Biochemical Pharmacology, Vol. 43, No. 8, pp. 1859–1864, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

RELATIONSHIPS BETWEEN ENERGY LEVEL AND INSULIN SECRETION IN ISOLATED RAT ISLETS OF LANGERHANS

MANIPULATION OF [ATP]/[ADP][P_i] BY 2-DEOXY-D-GLUCOSE

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(*Received* 21 *October* 1991; *accepted* 2 *December* 1991)

Abstract—Perifusion of islets with nominally phosphate-free buffer containing increasing concentrations of 2-deoxy-D-glucose (2.5 to 10 mM) produced increments in high α-ketoisocaproic acid-induced secretion of insulin beyond those observed in the absence of the sugar analogue. 3-*O*-methyl-D-glucose, a poorly metabolized sugar, was without effect. Insulin release evoked by 40 mM KCl was not altered by 2-deoxyglucose. The concentration of intracellular inorganic phosphate was lower in islets perifused with 2-deoxyglucose and declined to a lower level after addition of 20 mM α-ketoisocaproic acid. The enhancement of α-ketoisocaproic acid-induced hormone secretion by 2-deoxyglucose was not seen in islets perifused with medium containing 1.5 mM phosphate; instead a small inhibition was observed. It is postulated that conditions which lower intracellular [P_i] facilitate, either directly or indirectly, hormone release although the mechanism of this effect remains to be elucidated.

Recent experimental evidence suggests that a rise in the level of ATP, which closes the ATP-dependent K^+ channels and causes membrane depolarization, is an essential link between the stimulation of pancreatic islets by glucose and the subsequent release of insulin [1]. However, we have shown recently [2] that conditions can be found where secretion induced by metabolic secretagogues appears to correlate neither with [ATP] nor [ATP]/[ADP] but with the value of ΔG_{ATP} ($\Delta G_{ATP} = \Delta G'^{\circ}_{ATP} + RT \ln[ATP]/[ADP]\,[P_i]$). This intriguing observation suggests that alterations in the cytoplasmic phosphate concentration ([P_i]) may also contribute to the regulation of hormone release, because ΔG is determined not only by [ATP] and [ADP] but also by [P_i].

The effects of external phosphate on insulin secretion have been examined previously although the results obtained are not very consistent. In perfused rat pancreas, Campillo *et al.* [3] have found that omission of P_i from the medium decreases insulin secretion triggered by the addition of a high concentration of glucose but increases that caused by a high concentration of arginine [4]. In isolated islets, changes in the buffer phosphate of between 0.3 and 2 mM do not seem to influence high glucose-induced hormone release [5]. By contrast, in humans a decrease in the serum level of this anion from 1.3 to 1.03 mM is accompanied by greater increases in plasma insulin at 5, 10 and 20 min after an i.v. bolus of glucose [6]. Since P_i was not measured in any of the studies cited above, it is not clear whether, and to what extent, changes in the external concentration of the anion involve concomitant alterations in its internal level.

The object of the current study was to manipulate the concentration of intracellular phosphate directly using metabolic means, without the involvement of its transport across the plasma membrane. This was accomplished by perifusing islets with 2-deoxy-D-glucose (2-DG§), an analogue of glucose, which is phosphorylated inside cells [7, 8] and thus results in lowering of the cytoplasmic [P_i].

MATERIALS AND METHODS

Islet isolation and perifusion. Fed male Wistar rats, weighing between 200 and 250 g, were used in all studies. Animals were anesthetized with sodium pentobarbital (50 mg/kg injected i.p.). The methodology for islet isolation, perifusion studies and measurement of insulin has been published previously [9, 10].

Perifusion experiments. After 1 hr of culture in RPMI 1640 medium (supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin) in an atmosphere of humidified 95% air and 5% CO_2 at 37°, batches of 100 islets were perifused with a bicarbonate-buffered saline (pH 7.4) gassed continuously with 95% O_2/5% CO_2. The composition

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§ Abbreviations: 2-DG, 2-deoxy-D-glucose; 3-MG, 3-*O*-methyl-D-glucose; and 2-DGP, 2-DG phosphate.

of this medium was: NaCl, 115 mM; KCl, 5 mM; $CaCl_2$, 2.2 mM; $MgCl_2$, 1 mM; $NaHCO_3$, 24 mM. Bovine serum albumin, at a final concentration of 0.25%, was also added. Glucose was absent, but 2 mM lactate and 0.2 mM pyruvate (L/P) were included as the fuel.

