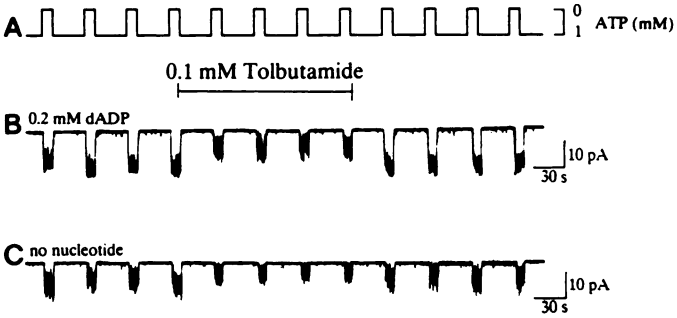

Fig. 2. Effect of dADP on tolbutamide-induced inhibition of K-ATP channels in an inside-out patch of a mouse pancreatic B cell. *Trace A*, schematic protocol of solution exchange, as in Fig. 1. *Traces B* and *C*, current traces are shown in the sequence of recording during the experiment. The inside-out patch was exposed to 0.2 mM dADP (*trace B*) or no dADP (*trace C*) during the pulses of ATP-free bath solutions, which were also supplemented with tolbutamide (0.1 mM) as indicated by the *horizontal bar above traces B and C*. In this experiment, tolbutamide inhibited the channel activity by 50% (*trace B*) and 51% (*trace C*).

TABLE 2

Effects of adenine and guanine nucleotides on tolbutamide-induced inhibition of K-ATP channel activity in excised inside-out patches from mouse B cells

Each experimental series consisted of n experiments using different membrane patches and testing the effects of tolbutamide (0.1 mM in series A–E; 0.03 mM in series F) with each patch in the absence and presence of nucleotide (for experimental design, see Fig. 2). The control (100%) was the mean of the amplitudes of the current responses during the four pulses (10 sec) of bath solution before and after application of tolbutamide. Results are presented as mean ± standard error.

Series	Nucleotide	n	Current amplitude in the presence of tolbutamide
	mM		% of control
A	None	8	46.2 ± 3.0
	dADP, 0.2		41.5 ± 3.5
B	None	6	51.2 ± 4.2
	dADP, 1		33.3 ± 4.1[a]
C	None	8	43.0 ± 2.9
	GDP, 0.2		39.7 ± 3.1
D	None	5	37.6 ± 7.4
	GDP, 1		44.8 ± 8.4
E	None	6	50.9 ± 4.4
	ADPβS, 0.1		28.6 ± 4.5[a]
F	None	6	46.1 ± 5.2
	AMP-PNP, 0.2		39.2 ± 7.4

[a] Significantly different from the other group of the series.