After 35 min of perifusion, a 2 M stock solution of either 2-DG or 3-*O*-methyl-D-glucose (3-MG) was added to the perifusion medium to attain the desired concentration. At 40 min of perifusion, the islets were stimulated with 20 mM α-ketoisocaproic acid or 40 mM KCl by the addition of an appropriate amount of a concentrated stock solution (2 M and 4 M, respectively).

Analogous experiments were performed in a medium containing 1.5 mM Na_2HPO_4.

Measurement of adenine nucleotides and inorganic phosphate. Two hundred, and in most experiments, five hundred islets were perifused as described above. The preparations were quenched either after a 40-min perifusion (control, non-stimulated) or after a 5-min stimulation (experimental) using the previous method [9, 10]. Islets were snap-frozen on dry ice, and then sonicated in 2 M $HClO_4$ at −10° with two bursts of 11 pulses. The power setting and duty cycle of the sonicator (Heat Systems Sonicator model W-385, Plainview, NY) were 3 and 50%, respectively. After 10 min in the −10° bath, $HClO_4$ extracts were centrifuged at 9,000 *g* for 5 min at 4° and the supernatant was neutralized by the addition of 2.5 M K_2CO_3. The potassium perchlorate precipitate was removed by centrifugation in a Beckman Microfuge B for 2 min at 4°. Supernatants were stored at −70°.

Analytic methods. After perifusion, the filters containing adhering islets were removed from the chambers and stored at −70° for determining DNA. The islets were extracted at room temperature in a saline solution (2 M NaCl, 50 mM sodium phosphate, 1.8 mM EDTA, pH 7.4) by sonicating with 80 pulses from the sonicator with a power setting of 3 and a 50% duty cycle. DNA was measured fluorometrically with Hoechst 33258 dye and calf thymus DNA (Sigma, St. Louis, MO) as the standard [11].

Nucleotides were analyzed by HPLC as described previously [9, 10] and inorganic phosphate was estimated fluorometrically by the method of Schulz *et al.* [12]. Their concentrations were calculated using DNA content and the conversion factor of 90 pL internal islet cell water/ng of DNA [9]. In these experiments DNA was measured in two batches of at least 20 islets selected at random from each preparation.

Integration of perifusion data to determine total insulin release was performed using the trapezoid rule. Statistical analysis was performed by *t*-test or multifactor ANOVA using Macintosh Statistical System software (Statsoft, Tulsa, OK).

RESULTS

Effect of 2-DG on insulin release. The effects of 2-DG on high α-ketoisocaproate-induced insulin secretion were investigated in islets supplied with lactate/pyruvate as the fuel because 2-DG competes with glucose for transport and metabolism. Figure 1 shows that the addition of increasing concentrations of 2-DG to the perifusion medium potentiated secretion of insulin caused by 20 mM α-ketoisocaproic acid. Maximal stimulation was attained with 5–10 mM 2-DG. To evaluate the enhancement more accurately, the three phases of secretion, the basal (non-stimulated phase, 35–40 min), the acute (40–45 min), and the late (45–70 min), were quantified separately by integration, and are displayed in Table 1. It can be seen that secretion in the acute phase was increased by 68% and that in the late phase was raised by 51%. Total release, which is dominated by the late phase, was 52% greater.

The enhancement by 2-DG of α-ketoisocaproic acid-induced insulin release was specific for both the sugar analogue and the secretagogue. Addition to the perifusion medium of 10 mM 3-MG instead of 2-DG (Table 2, Fig. 2) did not increase stimulated hormone release, while replacement of α-ketoisocaproic acid with 40 mM KCl produced secretion profiles that were the same with and without 2-DG (Fig. 2).

Nucleotide levels in islets perifused with 2-DG in the absence of P_i in the medium. The concentrations of ATP and ADP were measured in islets perifused either with or without 10 mM 2-DG before and after stimulation with α-ketoisocaproic acid (Table 2). The levels of ATP were 1.47–1.78 mM before stimulation and rose to 1.85–2.07 mM after addition of 20 mM α-ketoisocaproic acid. The concentrations of ADP behaved in the opposite manner; they were about 0.5 mM under basal condition and decreased to about 0.3 mM upon stimulation. The sums of ATP and ADP remained constant at 2.0–2.3 mM. The [ATP]/[ADP] ratios with lactate/pyruvate as the substrate were lower than with 5 mM glucose [2, 10]. Addition of 2-DG decreased the nucleotide ratio from 3.35 to 2.92, whereas addition of 20 mM α-ketoisocaproic acid resulted in a substantial rise which was essentially the same whether or not 2-DG was present.

3-MG had no effect on either the nucleotide levels or their ratios, both before and after stimulation with α-ketoisocaproic acid.

Concentration of inorganic phosphate in islets perifused with and without 2-DG. Inorganic phosphate was measured in the same samples in which the adenine nucleotides were determined. In control islets, the concentration of this anion was 4.72 mM and decreased to 3.44 mM when 2-DG was present. Upon addition of 20 mM α-ketoisocaproate, intracellular [P_i] fell and the reduction was greater in islets perifused with 2-DG (Table 2).

Effects of phosphate on insulin release and nucleotide levels. Experiments analogous to those presented in Fig. 1 were carried out in the presence of 1.5 mM phosphate in the perifusion buffer. Phosphate had no effect on either basal secretion or α-ketoisocaproic acid stimulated release. However, the potentiation by 2-DG, as illustrated in Fig. 3, was not only eliminated but converted to a small inhibition.

The content of ATP in islets perifused with phosphate-containing buffer and stimulated with a high concentration of α-ketoisocaproic acid was, within the limits of experimental error, the same as that in the medium without phosphate; the level of

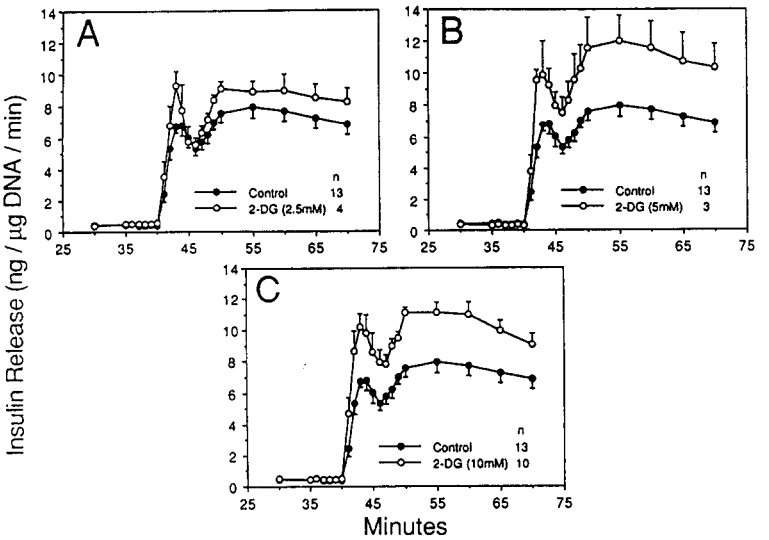

Fig. 1. Concentration dependence of 2-DG-induced enhancement of α-ketoisocaproic acid-evoked insulin release. Procedures of perifusion and stimulation are described in Materials and Methods. Values are means ± SEM for the number (n) of experiments indicated.

Table 1. Effects of 2-deoxy-D-glucose (2-DG) on insulin release in islets stimulated with 20 mM α-ketoisocaproic acid

Treatment	N	Basal (ng/35–40 min)	Acute (ng/40–45 min)	Late (ng/45–70 min)	Total (ng/35–70 min)
Control	14	1.99 ± 0.23	27.31 ± 1.25	181.93 ± 10.67	211.22 ± 11.46
2-DG (2.5 mM)	6	2.20 ± 0.48	35.46 ± 1.67*	197.14 ± 14.27	234.81 ± 14.50
2-DG (5 mM)	4	1.81 ± 0.30	46.72 ± 5.12*	265.40 ± 29.46*	313.93 ± 29.99*
2-DG (10 mM)	10	2.38 ± 0.23	42.53 ± 2.90*	255.56 ± 13.48*	300.46 ± 14.00*

Conditions of perifusion are described in detail in Materials and Methods. Values are means ± SEM for the number (N) of experiments indicated.