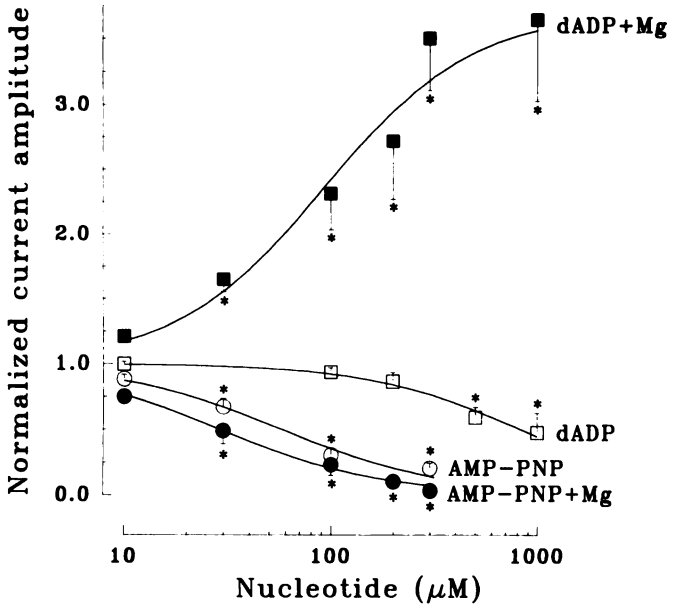

Fig. 3. Concentration-dependent effects of dADP (*squares*) and AMP-PNP (*circles*) on K-ATP channels in inside-out patches of mouse pancreatic B cells. The patches were exposed to pulses of ATP-free bath solutions, which contained no Mg^{2+} (*open symbols*) or 0.7 mM free Mg^{2+} (*closed symbols*) and were supplemented with the indicated total concentrations of nucleotide (logarithmic scale), for periods of 3-min duration. The mean of the amplitudes of the current responses during application of nucleotide was normalized to the mean of the amplitudes during the control periods (no nucleotide) before and after application of nucleotide in each single experiment. *Data points* represent the means ± standard errors for 3–17 separate experiments. The *curve* through the data points for dADP in the presence of Mg^{2+} (■) was fitted by eye. The other *curves* were fitted as described in Materials and Methods. The following values were obtained for EC_{50} values and Hill coefficients: 0.06 mM and −1.09 (○), 0.03 mM and −1.13 (●), 0.83 mM (□), and 0.09 mM (■). *, Differs significantly from the corresponding control (no nucleotide).

Table 3, groups A and B, with Fig. 3). The role of the phosphate chain of ADP was examined with 0.1 mM ADPβS and 0.2 mM AMP-PNP, which inhibited the K-ATP channel activity by 65% (Table 3, group D) and 90% (Fig. 3), respectively. The inhibitory effect of ADPβS, but not that of AMP-PNP, was accompanied by enhancement of the tolbutamide sensitivity of the K-ATP channel (Table 2, series E and F).

Three points led us to analyze whether enhancement of the tolbutamide sensitivity might require both inhibitory and stimulatory actions of nucleotides at separate receptors. Firstly, the overall effects of nucleotides on the activity of the K-ATP channel did not correlate with their effects on the tolbutamide sensitivity. Secondly, enhancement of the tolbutamide sensitivity could be obtained both with a channel-inhibiting nucleotide (ADPβS) and with a channel-activating nucleotide (dADP). Thirdly, bath solution supplemented with Mg^{2+} and ADP contained both free ADP, which inhibited the channel, and MgADP, which activated the channel (see Refs. 4 and 22 and Table 3, group G). We calculated that our bath solution supplemented with Mg^{2+} and 1 mM dADP or GDP contained 0.54 mM free nucleotide. When tested at 0.5 mM concentration in the absence of Mg^{2+}, dADP significantly inhibited the activity of the K-ATP channel (Fig. 3). In the absence of Mg^{2+}, GDP (0.5 mM) caused a slight but not significant stimulation of the channel (Table 3, group C). It has been reported previously that 0.1 mM ADPβS inhibited the K-ATP channels of insulin-secreting cells both in the absence and in the presence of Mg^{2+}

TABLE 3

Effects of adenine and guanine nucleotides on the K-ATP channel activity in excised inside-out patches from mouse B cells

Each group consisted of *n* experiments using different membrane patches and testing the effects of nucleotides with an experimental design similar to that in Fig. 1B. In each group, the control (100%) was the mean of the amplitudes of the current responses during the four pulses (10 sec) of nucleotide-free bath solution before and after application of nucleotide(s). In groups C, E, and F, the bath was perfused with solutions from which Mg^{2+} was omitted. Results are presented as mean ± standard error.

Group	Nucleotide(s)	*n*	Current amplitude in the presence of nucleotide(s)
	mM		*% of control*
A	GDP, 0.2	14	250.4 ± 38.6[a]
B	GDP, 1.0	11	352.5 ± 58.9[a]
C	GDP, 0.5; no Mg^{2+}	6	118.2 ± 4.8
D	ADPβS, 0.1	6	34.7 ± 6.5[a]
E	ADPβS, 0.05; no Mg^{2+}	6	38.8 ± 4.5[a]
F	ADPβS, 0.08; no Mg^{2+}	8	29.8 ± 4.1[a]
G	ADP, 0.1	29	166.6 ± 14.5[a]
H	dADP, 0.2, + AMP-PNP, 0.05	6	136.2 ± 11.8
I	dADP, 0.2, + AMP-PNP, 0.2	6	59.1 ± 4.8[a]
K	GDP, 0.2, + AMP-PNP, 0.2	6	52.2 ± 6.0[a]
L	GDP, 1.0, + AMP-PNP, 0.2	6	184.4 ± 31.9[b]
M	ADP, 0.1 + AMP-PNP, 0.2	6	59.2 ± 3.7[a]
N	ADP, 0.1, + ATP, 0.02	6	142.5 ± 30.0
O	ADP, 0.1, + ATP, 0.1	7	41.7 ± 7.4[a]
P	ADP, 0.1, + GDP, 1.0	10	279.2 ± 31.7[a,c]