* Statistical analysis: * $P < 0.05$ or better compared to control.

ADP was, however, significantly lower (Table 2). Consequently the [ATP]/[ADP] ratio was larger. Addition of 2-DG to the phosphate-containing perifusion buffer did not alter either the nucleotide levels or their ratios.

The concentration of P_i was not measured in these samples because contamination with phosphate-containing medium contributed an error which was too large to allow an accurate determination of changes.

DISCUSSION

An important contribution of the current study is the re-evaluation of the concentration of intracellular P_i in pancreatic islets using a quenching procedure that minimizes hydrolysis of the high energy phosphate compounds, as judged by the high [ATP]/[ADP] determined in these samples. Orthophosphate is a necessary component in the synthesis of ATP by both mitochondrial oxidative phosphorylation and glycolysis and an important regulator of several enzymes. Moreover, it is the key player in maintaining the proper balance of calcium. Yet the amount of information on the level of intracellular P_i in islets is very sparse. The only data we were able to find are the studies from the laboratory of Matschinsky [13–15] which report values between 30–50 mmol/kg of dry weight in the absence of extermal phosphate. Assuming a water content of 3 nL/µg of dry weight, one calculates a concentration of 10–17 mM. Our figure of 4.7 mM is considerably lower and not markedly different from the level of inorganic phosphate found in other types of cells although it is about twice as high as the value seen in creatine phosphate/creatine containing tissues [16]. This comparison indicates that there still may be some hydrolysis of the labile high-energy phosphate compounds during experimental manipulations. Alternately, it may indicate that there is in islets a subcompartment which contains a large amount of P_i and that this pool contributes significantly to the total cellular content of the anion. The secretory granules are the obvious candidate

Table 2. Effects of 2-deoxy-D-glucose (2-DG), 3-*O*-methyl-D-glucose (3-MG) and P_i on nucleotide levels, [ATP]/[ADP], P_i and insulin release in islets stimulated with 20 mM α-ketoisocaproic acid

	Treatment	N	ATP (mM)	ADP	[ATP]/[ADP]	P_i (mM)	Insulin (ng/μg DNA/3 min)
Basal	Control	9	1.61 ± 0.15	0.48 ± 0.04	3.35 ± 0.15	4.72 ± 0.46	1.23 ± 0.14
	2-DG (10 mM)	7	1.47 ± 0.19	0.51 ± 0.07	2.92 ± 0.11*	3.44 ± 0.14*	1.43 ± 0.14
	3-MG (10 mM)	3	1.78 ± 0.01	0.47 ± 0.00	3.78 ± 0.04	ND	0.91 ± 0.15
Stimulated	Control	10	2.02 ± 0.14	0.27 ± 0.02	7.54 ± 0.27†	3.01 ± 0.52*	14.77 ± 0.95
	P_i	3	2.07 ± 0.06	0.19 ± 0.01	10.90 ± 0.11†‡	ND	16.03 ± 1.33
	2-DG (10 mM)	9	1.85 ± 0.17	0.27 ± 0.03	7.09 ± 0.25†	1.83 ± 0.17*†‡	26.12 ± 2.00‡
	2-DG (10 mM) + P_i	5	2.14 ± 0.04	0.19 ± 0.01	11.19 ± 0.74†‡	ND	12.79 ± 1.20
	3-MG (10 mM)	4	2.04 ± 0.10	0.28 ± 0.01	7.40 ± 0.05†	ND	14.78 ± 1.40

Conditions of perifusion and analytical techniques for measurement of both nucleotides and insulin are described in detail in Materials and Methods. Basal and stimulated samples were snap-frozen at 40 and 45 min of perifusion, respectively. α-Ketoisocaproic acid (20 mM) was added at 40 min of perifusion. Insulin values are the amounts secreted either from 36 to 39 min (basal) or from 41 to 44 min (stimulated). Values are means ± SEM for the number (N) of experiments indicated, except for P_i which was measured in six experiments from each series. ND = not determined.
*–‡ Statistical analysis: * $P < 0.03$ compared to control (basal), † $P < 0.001$ compared to basal, and ‡ $P < 0.05$ compared to control (stimulated).