[a] Significantly different from the control of the group.
[b] Significantly different from group K.
[c] Significantly different from group G.

(10). In our bath solution containing Mg^{2+} and 0.1 mM ADPβS, 80% of ADPβS was free. We found 80 μM ADPβS to be slightly more inhibitory in Mg^{2+}-free solution than 100 μM ADPβS in Mg^{2+}-containing solution (Table 3, groups D and F). This observation is consistent with the idea that channel activation induced by MgADPβS partly antagonized the inhibitory effect of free ADPβS. However, in the case of AMP-PNP, the Mg^{2+}-bound nucleotide was even more inhibitory on the K-ATP channel than the free nucleotide (see Refs. 22 and 39 and Fig. 3).

The data presented so far support the view that enhancement of the tolbutamide sensitivity of the K-ATP channel requires the presence of a nucleotide that is effective at both inhibitory and stimulatory receptor sites of the B cell membrane. As a further test of this interpretation, we exposed inside-out patches to combinations of channel-inhibiting and channel-stimulating nucleotides. In conjunction with 0.2 mM dADP, AMP-PNP (0.05–0.2 mM) enhanced the tolbutamide sensitivity (Fig. 4; Table 4, series A and B). At these concentrations, both dADP and AMP-PNP were ineffective on the tolbutamide sensitivity when tested alone (Fig. 2; Table 2, series A and F). AMP-PNP enhanced the tolbutamide sensitivity of the K-ATP channel also in conjunction with GDP (Table 4, series C and D). Moreover, ADP-induced enhancement of the tolbutamide sensitivity was further increased by AMP-PNP (0.2 mM) or ATP (0.1 mM) but not by GDP (1 mM) (Table 4, series E and G–I). There was a trend that GDP even weakened the effect of ADP.

Discussion

In excised inside-out membrane patches of B cells, the opening activity of the K-ATP channels declines rapidly (run-down of the channels) unless cytosolic MgATP is applied (4). Phos-

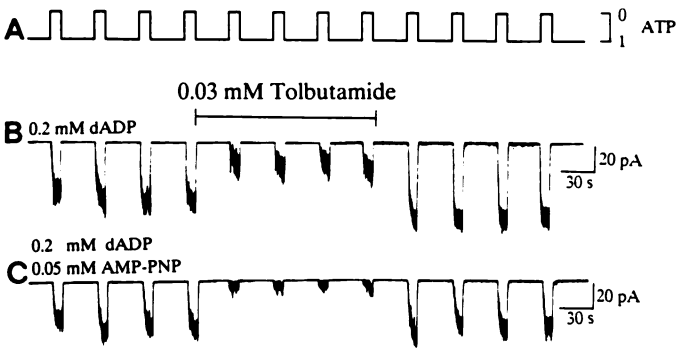

Fig. 4. Combined effect of dADP and AMP-PNP on tolbutamide-induced inhibition of K-ATP channels in an inside-out patch of a mouse pancreatic B cell. *Trace A*, schematic protocol of solution exchange, as in Fig. 1. *Traces B* and *C*, current traces are shown in the sequence of recording during the experiment. The inside-out patch was exposed to 0.2 mM dADP (*trace B*) or 0.2 mM dADP plus 0.05 mM AMP-PNP (*trace C*) during the pulses of ATP-free bath solutions, which were also supplemented with tolbutamide (0.03 mM) as indicated by the *horizontal bar above traces B and C*. In this experiment, tolbutamide inhibited the channel activity by 62% (*trace B*) and 86% (*trace C*).

TABLE 4

Combined effects of adenine and guanine nucleotides on tolbutamide-induced inhibition of K-ATP channel activity in excised inside-out patches from mouse B cells

Each experimental series consisted of *n* experiments using different membrane patches and testing the effects of tolbutamide (0.03 mM in series A–E, G, and H; 0.1 mM in series F and I) with each patch in the presence of one and two nucleotides (for experimental design, see Fig. 4). The control (100%) was the mean of the amplitudes of the current responses during the four pulses (10 sec) of bath solution before and after application of tolbutamide. Results are presented as mean ± standard error.

Series	Nucleotide(s)	*n*	Current amplitude in the presence of tolbutamide
	mM		% of control
A	dADP, 0.2	6	36.1 ± 3.5
	dADP, 0.2, + AMP-PNP, 0.05		20.2 ± 2.5[a]
B	dADP, 0.2	6	44.4 ± 2.4
	dADP, 0.2, + AMP-PNP, 0.2		10.1 ± 1.4[a,b]
C	GDP, 0.2	6	52.5 ± 5.4
	GDP, 0.2, + AMP-PNP, 0.2		24.9 ± 3.4[a]
D	GDP, 1	6	60.2 ± 5.7
	GDP, 1, + AMP-PNP, 0.2		23.4 ± 4.9[a]
E	ADP, 0.1	6	30.8 ± 6.9
	ADP, 0.1, + AMP-PNP, 0.2		11.0 ± 1.9[a]
F	ADP, 0.1	6	10.5 ± 0.7
	ADP, 0.1, + ATP, 0.02		10.9 ± 1.0
G	ADP, 0.1	7	32.9 ± 7.2
	ADP, 0.1, + ATP, 0.1		16.8 ± 2.7[a]
H	ADP, 0.1	5	26.0 ± 2.9
	ADP, 0.1, + GDP, 1		39.1 ± 7.3
I	ADP, 0.1	5	17.4 ± 1.6
	ADP, 0.1, + GDP, 1		24.0 ± 3.5

[a] Significantly different from the other group of the series.
[b] Significantly different from dADP, 0.2 mM, + AMP-PNP, 0.05 mM, in series A.

phorylation of membrane proteins appears to be necessary to maintain the available channels. In the experiments performed in our study, 0.86 mM MgATP (1 mM total ATP) was present as substrate for protein kinases except for 10-sec test periods. During these periods, significant run-down of the channels was not observed (7). The results of our study thus mainly reflect the function of the phosphorylated states of the K-ATP channel and/or regulatory proteins.

The data documented here support the previous conclusion that a distinct receptor at the cytoplasmic face of the B cell membrane mediates activation of the K-ATP channel by the Mg^{2+} complexes of ADP and some related nucleoside diphosphates (4, 10). This receptor is obviously stimulated by dADP too. The failure to demonstrate activation of the channel by ADPβS in the presence of Mg^{2+} is probably due to the small percentage of Mg^{2+} complex formed in our bath solution. The response to MgADPβS seems to be concealed by the strong channel-inhibiting effect of free ADPβS. This interpretation is supported by the observation that ADPβS (in the presence of Mg^{2+}) opened K-ATP channels blocked by ATP (11). Unlike nucleotide-induced channel activation, channel inhibition does not require the presence of Mg^{2+} (4). Moreover, structure-activity relationships, too, argue in favor of separate receptor sites for activation and inhibition. Firstly, MgAMP-PNP does not activate but inhibits even more strongly than does free AMP-PNP (Fig. 3) (39). Secondly, 0.1 mM dADP and 0.5 mM GDP do not inhibit in the absence of Mg^{2+} but strongly activate in the presence of Mg^{2+} (Fig. 3; Table 3).

Cytoplasmic ADP enhances the sensitivity of the K-ATP channel to both tolbutamide and meglitinide (21, 22) (this study). Therefore, it seems likely that the sulfonylurea receptor that is characteristic of the K-ATP channel is involved in these actions of ADP. The effects of ADP are only seen in the presence of Mg^{2+} (22) and do not depend critically on the concentration of free Mg^{2+} ions (this study). Hence, these findings indicate that ADP-induced enhancement of the sulfonylurea sensitivity requires the formation of MgADP.