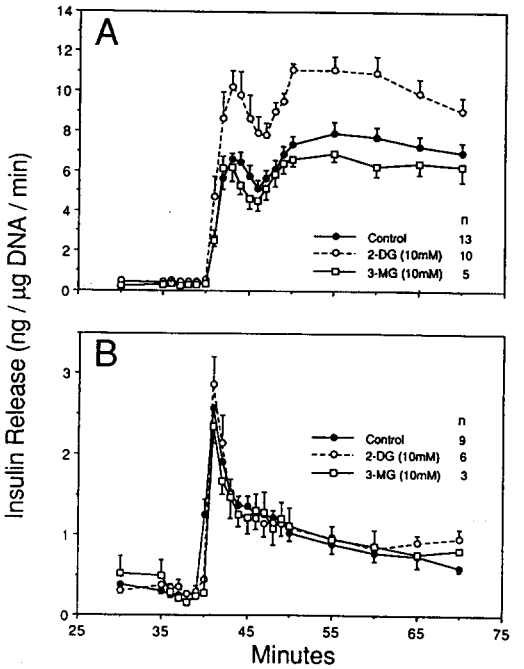

Fig. 2. Sugar and secretagogue dependence of enhanced insulin release. (A) Comparison of effects of 2-DG and 3-MG on α-ketoisocaproic acid-induced release. (B) Effect of 2-DG on KCl-triggered secretion. Experimental details are given in Materials and Methods. The concentration of α-ketoisocaproate in A was 20 mM and that of KCl in B was 40 mM. Values are means ± SEM for the number (n) of experiments indicated.

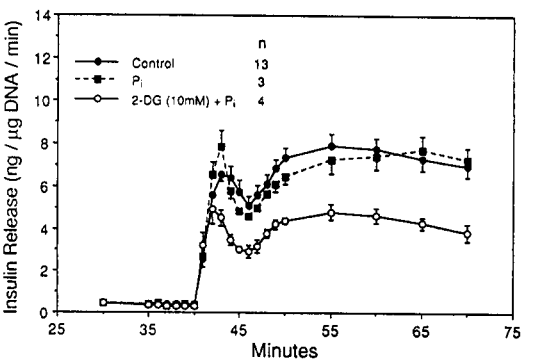

Fig. 3. Effect of inorganic phosphate (P_i) on the 2-DG-induced enhancement of insulin release evoked by 20 mM α-ketoisocaproate. Experimental details are provided in Materials and Methods. The concentration of orthophosphate was 1.5 mM. Values are means ± SEM for the number (n) of experiments indicated.

because they were reported to possess about 130 nmol phosphate/mg protein [17]. Hence, if a substantial proportion of P_i is indeed sequestered in these organelles, the cytosolic concentration of the anion may be significantly lower than 4.7 mM and more in line with values of 2–3 mM found in other creatine phosphate/creatine containing tissues such as heart or brain [18].

The results presented in the current study lead to the following conclusions. First, perifusion of islets with 2-DG in a phosphate-free medium containing lactate plus pyruvate as the fuel results in a decrease in the level of intracellular inorganic phosphate both under basal conditions and in islets stimulated with the secretagogue, α-ketoisocaproic acid. Second, in islets stimulated with a high concentration of α-ketoisocaproic acid, 2-DG potentiates the release of insulin. Third, potentiation of α-ketoisocaproate-evoked hormone release by 2-DG occurs concomitantly with a decrease in the concentration of intracellular inorganic phosphate to a level which is lower than that in control, stimulated islets. Fourth, enhancement of stimulated insulin release can be prevented by addition of physiologic concentrations of inorganic phosphate to the perifusion buffer.