MgADP is not sufficient for potent inhibition of the K-ATP channel by sulfonylureas. The evidence for this stems from experiments with ADP analogues. The sulfonylurea sensitivity is not enhanced in bath solutions containing channel-activating concentrations of MgdADP or MgGDP but lacking channel-inhibiting concentrations of free nucleotides. However, these solutions become effective by further addition of AMP-PNP. When applied alone, AMP-PNP does not enhance the sulfonylurea sensitivity of the K-ATP channel. Moreover, AMP-PNP or ATP increases the response to sulfonylureas observed in the presence of a submaximally effective ADP concentration. These results strongly suggest that nucleotides must occupy both channel-activating and channel-inhibiting receptors in order to enhance the sulfonylurea sensitivity. This interpretation readily explains why ADP, dADP (at 1 mM concentration), and ADPβS enhance the sulfonylurea sensitivity when tested as individual nucleotides.

Maximum effectiveness of sulfonylureas does not require maximum effects of channel-activating Mg^{2+}-nucleotide complexes. Firstly, in the presence of 0.2 mM AMP-PNP, there is no difference between the effects of 0.2 or 1 mM GDP on the sulfonylurea sensitivity, although 0.2 mM GDP activates the K-ATP channel much less than does 1 mM GDP (Tables 3 and 4). Secondly, in the presence of 0.1 mM ADP, channel activity, but not sulfonylurea sensitivity, is enhanced by GDP. However, maximum effectiveness of sulfonylureas (80–90% inhibition of channel activity by 0.03 mM tolbutamide; see Ref. 22 and Tables 1 and 4) seems to require occupation of nearly all nucleotide receptors mediating channel inhibition. Firstly, 90% inhibition of the K-ATP channel by 0.2 mM AMP-PNP coincides with stronger enhancement of the sulfonylurea sensitivity (in the presence of MgdADP) than does 65% inhibition by 0.05 mM AMP-PNP (Fig. 3; Table 4). Secondly, in the presence of Mg^{2+}, 0.3 mM ADP is required for maximum sulfonylurea sensitivity

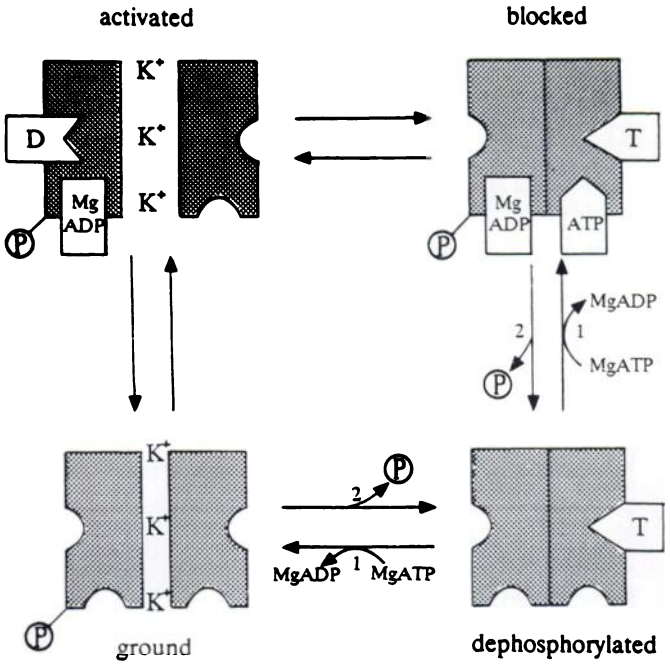

Fig. 5. Simplified model for regulation of the K-ATP channel by nucleotides, tolbutamide (*T*), and diazoxide (*D*). The protein assembly organizing the K-ATP channel is thought to exist in four conformations. In the activated state, the channel opens much more frequently than in the ground state. The activated state is stabilized by MgADP and diazoxide. The nonconductive blocked state is stabilized by free ATP and tolbutamide. Maximum effectiveness of tolbutamide requires the simultaneous binding of both MgADP and free ATP. Ground, activated, and blocked state are conformations of the phosphorylated (*P*) K-ATP channel. It is suggested that an imbalance between protein kinase (*1*) and phosphatase (*2*) reactions induces the dephosphorylated state. This state shows affinity for tolbutamide but not for diazoxide and remains nonconductive in the absence of any ligands.