2-DG is actively phosphorylated in most cells but its further utilization is very slow [7, 19, 20]. The source of phosphate is ATP and thus, if the rate of 2-DG phosphate (2-DGP) formation is faster than that of ATP resynthesis, a fall in the adenine nucleotide is a likely consequence. Moreover, since 2-DGP accumulates inside cells, it may lower $[P_i]_i$ unless transport from outside compensates for the reduction. Consistent with these predictions, incubation of ascites tumor cells with 2-DG results in a decrease in [ATP] and a simultaneous uptake of phosphate [7], whereas exposure of hepatocytes to either fructose or glycerol (two sugars that are rapidly phosphorylated inside liver cells) decreases both [ATP] and $[P_i]_i$ [21]. A similar response was seen in islets perifused without phosphate, where a small decline in [ATP] (Table 2) was accompanied by a fall in intracellular inorganic phosphate. This reduction in $[P_i]_i$ persists and may be even more pronounced when islets are stimulated with 20 mM α-ketoisocaproate. It should be pointed out that differences in the concentrations of cytosolic orthophosphate between the control and 2-DG-treated cells are likely to be greater than those measured here because they are superimposed on a large background of a rather inert pool which is sequestered in the secretory granules and not available for the formation of 2-DGP. However, whether and to what extent depletion of the internal phosphate is reversed in the anion-containing perifusion medium cannot be answered definitely by the present study because under such conditions we could not measure the anion level with sufficient accuracy. Nevertheless, the observation that with external phosphate present 2-DG does not potentiate release of insulin induced by a high concentration of α-ketoisocaproate, and may even inhibit it, might suggest that transport from outside is sufficiently rapid to compensate for the rate of depletion.

The potentiation by 2-DG of insulin release caused by 20 mM α-ketoisocaproic acid was originally described by Zawalich *et al.* [20]. These authors reported that addition of 80 mM 2-DG to a perifusion buffer containing no phosphate increased by 47% hormone release caused by 10 mM α-ketoisocaproic acid. No explanation, however, was offered for this interesting finding. The observation was confirmed and extended in the current study which shows that the effect is specific for a sugar analogue that can be phosphorylated, is dependent on its concentration, and requires the presence of a metabolic secretagogue. The discrepancy seen in the two studies between the concentrations of 2-DG necessary to achieve the same stimulation may be attributed to a difference in the experimental conditions. Whereas Zawalich *et al.* used 2.75 mM glucose as the fuel, the present work used lactate plus pyruvate.

The higher release of insulin evoked by α-ketoisocaproic acid in islets perifused with 2-DG coincided with a decline in the concentration of intracellular inorganic phosphate to a level that was lower than that seen in the absence of the sugar analogue. This indicates that reducing the intracellular concentration of the anion, or consequences thereof, facilitates in some manner hormone secretion. It has been known for a long time that stimulation of insulin release by metabolic secretagogues is accompanied by a decrease in intracellular $[P_i]$, a so-called "phosphate flush" [13, 22, 23]. Omission of bicarbonate from the perifusion medium delays efflux of phosphate [23] and simultaneously retards the first phase of glucose-enhanced insulin release [24]. Although the mechanism of "phosphate flush" has not been elucidated, its occurrence and properties appear to suggest that it may be causally linked to hormone secretion. Studies in humans with reduced plasma $[P_i]$ [6] are consistent with this suggestion while experiments reported here provide independent support. It must be remembered, however, that neither the older nor the current work allows distinction between the effect of P_i itself or its indirect action via the cellular energy level. Since ΔG is dependent not only on the ATP/ADP but also on the P_i, all information thus far available can be interpreted as being caused by a change in ΔG_{ATP}.

Our data show that in the presence of external phosphate the potentiating effect of 2-DG on stimulated insulin release was replaced by a small inhibition. This finding seems to indicate, consistent with the considerations above, that transport of P_i across the plasma membrane is rapid and that 2-DG cannot decrease the anion level upon stimulation with high α-ketoisocaproate to the same extent as it does without phosphate in the medium.

Finally we wish to point out that an interesting and physiologically relevant consequence of the presence of phosphate in the external environment was a rise in cellular ATP/ADP (Table 2). Since one would expect that under such conditions the intracellular phosphate also rises, a positive correlation exists between the nucleotide ratio and the P_i level. A similar behavior was observed by us earlier in yeast, liver [21] and perfused heart [25] and was interpreted to mean that cellular homeostatic mechanisms are designed to maintain a constant ΔG_{ATP} (which is dependent not only on the [ATP]/[ADP] but also on the concentration of inorganic phosphate).

Acknowledgements—The authors would like to thank June Nelson and Jen H. Yu for their expert technical assistance. This study was supported by National Institutes of Health Grants DK-35808 and DK-19525.

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