(22). The ADP concentration (0.16 mM) free under these conditions inhibits the K-ATP channel by 80–90%.

Fig. 5 summarizes how we envisage the regulation of the K-ATP channel by nucleotides and drugs. It is assumed that the binding sites for ligands are located on the proteins organizing the K^+ channel. However, it may well be that the ligands bind to regulatory proteins not permanently associated with the K^+ channel. In excised inside-out membrane patches from B cells, the run-down of the K-ATP channel is believed to indicate the formation of a dephosphorylated nonconductive state (4). Experiments with B cell microsomes suggest that the dephosphorylated state binds sulfonylureas with affinities similar to those observed in intact B cells (40). This state does not bind diazoxide. In the presence of MgATP, protein kinases induce phosphorylated states of the channel (4). In our experiments, the current amplitudes during the test pulses with ligand-free solutions reflect the opening activity of the ground state. States showing higher opening activity (activated states) can be stabilized by binding of diazoxide or MgADP (or some related Mg^{2+}-nucleotide complexes). Evidence has been presented that diazoxide-induced activation of the K-ATP channel requires phosphorylation of membrane proteins (41, 42). The reason for this seems to be that diazoxide binds only to phosphorylated receptors (40). It is unclear whether the state stabilized by diazoxide is identical to the state stabilized by MgADP. It is unlikely, however, that MgADP-induced channel activity is specific to the dephosphorylated state, as suggested for ventricular myocytes (43). This is because the present study was carried out using an experimental protocol favoring protein phosphorylation. Furthermore, in excised inside-out B cell membranes, run-down of the K-ATP channels proceeded in the presence of MgADP (7). Finally, in the experiments with myocyte membranes, phosphorylation of the K-ATP channels was not excluded (43). $MgCl_2$ plus millimolar concentrations of nucleoside diphosphates were applied to the cytoplasmic side of inside-out patches. These patches are membrane-covered blebs of myocyte cytoplasm that include organelles (44, 45). Therefore, protein phosphorylation can be produced by nucleoside diphosphates via transphosphorylation reactions, yielding nucleoside triphosphates (46).

Cytosolic ATP is able to block the K-ATP channel in the absence of any other ligand. When MgADP, too, is bound to its receptor, the conformation of the ATP-blocked state appears to be shifted to a conformation showing channel openings. This is because MgADP relieves channel block induced by maximally effective concentrations of free ADP or ATP (11, 22). Sulfonylureas, too, are able to inhibit the K-ATP channel in the absence of any other ligand, but their potency is very low. This finding may be due to inadequate binding of sulfonylureas to their phosphorylated receptor, as suggested by binding experiments with B cell microsomes (40). It may be speculated that binding of channel-blocking nucleotides (e.g., ATP) converts the channel into a state strongly binding sulfonylureas. In this case, interaction of sulfonylureas with channels already blocked by the sole binding of inhibitory nucleotides would not be reflected in alteration of channel activity. However, sulfonylureas would effectively inhibit the opening activity of the conformational state that is induced by the simultaneous binding of inhibitory and stimulatory nucleotides (Fig. 5). This hypothesis is in agreement with the aforementioned conclusion that maximum sensitivity to sulfonylureas requires occupation of nearly all inhibitory nucleotide receptors but does not require maximum effects of stimulatory nucleotides.

In summary, the present data support the view that nucleotides inhibit and activate the K-ATP channel by interaction with two separate receptor sites at the cytoplasmic face of the B cell membrane (4, 10). The two sites recognize different structural features of the nucleotides. Effective inhibition of the channel openings by sulfonylureas results from the simultaneous occupation of both sites by appropriate nucleotides.

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